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Expanding Aminoglycoside Resistance Enzyme Regiospecificity by Mutation and Truncation

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Abstract

Aminoglycosides (AGs) are broad-spectrum antibiotics famous for their antibacterial activity against Gram-positive and Gram-negative bacteria, as well as mycobacteria. In the United States, the most prescribed AGs, including amikacin (AMK), gentamicin (GEN), and tobramycin (TOB), are vital components of the treatment for resistant bacterial infections. Arbekacin (ABK), a semisynthetic AG, is widely used for the treatment of resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* in Asia. However, the rapid emergence and development of bacterial resistance are limiting the clinical application of AG antibiotics. Of all bacterial resistance mechanisms against AGs, the acquisition of AG-modifying enzymes (AMEs) by bacteria is the most common. It was previously reported that a variant of a bifunctional AME, the 6'-N-AG acetyltransferase-Ie/2''-O-AG phosphotransferase-Ia [AAC(6')-Ie/APH(2'')-Ia], containing a D80G point mutation and a truncation after amino acid 240 modified ABK and AMK at a new position, the 4'''-amine, therefore displaying a change in regiospecificity. In this study, we aimed to verify the altered regiospecificity of this bifunctional enzyme by mutation and truncation for the potential of derivatizing AGs with chemoenzymatic reactions. With the three variant enzymes in this study that contained either mutation only (D80G), truncation only (1–240), or mutation and truncation (D80G-1–240), we characterized their activity by profiling their substrate promiscuity, determined their kinetics parameters, and performed mass spectrometry to determine how and where ABK and AMK were acetylated by these enzymes. We found that the three mutant enzymes possessed distinct acetylation regiospecificity compared to that of the bifunctional AAC(6')-Ie/APH(2'')-Ia enzyme and the functional AAC(6')-Ie domain [AAC(6')/APH(2'')-1–194].

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ASSOCIATED CONTENT

Supporting Information

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Three tables listing the primers used for the PCR amplification of the *aac(6')/aph(2'')* gene for various constructs (Table S1), SOE PCR strategies for generating all new *aac(6')/aph(2'')* constructs used in this study (Table S2), purification yields of AAC(6')/APH(2'') and mutant proteins (Table S3), and five figures displaying a Coomassie blue-stained 15% Tris-HCl SDS-PAGE gel showing the purified proteins used in this study (Figure S1), kinetic curves for AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1–240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1–240 (NH₂) with various AGs with insets including the K_m and k_{cat} values from a nonlinear regression fit to the Michaelis-Menten equation (Figures S2–S5), and mass spectra of acetylated AMK (Figures S6 and S7) (PDF)

The authors declare no competing financial interest.

Graphical Abstract

The broad-spectrum aminoglycoside (AG) antibiotics are active against Gram-positive and Gram-negative bacteria, as well as mycobacteria.^{1–4} Their broad-spectrum feature has given AGs a vital position in clinical settings for the treatment of severe bacterial infections. Amikacin (AMK), gentamicin (GEN), and tobramycin (TOB) are currently approved in the United States for systemic administration (Figure 1). On the other hand, the systemic use of kanamycin (KAN) is limited to the treatment of multidrug-resistant (MDR) tuberculosis. Neomycin B (NEO) is most commonly added to antibiotic ointments for topical applications. In addition to being used as antibiotics, AGs are also currently being thoroughly investigated as antifungal agents,^{5–10} and as potential treatments for genetic disorders caused by premature termination codons, including, but not limited to, cystic fibrosis and Duchene muscular dystrophy.^{3,11–15}

Arbekacin [ABK (Figure 1)], although not currently prescribed in the United States, has been widely used in Asia for decades because of its excellent efficacy against MDR strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and methicillin-resistant *Staphylococcus aureus* (MRSA).¹⁶ ABK is a semisynthetic AG that has regained popularity over the past few decades. When an (*S*)-4-amino-2-hydroxybutyryl (AHB) group was appended to its precursor dibekacin, ABK was shown to possess antimicrobial activity better than that of AMK and GEN and to be less prone to the development of bacterial resistance than other AHB-lacking AGs.

As with most antibiotics, the extensive use of AGs has led to the accelerated development of bacterial resistance to these drugs. Bacteria can develop resistance to AGs by (i) changing their cell envelope composition, resulting in reduced drug permeability, (ii) upregulating their efflux pumps, resulting in a decreased intracellular drug concentration, (iii) modifying the ribosome, preventing binding of AGs to this target, and (iv) acquiring AG-modifying enzymes (AMEs), also preventing binding of the chemically modified drugs to the ribosome target.^{17–22} AMEs, which represent the most common mechanisms for resistance to AGs, can be classified into three families based on the distinct chemistry they catalyze: AG *N*-acetyltransferases (AACs), AG *O*-phosphotransferases (APHs), and AG *O*-nucleotidyltransferases (ANTs). AACs, the most common AMEs detected in bacteria that are resistant to AGs, catalyze the transfer of an acetyl group from acetyl-coenzyme A (AcCoA) onto the amine functionalities present on AG molecules. Studies have revealed that most AACs are regiospecific and modify only one amine moiety on AGs, as often designated in their names.²³ One exception to this rule is Eis (enhanced intracellular survival), an AG multiacetylating protein upregulated in strains of *Mycobacterium*

tuberculosis displaying resistance to KAN.^{24–31} Research has shown that some acetylated AGs, after being “inactivated” by AACs, still retain antibacterial activity. Therefore, having additional resistance enzymes that further modify AGs at different positions can help bacteria to completely inactivate AGs, avoiding toxic effects from these AG metabolites. The ability of Eis to multiacetylate a variety of AGs at various positions poses special challenges when it comes to combating bacterial resistance. Eis protein homologues are also found in other mycobacterial strains²⁹ as well as *Anabaena variabilis*,³⁰ *Bacillus anthracis*,³² *Gordonia bronchialis*,³³ *Kocuria rhizophila*,³³ and *Tsukamurella paurometabola*.³³

Research efforts were inspired by the discovery of a new ABK metabolite in an ABK-resistant MRSA strain in Japan and subsequently led to the identification of a variant of the AAC(6′)-Ie/APH(2′′)-Ia enzyme, hereafter denoted as AAC(6′)/APH(2′′) for the sake of simplicity.^{34–37} The reported variant enzyme contained a D80G point mutation in the AAC(6′) domain of this bifunctional protein, which was reported to introduce a natural proteolytic cleavage after amino acid 240, resulting in a mutated and truncated protein. Compared to the wild-type (wt) protein that acetylates only at position 6′, the mutant truncated protein displayed a switch in regiospecificity to the 4′′-amine of ABK and AMK, which has not been previously observed in AACs.^{35,36} The unique regiospecificity of the variant enzyme could provide special protection to the bacteria under exposure to AGs, as it would modify and inactivate AG antibiotics at a special position that is distinct from what the common resistance enzymes modify.

Herein, we set off to verify the change in regiospecificity and examine whether both the D80G mutation and truncation are necessary for this change in regiospecificity. We first cloned, expressed, and purified various enzymes, including AAC(6′)/APH(2′′)-wt (previously cloned),³⁸ AAC(6′)/APH(2′′)-D80G (with the mutation only), AAC(6′)/APH(2′′)-1–240 (with the truncation only), and AAC(6′)/APH(2′′)-D80G-1–240 (with the mutation and truncation) (Tables S1–S3, Figure 2A, and Figure S1). We then determined the substrate profiles of these enzymes with a panel of 14 AGs [ABK, AMK, apramycin (APR), geneticin (G418), GEN, hygromycin (HYG), KAN, neamine (NEA), NEO, netilmicin (NET), paromomycin (PAR), ribostamycin (RIB), sisomicin (SIS), and TOB] and one fluoroquinolone [ciprofloxacin (CIP)] (Figure 1 and Table 1). We also determined the kinetic parameters for AAC(6′)/APH(2′′)-wt and the three different mutant enzymes with seven selected AGs (ABK, AMK, KAN, NEA, NEO, SIS, and TOB) (Table 2 and Figures S2–S5). After seeing the difference in the enzymatic regiospecificity between AAC(6′)/APH(2′′)-wt and mutant enzymes by performing mass spectrometry (MS) [and tandem mass spectrometry (MS²)], we cloned an additional AAC(6′)/APH(2′′)-1–194 protein, which was previously reported to be the functional AAC(6′)-Ie domain (Tables S1–S3 and Figure 2A).³⁹ We compared the activity of AAC(6′)/APH(2′′)-wt, AAC(6′)/APH(2′′)-1–240, AAC(6′)/APH(2′′)-D80G, AAC(6′)/APH(2′′)-D80G-1–240, and AAC(6′)/APH(2′′)-1–194 on purified reaction mixtures containing acetylated ABK and AMK to see how and where these AG molecules were being modified by AAC(6′)/APH(2′′)-wt and its mutants (Table 3 and Figures 3–5).

MATERIALS AND METHODS

Materials and Instrumentation

The pET28 vector was purchased from Novagen (Gibbstown, NJ). TOP10 and BL21 (DE3) chemically competent *Escherichia coli* cells were purchased from Invitrogen (Carlsbad, CA). Reagents for cloning, including restriction endonucleases, T4 DNA ligase, and Phusion DNA polymerase (accompanied by appropriate buffers), were purchased from New England BioLabs (Ipswich, MA). DNA primers for polymerase chain reaction (PCR) were purchased from Sigma-Aldrich (Milwaukee, WI) and Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed by Eurofins Genomics. AcCoA and DTNB were purchased from Sigma-Aldrich. AMK, GEN, KAN, NEA, NET, SIS, and TOB were purchased from AK Scientific (Mountain View, CA). APR, G418, HYG, PAR, and RIB were purchased from Sigma-Aldrich. NEO was purchased from Tokyo Chemical Industry Co. Ltd. ABK was a generous gift from S. Vakulenko (University of Notre Dame, Notre Dame, IN). CIP was purchased from Sigma-Aldrich. Ni^{II}-NTA used for affinity chromatography was purchased from Qiagen.

E. coli cultures were grown in Thermo Scientific MaxQ 6000 incubators, and OD measurements were taken on a Thermo Spectronic Genesys 20 instrument. Centrifugation was performed by using a Thermo Sorvall RC 6 Plus centrifuge. Cell disruption was achieved by sonication using a Qsonica (Newtown, CT) sonicator ultrasonic processor. UV-vis assays for the determination of kinetic parameters and substrate profiling were performed on a SpectraMax M5 plate reader. Mass spectra were recorded in positive mode using an AB SCIEX TripleTOF 5600 (AB SCIEX, Redwood City, CA) mass spectrometer and a Shimadzu HPLC system equipped with a DGU-20A/3R degasser, LC-20AD binary pumps, a CBM-20A controller, and a SIL-20A/HT autosampler (Shimadzu, Kyoto, Japan).

Cloning of N-His₆-Tagged *aac(6')*/*aph(2'')*-1-240, *aac(6')*/*aph(2'')*-D80G, *aac(6')*/*aph(2'')*-D80G-1-240, and *aac(6')*/*aph(2'')*-1-194 Genes in pET28a

The wt AAC(6')/APH(2'') DNA was previously cloned into pET22b and pET28a vectors to generate a plasmid encoding C-His₆- and N-His₆-tagged proteins.³⁸ Here, the *aac(6')*/*aph(2'')*-1-240, *aac(6')*/*aph(2'')*-D80G, *aac(6')*/*aph(2'')*-D80G-1-240, and *aac(6')*/*aph(2'')*-1-194 genes were cloned into pET28a using the primers and cloning strategies listed in Tables S1 and S2 and Figure 2A, respectively. All genes were amplified by PCR using the AAC(6')/APH(2'')-wt DNA from the pAAC(6')/APH(2'')-pET22b vector. PCR products were confirmed via agarose gel electrophoresis and purified by gel extraction (Qiagen QIAquick Gel Extraction Kit). The PCR-amplified genes and empty pET28a vector were digested by using *Nde*I and *Xho*I for 4 h at 37 °C and purified using the Qiagen QIAquick PCR purification kit. The purified inserts and digested vector were ligated using T4 DNA ligase (5:1 insert:vector ratio) overnight at room temperature to generate plasmids pAAC(6')/APH(2'')-1-240-pET28a, pAAC(6')/APH(2'')-D80G-pET28a, pAAC(6')/APH(2'')-D80G-1-240-pET28a, and pAAC(6')/APH(2'')-1-194-pET28a, which were directly transformed into *E. coli* TOP10 chemically competent cells for DNA isolation (Qiagen QIAprep Spin Miniprep Kit). After confirmation of the existence of the desired gene insert into the pET28a vector by double digestion of the isolated DNA with *Nde*I and

*Xho*I and DNA sequencing (Eurofins MWG Operon), the isolated plasmids were transformed into *E. coli* BL21 (DE3) chemically competent cells for protein expression and purification.

Overexpression and Purification of AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, AAC(6')/APH(2'')-D80G-1-240, and AAC(6')/APH(2'')-1-194 N-His₆-Tagged Proteins

The *E. coli* BL21 (DE3) cells transformed with the pAAC(6')/APH(2'')-1-240-pET28a, pAAC(6')/APH(2'')-D80G-pET28a, pAAC(6')/APH(2'')-D80G-1-240-pET28a, and pAAC(6')/APH(2'')-1-194-pET28a constructs were inoculated into 2 × 1 L of LB broth supplemented with 50 µg/mL KAN and stirred at 200 rpm and 37 °C until the attenuation at 600 nm reached 0.5. Then, each culture was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (final concentration) and incubated at 20 °C overnight (200 rpm). Cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C and lysed in buffer A [50 mM Na₂HPO₄ (pH 8.0, adjusted at room temperature), 300 mM NaCl, and 10% glycerol] by sonication 3 × 2 min cycles, alternating between 2 s “on” and 10 s “off” for each cycle, resting for 2 min between cycles, on ice. After removal of the cell debris by centrifugation at 16000 rpm for 45 min at 4 °C, each enzyme was purified via Ni^{II}-NTA affinity chromatography by washing and eluting with buffer A containing imidazole in a gradient fashion (10 mL of 5 mM imidazole, 3 × 5 mL of 20 mM imidazole, 3 × 5 mL of 40 mM imidazole, and 3 × 5 mL of 250 mM imidazole). After analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fractions containing pure protein were combined and dialyzed in 50 mM HEPES (pH 7.5, adjusted at room temperature) and 10% glycerol and stored at 4 °C or flash-frozen at –80 °C. By comparing the enzyme activity from the 4 and –80 °C protein stocks, we found that being stored at 4 °C does not result in a noticeable loss of enzymatic activities of the enzymes involved in this study compared to those of the enzymes stored at –80 °C. A sample SDS-PAGE gel of AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1-240 is shown in Figure S1 with yields provided in Table S3.

Establishing the Substrate Profile of AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1-240 by UV-Vis Assays

To test whether the AAC(6')/APH(2'')-wt and mutant enzymes could modify various AGs, we established their substrate profiles by UV-vis assays. We tested a panel of 14 AGs (ABK, AMK, APR, G418, GEN, HYG, KAN, NEA, NEO, NET, PAR, RIB, SIS, and TOB) and one fluoroquinolone (CIP). Acetylation reactions were monitored by using a colorimetric UV-vis assay in 96-well plates at 37 °C. Each reaction mixture (total volume of 200 µL) contained 50 mM MES (pH 6.6, adjusted at room temperature), 2 mM DTNB, 150 µM AcCoA, and various AG substrates at 100 µM, and each reaction was initiated by the addition of 5 µM enzyme [stored in 50 mM HEPES (pH 7.5) and 10% glycerol]. The reaction progress was monitored by recording the absorbance at 412 nm every 20 s for 20 min. As presented in Table 1, the question of whether the AG (or CIP) tested was a great, moderate, or poor substrate or not a substrate was arbitrarily answered in this study on the basis of the absorbance at 412 nm (an absorbance of >0.8 categorized a great substrate, an

absorbance of 0.4–0.8 a moderate substrate, an absorbance of 0.1–0.4 a poor substrate, and an absorbance of <0.1 a compound that was not a substrate).

Determination of Kinetic Parameters for AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1-240

To determine the kinetic parameters of various enzymes with respect to AGs, we tested seven selected AGs that were tested to be good substrates of the four enzymes in a previous substrate profiling experiment (ABK, AMK, KAN, NEA, NEO, SIS, and TOB) by UV-vis assays in 96-well plates for 15 min at 37 °C. Reaction mixtures contained 50 mM MES (pH 6.6, adjusted at room temperature), 2 mM DTNB, 0.05 µM enzyme, and AcCoA (the concentration of AcCoA in the determination of kinetic parameters for each AG equals 1.5 equiv of the highest AG concentration), and each reaction was initiated by the addition of 50 µL of AG substrate of various concentrations to make the total reaction volume 200 µL. All experiments were performed in triplicate, and the kinetic parameters were then calculated on the basis of Lineweaver-Burke plots generated by using Sigma Plot version 9.0 (Table 2 and Figures S2–S5).

Mass Spectrometry

To confirm the number of acetylation events and their positions, we subjected purified enzymatic reactions to mass spectrometry and tandem mass spectrometry. We performed reactions with 1 equiv of ABK or AMK modified by a single enzyme generated in this study with 1.5 equiv of AcCoA to determine the preferred position of each enzyme (1.5 equiv of AcCoA allowed for acetylation of one position on AG by the enzyme to be visualized by mass spectrometry) or with 3 equiv of AcCoA to determine whether each enzyme is capable of transferring multiple acetyl groups onto one AG molecule. We then performed sequential modification reactions where we set up reactions with 1 equiv of ABK or AMK with 3 equiv of AcCoA and a first enzyme (either the wt or any of the three mutants generated in this study). After completion of the reaction (24 h at 37 °C), we added a second enzyme (either any of the three mutants or the wt enzyme) and let the reaction proceed for an additional 24 h at 37 °C to determine whether the wt and mutants have different preferences for the site of modification/acetylation on ABK and AMK. Each reaction mixture (total volume of 50 µL) contained MES (pH 6.6) (50 mM), AGs (100 µM) (either ABK or AMK), AcCoA (150 or 300 µM), and various enzymes by themselves or in sequential order (5 µM each). Reaction mixtures were subjected to cation solid phase extraction (Alltech, catalog no. 209800) and eluted with 2% NH₄OH. Elution fractions were dried in a vacuum centrifuge and redissolved in doubly distilled H₂O (50 µL). Fifteen microliters of each sample was directly injected using the ESI method with a mobile phase composed of 0.1% formic acid in H₂O onto the mass spectrometer. Species between m/z 300 and 800 were collected by MS (Figures 4 and 5). Species of interest with specific m/z ratios, including ABK (exact mass of 552.3119 Da), monoacetylated ABK (exact mass of 594.3225 Da), diacetylated ABK (exact mass of 636.3330 Da), AMK (exact mass of 585.2857 Da), monoacetylated AMK (exact mass of 627.2963 Da), and diacetylated AMK (exact mass of 669.3069 Da), were collected and fragmented via tandem MS, as presented in Table 3, Figure 3, and Figures S6 and S7.

Disk Diffusion Assays

To assess the antibacterial activity of AME-modified ABK metabolites, we performed disk diffusion assays with reaction mixtures containing ABK modified by either a single enzyme individually [i.e., AAC(6')/APH(2'')-wt, AAC(6')/APH(2'')-1-240, or AAC(6')/APH(2'')-D80G-1-240] or two enzymes sequentially [i.e., AAC(6')/APH(2'')-1-240 followed by AAC(6')/APH(2'')-wt or AAC(6')/APH(2'')-D80G-1-240 followed by AAC(6')/APH(2'')-wt]. Reaction conditions were as described for the MS experiments. After completion of the reactions (24 h at 37 °C), 15 µL portions of the reaction mixtures were spotted onto sterile filter disks (~0.6 cm), air-dried for 15 min, and then placed on the LB-agar surface. A fresh dilution of an overnight culture of *E. coli* MC 1061 strain in soft LB-agar (100 µL culture in 10 mL of soft LB-agar) was thinly layered onto the hard LB-agar surface. The presence or absence of zones of inhibition was visually assessed after overnight incubation at 37 °C.

RESULTS AND DISCUSSION

Molecular Cloning and Expression of AAC(6')/APH(2'')-wt and Its Mutant Enzymes

As postulated in previous studies,^{34–37} the change in regiospecificity of an AAC(6')/APH(2'') mutant enzyme from position 6' to 4'' could result from a D80G mutation and a D80G-induced truncation at amino acid 240. According to the published AAC(6') domain structure (PDB entry 4QC6)⁴⁰, D80 is a residue that lies on the surface of this globular protein and belongs to the beginning of an α -helix (Figure 2B,C). Mutation of this aspartate to a glycine was predicted to introduce a strong α -helix break, which in turn was suggested to abruptly change the protein conformation during folding, thereby introducing a proteolytic cleavage after amino acid 240 in the APH(2'') domain of the bifunctional enzyme.³⁶ Previous research on domain dissecting of this bifunctional AME³⁹ and crystallographic knowledge of each of the two domains^{40–41}, have revealed that the functional AAC(6') and APH(2'') domains span amino acids 1–194 and 175–479, respectively. The overlapped linker region between the two domains (amino acids 175–194) had been proven to be critical for the activities for both domains.³⁹ Therefore, the resulting truncated protein would contain the linker domain and 46 residues from the APH(2'') domain in addition to the AAC(6') domain. Even though we were not successful in determining crystal structures to demonstrate how the D80G mutation and/or 1–240 truncation would impact the protein conformation, we postulate that if the D80G mutation were to shift the rest of the protein conformation during folding, the rest of the protein after the D80 residue, as represented by the pale turquoise portion, would change, which would impact the shape and size of the substrate binding pocket, as the residues after D80 compose half of the AG binding site and the entire CoA binding site. Although structures of the bifunctional enzyme and of the AAC(6') domain have been modeled and/or determined, attempts to crystallize AAC(6')/APH(2'')-D80G-1-240 have proven to be unsuccessful so far. It is therefore difficult to prove how the protein would fold as a result of the mutation and/or truncation, especially because the D80G mutation occurred so early in the protein sequence.

To verify whether both mutation and truncation are required for the change in AAC regiospecificity, we cloned three mutant enzymes that contained the mutation only [AAC(6')/APH(2'')-D80G], the truncation only [AAC(6')/APH(2'')-1–240], and both the mutation and the truncation [AAC(6')/APH(2'')-D80G-1–240] with an N-His₆ tag. Expression in BL21 (DE3) cells and purification with Ni^{II}-NTA resin yielded soluble proteins with yields between 6.3 and 13.9 mg/L of LB broth culture (Tables S1–S3, Figure 2A, and Figure S1).

Establishment of the Substrate Profiles of AAC(6')/APH(2'')-wt and Its Three Mutant Enzymes by UV-vis Assays

To determine whether AAC(6')/APH(2'')-wt and its three mutant enzymes have different or similar substrate profiles, we performed UV-vis assays with 14 AGs (ABK, AMK, APR, G418, GEN, HYG, KAN, NEA, NEO, NET, PAR, RIB, SIS, and TOB) and one fluoroquinolone (CIP). We found that AAC(6')/APH(2'')-wt and its mutant enzymes were all most active against AMK, KAN, NEA, and TOB and mildly active against ABK, GEN, NEO, NET, RIB, and SIS. These enzymes were not active against APR, G418, HYG, PAR, or CIP (Table 1). These results contradict the previous reports on AAC(6')/APH(2'')-D80G-1–240 stating that this specific mutant is devoid of AAC(6') activity,³⁶ as we found that AGs possessing 6'-amines are substrates of our mutant enzymes whereas the AGs that do not possess 6'-amines, but instead comprise hydroxyl groups at position 6', are not substrates of these mutant enzymes, indicating the mutant enzymes are likely to possess 6'-acetyltransferase activity. Additionally, AAC(6')/APH(2'')-wt was previously reported to be capable of O-acetylating AGs, such as PAR, that lack 6'-amine.⁴² However, we did not observe such activity in our study. Given that ABK and AMK were the only AGs that possessed AHB groups, it was not clear whether acetylation was happening at position 6' or 4' or another amine position. We therefore later relied on MS and MS² to decipher the position(s) modified by all of our enzymes (see below).

Determination of Kinetic Parameters for AAC(6')/APH(2'')-wt and Its Three Mutant Enzymes

Next, we determined the kinetic parameters for AAC(6')/APH(2'')-wt and its three mutant enzymes to gain insights into potential differences in enzyme behavior. We selected seven AGs (ABK, AMK, KAN, NEA, NEO, SIS, and TOB) that were assessed to be good substrates of AAC(6')/APH(2'')-wt and its mutants in our substrate profiling experiments and determined the K_m , k_{cat} , and k_{cat}/K_m values (in units of μM , min^{-1} , and $\mu\text{M}^{-1} \text{min}^{-1}$, respectively) by fitting to a nonlinear regression Michaelis-Menten equation (Table 2 and Figures S2–S5). In general, we found that for AAC(6')/APH(2'')-wt, the substrates that showed the lowest and highest K_m values were KAN ($2.98 \pm 0.67 \mu\text{M}$) and NEO ($102 \pm 20 \mu\text{M}$), respectively. While KAN consistently displayed the lowest K_m values with all four enzymes, the substrates that displayed the highest K_m values were NEO ($102 \pm 20 \mu\text{M}$) for AAC(6')/APH(2'')-wt, ABK ($146 \pm 17 \mu\text{M}$) for AAC(6')/APH(2'')-1–240, NEO ($307 \pm 51 \mu\text{M}$) for AAC(6')/APH(2'')-D80G, and ABK ($331 \pm 108 \mu\text{M}$) for AAC(6')/APH(2'')-D80G-1–240. As implied by the definition of K_m values, the full-length enzymes shared similar preferences in terms of binding efficiencies, as did the two truncated enzymes. As for the enzyme turnover rate (k_{cat}), the values generally did not change drastically from

AAC(6')/APH(2'')-wt to its three mutants. Combining the binding affinity and enzyme turnover rate, we found that in some cases, i.e., with AMK as a substrate, mutation seemed to have slightly improved enzyme efficiencies. In other cases, with TOB as a substrate, mutation did not decrease the overall enzyme efficiencies but truncation did.

The enzyme activities we observed in this study were overall consistent with those presented in previously reported studies of the wt and truncated AAC(6')/APH(2'') enzyme kinetics.^{38,39,42,43} The difference observed in the mutant enzymes with the occurrence of mutation and/or truncation was not sufficiently significant to indicate a substantial difference between enzyme mechanisms. It was interesting to note that despite the fact that ABK would be considered as “the natural substrate” for the AAC(6')/APH(2'')-D80G-1–240 enzyme, we found all four enzymes display low efficiency toward ABK as a substrate. The D80G mutant enzyme also showed potential indications of slight substrate inhibition in the Michaelis–Menten plots, which might suggest that the mutation could facilitate alternative modes of binding to the enzyme that interfere with catalysis (Figure S4).

Determination by MS and MS² of the Position(s) Acetylated on ABK and AMK by AAC(6')/APH(2'')-wt and Its Three Mutant Enzymes

Because the substrate profile and the kinetic parameters of AAC(6')/APH(2'')-wt and D80G mutant and/or 1–240 truncated enzymes did not reveal significant differences among the four enzymes, we further looked into more sensitive techniques such as MS and MS² to decipher the potential switch in regioselectivity of these enzymes. We first incubated ABK with a single enzyme [AAC(6')/APH(2'')-wt, -1–240, -D80G, or -D80G-1–240] and 1.5 or 3 equiv of AcCoA. Theoretically, 1.5 equiv of AcCoA would be sufficient for at least one acetylation and 3 equiv of AcCoA sufficient for at least two acetylations on each AG molecule.

As summarized in Table 3 and Figures 3–5, we found that the AAC(6')/APH(2'')-wt enzyme modifies ABK solely at position 6' with either 1.5 or 3 equiv of AcCoA, indicated by only an AcABK peak at m/z 595.3297 and a fragmentation pattern aligning with that of 6'-AcABK in MS². This strict regiospecificity displayed by AAC(6')/APH(2'')-wt agreed with previous findings. ABK modified by AAC(6')/APH(2'')-1–240, -D80G, or -D80G-1–240 with 1.5 equiv of AcCoA resulted in only 6'-AcABK according to MS and MS². However, each reaction with 3 equiv of AcCoA yielded not only 6'-AcABK but also 6',4''-diAcABK (m/z 637.3403). This suggested that AAC(6')/APH(2'')-1–240, -D80G, and -D80G-1–240 enzymes preferred position 6' but were also able to modify the 4''-amine given an excess of AcCoA, expanding their original regiospecificity to a novel position, the 4''-amine. Similar activities were observed among the three mutant enzymes.

Next, we incubated ABK with two different enzymes sequentially with either AAC(6')/APH(2'')-wt as enzyme 1 (E1) followed by AAC(6')/APH(2'')-1–240, -D80G, or -D80G-1–240 as enzyme 2 (E2) or one of the mutant enzymes as E1 and wt as E2. The results were similar. We found that most of the ABK had been converted to 6',4''-diAcABK. Although previous research reported that the acetylation of AGs at a certain position can hinder further acetylation at other positions, i.e., it was reported that acetylation of NEO and SIS at position 3 hindered the subsequent acetylation at position 6',³⁸ we did not observe such

phenomenon in this study regarding the modification of ABK at positions 6' and 4''. The fact that we observed similar activity among all three mutant enzymes, either by one enzyme or by two enzymes sequentially, suggested that the D80G mutation and the 1–240 truncation are not both required for the enhanced regiospecificity. Even though the D80G-1–240 enzyme could be the naturally occurring variant in the producing bacteria due to the D80G mutation, enzymes with either mutation or truncation alone, when expressed heterologously, can show altered regiospecificity, as indicated by MS and MS² results.

Structurally, the D80G mutation was first postulated to significantly change protein conformation.^{35–37} If the D80G mutation were to cause the protein conformation to be significantly altered during protein folding [i.e., five-sixths of AAC(6')/APH(2'')-D80G will fold in a manner different from that of the wt protein conformation], the AAC(6')/APH(2'')-D80G conformation could have an expanded active site to accommodate the substrate, ABK, to bind in various orientations and be modified at two distinct amine appendages. According to the published modeling and structure of AAC(6')/APH(2''), the substrate binding cavities of AAC(6') and APH(2'') are reported to lie on the same side of the protein. Such a close arrangement inspired suspicions that the two active sites facilitate substrate binding and/or channeling cooperatively. Given such a rigid arrangement, the proteolytic cleavage at amino acid residue 240 that resulted from the mutation in the distant D80 residue might not in fact be random. The fact that the D80G mutation possibly altered the protein conformation that rearranged the two active sites relative to each other might account for the alternative binding of ABK in the AAC(6') active site.⁴⁰ Similarly, because AAC(6')/APH(2'')-1–240 would contain an extra portion of the linker domain and the 46 residues from the APH(2'') domain, it is highly possible that this fairly large segment could also disrupt the native protein conformation of the AAC(6') portion by interacting with amino acid residues in the AAC(6') domain. Therefore, we postulated that the structural change in protein folding resulting from either the D80G mutation, the appendage of the linker region and the 46 residues from APH(2''), or a combination of both could enlarge the enzymes' substrate binding sites to allow the acetylation of ABK at position 4''.

To test this hypothesis, we cloned and expressed the functional AAC(6') domain, which included residues 1–194 from the original AAC(6')/APH(2'') bifunctional enzyme, herein denoted AAC(6')/APH(2'')-1–194 to test whether the AAC(6') domain alone would show the expanded regiospecificity. We included the linker region (residues 175–194) in this construct to obtain a functional AAC(6') but omitted the 46 residues from the APH domain. We first modified ABK with AAC(6')/APH(2'')-1–194 with 1.5 equiv of AcCoA and observed only 6'-AcABK in MS and MS² (Figures 4 and 5). The reaction mixture containing ABK, AAC(6')/APH(2'')-1–194, and 3 equiv of AcCoA still yielded mostly 6'-AcABK. Sequentially modifying ABK with either AAC(6')/APH(2'')-wt followed by 1–194 or AAC(6')/APH(2'')-1–194 followed by wt resulted in both 6'-AcABK and 6',4''-diAcABK as shown by MS and MS². The results generated by using AAC(6')/APH(2'')-1–194 were considerably different from those generated with AAC(6')/APH(2'')-1–240, -D80G, and -D80G-1–240 enzymes. We found that the ability of AAC(6')/APH(2'')-1–194 to acetylate ABK at position 4'' was significantly weaker than those of the other three mutant enzymes, confirming that the extra 46 residues from the APH(2'') domain in our

truncated enzymes indeed assisted the expansion of regiospecificity, as did the D80G mutation.

Because of the structural similarities between ABK and AMK, we next obtained the MS and MS² spectra for the same reactions with AMK instead of ABK to determine whether the expansion of regiospecificity applies to AMK (Table 3, Figure 3, and Figures S6 and S7). We found that AMK, when modified by a single enzyme [AAC(6')/APH(2'')-wt, -1-240, -D80G, -D80G-1-240, or -1-194] with 1.5 equiv of AcCoA, was converted to 6'-AcAMK only (*m/z* 628.3036). When AMK was co-incubated with a single enzyme [AAC(6')/APH(2'')-wt, -1-240, -D80G, or -D80G-1-240] and 3 equiv of AcCoA, we identified the majority of the product as 6'-AcAMK and a trace amount of 6',4''-diAcAMK (*m/z* 670.3141), whereas AAC(6')/APH(2'')-1-194 with 3 equiv of AcCoA converted AMK to 6'-AcAMK only. This agreed with the previous observation that AAC(6')/APH(2'')-1-194 was more limited in its ability to acetylate AGs at various amine positions compared than the AAC(6')/APH(2'')-1-240, -D80G, and -D80G-1-240 enzymes were.

At the same time, we used two different enzymes to sequentially modify AMK. We realized that no matter whether we used AAC(6')/APH(2'')-wt as E1 and AAC(6')/APH(2'')-1-240, -D80G, or -D80G-1-240 as E2, or AAC(6')/APH(2'')-1-240, -D80G, or -D80G-1-240 as E1 and AAC(6')/APH(2'')-wt as E2, the results were similar. We observed 6'-AcAMK as a major product and 6',4''-diAcAMK as a minor product. No significant difference was observed among the three different mutant enzymes. However, when we used AAC(6')/APH(2'')-wt as E1 and AAC(6')/APH(2'')-1-194 as E2, or AAC(6')/APH(2'')-1-194 as E1 and AAC(6')/APH(2'')-wt as E2, we observed different results: most of the AMK was converted into 6'-AcAMK, and only a trace amount of 6',4''-diAcAMK was detected. Once again, this supported our previous observation that AAC(6')/APH(2'')-1-240, -D80G, and -D80G-1-240 were much more efficient at acetylating position 4'' after the enzyme regiospecificity had been increased. In addition, the different results observed between the acetylation of ABK and AMK by the same enzyme revealed an interesting phenomenon. Even though ABK and AMK structurally differed only in their substitutions at positions 2', 3', and 4' on ring I, the enzymes were able to discriminate one AG from the other, as the expanded acetylation activity at position 4'' for ABK was much more efficient compared to that for AMK.

In addition to MS and MS², nuclear magnetic resonance (NMR) spectroscopy would also be a great tool for elucidating where and how ABK and AMK are being acetylated by AAC(6')/APH(2'')-wt and its various mutant enzymes. However, with the limited amount of ABK that we received as a gift from S. Vakulenko (University of Notre Dame) and the inability to obtain ABK in the United States, MS remained the better and only instrument available to us for elucidating various enzyme behaviors with respect to ABK as a substrate.

Acetylation of AGs by various AACs usually abolishes the antibacterial effect of these antibiotics. Therefore, acquiring these resistance enzymes can provide bacteria with advantages in surviving the presence of a xenobiotic. However, previous research has also demonstrated that some AG metabolites, even after being chemically modified by AMEs, can still retain antibacterial activities. For instance, 6'-AcNEO has been shown to still

possess antibacterial activity against *Bacillus subtilis*.³⁸ Various ABK metabolites, including 2'-, 6'-, and 3''-AcABK, have also been reported to retain weak to moderate antibacterial activities.⁴⁴⁻⁴⁶ In such a circumstance, a single acetylation does not completely eliminate the antibacterial effect of AGs. Therefore, possessing any additional enzymes that are capable of acetylating at a secondary site on AGs and further inactivate these drugs can provide the bacteria with substantial survival advantages. In the case of AAC(6')/APH(2'')-D80G-1-240, MRSA acquired and/or developed this mutant enzyme that further modifies the 4'''-amine in addition to the 6'-amine. As a result, the bacteria can better survive ABK treatment in case the 6'-acetylated ABK still retains any antibacterial activity.

In our study, we also used disk diffusion assays to examine whether ABK metabolites by various enzyme constructs generated in this study possess any antibacterial effect against a susceptible strain of *E. coli* MC 1061. We found that ABK metabolites modified with one enzyme alone [AAC(6')/APH(2'')-wt, -1-240, or -D80G-1-240] or with two enzymes sequentially [AAC(6')/APH(2'')-1-240 followed by wt, or D80G-1-240 followed by wt] displayed no visible zones of inhibition, suggesting that these ABK metabolites retained no detectable antibacterial activity against this strain of bacteria evaluated in this assay. Another possibility to consider is that the acetylated ABK metabolites (6'-AcABK and 6',4'''-diAcABK) may become less membrane permeable because of the chemical modification on the molecules by acetylation. If so, the ABK metabolites might become unable to cross the bacteria cell envelope and membrane to reach their target in the cytoplasm even if the ABK metabolites might still be able to act on the ribosome and interfere with protein translation. In such a scenario, we would not be able to observe any zones of inhibition produced by these ABK metabolites despite their inhibitory activity on the ribosome. The absence of a zone of inhibition with these ABK metabolites also suggested no product inhibition of these AAC(6')/APH(2'') enzymes.

CONCLUSIONS

In this study, we explored the possibility of expanding AAC(6')/APH(2'')-wt regiospecificity by introducing a D80G point mutation and/or 1-240 truncation (Tables S1-S3, Figure 2, and Figure S1). Even though AAC(6')/APH(2'')-D80G-1-240 may be the naturally occurring mutant enzyme as a result of the D80G mutation in the MRSA strain where this mutant enzyme was originally discovered, we found that the three mutant enzymes we cloned in this study [AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1-240] showed similar substrate promiscuity (Table 1) and kinetic efficiency against various AGs (Table 2 and Figures S2-S5). MS and MS² with purified reaction mixtures with ABK as a substrate revealed that the three mutant enzymes showed a preference for acetylating AGs at their 6'-amine positions but can also modify position 4''' given an excess of AcCoA (Table 3 and Figures 3-5). This was not a complete switch of regiospecificity from position 6' to 4''' as reported previously,³⁶⁻³⁷ but rather an expansion, which the presence of the mutation only or the truncation only can also alter the protein catalysis in a such way described above. We postulated the D80G mutation and/or 1-240 truncation could cause alteration of the protein conformation during protein folding, resulting in an enlarged AG binding site that would accommodate ABK binding from various orientations.

Moreover, we also found that these three mutant enzymes did not display the same activity with AMK as a substrate as they did with ABK (Table 3, Figure 3, and Figures S6 and S7). Even though the mutant enzymes still displayed a preference for the 6'-amine of AMK, the ability to modify AMK at position 4'' was significantly weaker than that when modifying ABK. This suggested an uncharacterized mechanism by which the enzymes were able to discriminate between ABK and AMK even though the two AG structures are extremely similar.

Overall, bacteria evolved to possess AAC(6')/APH(2'')-D80G-1-240, a variant enzyme of AAC(6')/APH(2'') with expanded regiospecificity, to further protect the bacteria from any AG metabolites that might retain antibacterial activity after chemical modification and inactivation by AACs. The findings in this study can help us better understand the bacterial resistance mechanisms associated with AG antibiotics to better combat resistant bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AAC	aminoglycoside <i>N</i> -acetyltransferase
ABK	arbekacin
AcCoA	acetyl-coenzyme A
AG	aminoglycoside
AHB	(<i>S</i>)-4-amino-2-hydroxybutyryl
AME	aminoglycoside-modifying enzyme
AMK	amikacin
ANT	aminoglycoside <i>O</i> -nucleotidyl-transferase
APH	aminoglycoside <i>O</i> -phosphotransferase
APR	apramycin
CIP	ciprofloxacin
GEN	gentamicin

HYG	hygromycin
KAN	kanamycin
MDR-TB	multidrug-resistant tuberculosis
MRSA	methicillin-resistant <i>S. aureus</i>
NEA	neamine
NEO	neomycin B
NET	netilmicin
PAR	paromomycin
PDB	Protein Data Bank
SIS	sisomicin
TOB	tobramycin

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4,6-Disubstituted AG:

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈
Amikacin (AMK)	OH	OH	OH	AHB	CH ₂ OH	OH	H	H
Arbekacin (ABK)	NH ₂	H	H	AHB	CH ₂ OH	OH	H	H
Gentamicin (GEN)	NH ₂	H	H	H	H	CH ₃	OH	CH ₃
Kanamycin A (KAN)	OH	OH	OH	H	CH ₂ OH	OH	H	H
Tobramycin (TOB)	NH ₂	H	OH	H	CH ₂ OH	OH	H	H

4,5-Disubstituted AG:

	R ₁	R ₂	R ₃
Neamine (NEA)	NH ₂	OH	H
Neomycin B (NEO)	NH ₂	OH	H
Paromomycin (PAR)	OH	OH	H
Ribostamycin (RIB)	NH ₂	OH	H

Other substitution patterns:

Figure 1.
Structures of the various AGs and CIP tested in this study.

Figure 2.

(A) Schematic diagram of all mutant enzyme constructs generated and used in this study, including AAC(6')/APH(2'')-wt (green) as well as truncated AAC(6')/APH(2'')-1–240 (yellow), mutated AAC(6')/APH(2'')-D80G (turquoise), mutated and truncated AAC(6')/APH(2'')-D80G-1–240 (orange), and truncated AAC(6')/APH(2'')-1–194 (purple). A red X denotes the D80G single-point mutation. (B and C) Crystal structures of the AAC(6') domain (PDB entry 4QC6)⁴⁰ of the bifunctional enzyme AAC(6')/APH(2'') in surface and cartoon representations, respectively, with amino acids 1–79 colored off white, the D80

residue colored red, and amino acids 81–179 colored pale turquoise. The substrate KAN (green) is represented in its stick form with nitrogen and oxygen atoms colored blue and red, respectively. The CoA derivative (black) is on the back side of the structure.

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Figure 3.

Structures of (A) ABK and (B) AMK with the potential acetylation sites of interest in this study (6'- and 4'''-amine positions) denoted with red arrows. The exact masses of the parent ABK and AMK, monoacetylated ABK (AcABK) and AMK (AcAMK), and diacetylated ABK (diAcABK) and AMK (diAcAMK) are also listed under the structure.

Figure 4.

Representative mass spectra of ABK control and ABK modified with various amounts of AcCoA by various enzymes generated in this study either individually or sequentially: (A) ABK control, (B) ABK, AAC(6′)/APH(2′)-wt, and AcCoA (3 equiv), (C) ABK, AAC(6′)/APH(2′)-D80G-1–240, and AcCoA (1.5 equiv) (note that using other mutant enzymes gave the same spectra), (D) ABK, AAC(6′)/APH(2′)-D80G-1–240, and AcCoA (3 equiv) (note that using different mutant enzymes gave the same spectra), (E) ABK and AAC(6′)/APH(2′)-D80G-1–240 followed by wt and AcCoA (3 equiv) (note that using various mutant

enzymes first followed by wt and using the wt enzyme followed by various mutant enzymes gave the same spectra), (F) ABK, AAC(6')/APH(2'')-1-194, and AcCoA (1.5 equiv), (G) ABK, AAC(6')/APH(2'')-1-194, and AcCoA (3 equiv), and (H) ABK and AAC(6')/APH(2'')-1-194 followed by wt and AcCoA (3 equiv) (note that using the wt enzyme first followed by the 1-194 enzyme gave the same spectra). The expected m/z ratios for ABK, AcABK, and diAcABK $[M + H]^+$ were m/z 553.3192, 595.3297, and 637.3403, respectively.

Figure 5.

Representative tandem mass spectra (MS^2) of (A) AcABK and (B) diAcABK with predicted fragmentation. Fragments with m/z ratios between 100 and 600 for AcABK and between 100 and 700 for diAcABK were collected. The fragments of interest in each panel that indicated the position of acetylation were labeled both in the spectra and in the structure of AcABK and diAcABK with their calculated m/z ratios. Note that each predicted fragment above or below a dashed line was indicated with the corresponding calculated m/z ratio placed above or below the dashed line, respectively.

Table 1

Substrate Promiscuity for AAC(6')/APH(2'')-wt and Its Mutant Constructs, Including AAC(6')/APH(2'')-1–240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1–240^a

AG	wt	1–240	D80G	D80G-1–240
ABK	~	--	~	--
AMK	✓	✓	✓	✓
APR	×	×	×	×
G418	×	×	×	×
GEN	--	--	~	~
HYG	×	×	×	×
KAN	✓	✓	✓	✓
NEA	✓	✓	✓	✓
NEO	~	~	~	~
NET	--	--	--	--
PAR	×	×	×	×
RIB	--	--	--	--
SIS	~	~	~	~
TOB	✓	✓	✓	✓
CIP	×	×	×	×

^aActivity is based on the absorbance observed at 412 nm. All enzymes used in this assay are N-His₆-tagged constructs: ×, not a substrate; --, poor substrate; ~, moderate substrate; ✓, great substrate.

Table 2

Kinetic Parameters Determined for AAC(6')/APH(2'')-wt and Its Mutant Enzymes, Including AAC(6')/APH(2'')-1-240, AAC(6')/APH(2'')-D80G, and AAC(6')/APH(2'')-D80G-1-240, with Various AGs^a

AG	AAC(6')/APH(2'')-wt	AAC(6')/APH(2'')-1-240	AAC(6')/APH(2'')-D80G	AAC(6')/APH(2'')-D80G-1-240
ABK				
K_m	77.9 ± 21.4	146 ± 17	109 ± 58	331 ± 108
k_{cat}	2.43 ± 0.14	2.55 ± 0.11	2.12 ± 0.36	5.16 ± 0.76
k_{cat}/K_m	0.031 ± 0.009	0.017 ± 0.002	0.019 ± 0.011	0.016 ± 0.006
AMK				
K_m	30.7 ± 5.6	65.8 ± 6.6	31.1 ± 3.5	26.4 ± 5.2
k_{cat}	3.16 ± 0.20	7.75 ± 0.37	9.17 ± 0.34	6.59 ± 0.53
k_{cat}/K_m	0.103 ± 0.020	0.118 ± 0.013	0.295 ± 0.035	0.250 ± 0.053
KAN				
K_m	2.98 ± 0.67	1.11 ± 0.28	0.779 ± 0.286	1.24 ± 0.35
k_{cat}	8.02 ± 0.57	5.64 ± 0.45	6.16 ± 0.73	9.09 ± 0.90
k_{cat}/K_m	2.69 ± 0.64	5.10 ± 1.35	7.91 ± 3.05	7.33 ± 2.20
NEA				
K_m	8.12 ± 1.56	7.63 ± 0.57	11.7 ± 1.3	7.21 ± 1.14
k_{cat}	12.3 ± 0.7	8.33 ± 0.18	8.72 ± 0.29	6.82 ± 0.30
k_{cat}/K_m	1.51 ± 0.30	1.09 ± 0.09	0.744 ± 0.084	0.946 ± 0.155
NEO				
K_m	102 ± 20	107 ± 19	307 ± 51	140 ± 19
k_{cat}	13.9 ± 1.1	7.33 ± 0.54	22.4 ± 2.0	11.9 ± 0.7
k_{cat}/K_m	0.136 ± 0.029	0.068 ± 0.013	0.073 ± 0.014	0.084 ± 0.013
SIS				
K_m	49.9 ± 6.0	13.9 ± 3.7	67.9 ± 9.1	45.8 ± 5.6
k_{cat}	12.9 ± 0.6	4.15 ± 0.30	11.7 ± 0.7	10.5 ± 0.5
k_{cat}/K_m	0.259 ± 0.033	0.298 ± 0.082	0.172 ± 0.025	0.229 ± 0.030
TOB				
K_m	15.0 ± 4.3	16.4 ± 1.7	7.46 ± 1.87	15.2 ± 3.0
k_{cat}	15.3 ± 1.7	10.7 ± 0.7	11.1 ± 0.9	9.38 ± 1.00
k_{cat}/K_m	1.02 ± 0.32	0.651 ± 0.080	1.48 ± 0.39	0.618 ± 0.140

^a All AGs presented in this table were found to be good substrates of the different AAC(6')/APH(2'') (N-His) constructs according to our substrate profiling experiment results. K_m , k_{cat} , and k_{cat}/K_m values are presented in units of μM , min^{-1} , and $\mu\text{M}^{-1} \text{min}^{-1}$, respectively.

Table 3

Summary of Mass Spectrometry Results Obtained by Using a Single Enzyme or Sequentially Using Two Enzymes^a

AG	enzyme 1	enzyme 2	AcCoA (equiv)	AG	6'-AcAG	6',4''-diAcAG
ABK	×	×	×			
	wt	×	1.5			
	wt	×	3			
	1-240	×	1.5			
	1-240	×	3		++	
	D80G	×	1.5			
	D80G	×	3		++	
	D80G-1-240	×	1.5			
	D80G-1-240	×	3		++	
	1-194	×	1.5			
	1-194	×	3		++	
	wt	1-240	3		--	++
	wt	D80G	3		--	++
	wt	D80G-1-240	3		--	++
	wt	1-194	3		++	++
AMK	1-240	wt	3		--	++
	D80G	wt	3		--	++
	D80G-1-240	wt	3		--	++
	1-194	wt	3		++	++
	×	×	×			
	wt	×	1.5			
	wt	×	3		+	--
	1-240	×	1.5			
	1-240	×	3		+	--
	D80G	×	1.5			
	D80G	×	3		+	--
	D80G-1-240	×	1.5			
	D80G-1-240	×	3		+	--

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AG	enzyme 1	enzyme 2	AcCoA (equiv)	AG	6'-AcAG	6',4''-diAcAG
	1-194	×	1.5			
	1-194	×	3			
	wt	1-240	3	++		+
	wt	D80G	3	++		+
	wt	D80G-1-240	3	++		+
	wt	1-194	3	++		--
	1-240	wt	3	++		+
	D80G	wt	3	++		+
	D80G-1-240	wt	3	++		+
	1-194	wt	3	++		--

^aReactions with a single enzyme were performed for 24 h prior to processing, whereas sequential reactions were performed by reacting with enzyme 1 for 24 h followed by enzyme 2 for 24 h. Legend: ×, enzyme or AcCoA not used in the reaction; ′, the only species detected; ++, highly abundant species; +, moderately abundant species; --, poorly abundant species.