

Mutant APH(2'')-IIa Enzymes with Increased Activity against Amikacin and Isepamicin[∇]

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Received 12 October 2009/Returned for modification 22 December 2009/Accepted 28 January 2010

Directed evolution by random PCR mutagenesis of the gene for the aminoglycoside 2''-IIa phosphotransferase generated R92H/D268N and N196D/D268N mutant enzymes, resulting in elevated levels of resistance to amikacin and isepamicin but not to other aminoglycoside antibiotics. Increases in the activities of the mutant phosphotransferases for isepamicin are the result of decreases in K_m values, while improved catalytic efficiency for amikacin is the result of both a decrease in K_m values and an increase in turnover of the antibiotic. Enzymes with R92H, D268N, and D268N single amino acid substitutions did not result in elevated MICs for aminoglycosides.

Since the introduction of streptomycin into clinical use in 1944, aminoglycoside antibiotics have been used for treatment of serious bacterial infections for more than 70 years (8, 14). In response to their extensive use, bacterial isolates acquired resistance to many aminoglycosides, rendering some of them obsolete (15, 19). The major mechanism of resistance to aminoglycoside antibiotics in clinical bacterial isolates is the production of three types of aminoglycoside-modifying enzymes, aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs). The APHs and ANTs are the bisubstrate enzymes that facilitate transfer of the γ -phosphate and nucleotide monophosphate, respectively, from a nucleotide substrate to the hydroxyl groups of aminoglycoside antibiotics, while AACs acetylate amino groups derived from acetyl coenzyme A (acetyl-CoA) (11, 15, 21). Such modifications result in resistance, as the modified aminoglycosides have decreased affinity for their target, the bacterial ribosome (12, 20). Aminoglycoside 2''-phosphotransferases [APH(2'')s] phosphorylate the 2'' hydroxyl of aminoglycoside antibiotics. Four distantly related enzymes of this type, APH(2'')-Ia, -Ib, -Ic, and -Id, sharing between 28 and 32% amino acid sequence identity, have been identified (4–6, 9, 10, 18). Recently, detailed enzymological characterization of these phosphotransferases revealed that contrary to the established dogma, two of these enzymes utilize GTP and not ATP as the nucleotide substrate. Moreover, the aminoglycoside substrate profiles of these enzymes are not identical. These findings resulted in a revised nomenclature for the APH(2'') enzymes (16), whereby the APH(2'')-Ib, -Ic, and -Id enzymes were renamed APH(2'')-IIa, -IIIa, and -IVa, respectively. The APH(2'') enzymes are widely disseminated among Gram-positive enterococcal and staphylococcal isolates. APH(2'')-IIa is the only 2'' aminoglycoside

phosphotransferase that has been identified also in a Gram-negative isolate, *Escherichia coli* (5). While the extensive use of β -lactam antibiotics resulted in the emergence of hundreds of mutant β -lactamases with extended-spectrum activity against β -lactam antibiotics, no mutant derivatives of aminoglycoside-modifying enzymes have been reported in clinical isolates despite the long-term use of aminoglycosides. In this paper we describe PCR-generated mutant derivatives of the APH(2'')-IIa enzyme that produce enhanced levels of resistance to two clinically important aminoglycoside antibiotics, amikacin and isepamicin.

MATERIALS AND METHODS

Random PCR mutagenesis. We utilized the gene for APH(2'')-IIa phosphotransferase cloned between the NdeI and HindIII sites of the pHF022 vector as the template for random PCR mutagenesis. The vector pHF022 was constructed by combining the pBR origin of replication, an ampicillin resistance gene from the pUC19 vector, and the strong constitutive expression promoter of the D-amino acid aminotransferase gene of *Geobacillus toebii* (13). The pBR origin of replication was PCR amplified from the pET24a(+) vector using the oligonucleotide primers oHF014 and oHF016 (Table 1). The gene for the ampicillin antibiotic selection marker was PCR amplified from the pUC19 vector using the primers oHF021 and oHF022 (Table 1). These two fragments were digested with EcoRV and PstI and ligated together to generate the plasmid pHF018. A DNA fragment encoding the constitutive promoter of the D-amino acid aminotransferase gene, a multicloning site, and the termination signal from the T2 element of the *Escherichia coli rmb* gene was custom synthesized (Celtek Genes). PstI sites were included at the 5' and 3' ends of this synthetic DNA fragment. Following digestion with PstI, the synthetic fragment was cloned into the unique PstI site of pHF018, resulting in the plasmid pHF022. The sequence of the entire vector was verified by DNA sequencing. Random PCR mutagenesis of the gene for the APH(2'')-IIa was performed with the GeneMorph II random mutagenesis kit (Stratagene catalog no. 200550) according to the manufacturer's recommendations, utilizing conditions that generate a low mutation rate (0 to 4 mutations per target gene). Briefly, the gene for APH(2'')-IIa in the pHF022 vector was amplified by PCR using Mutazyme II DNA polymerase and two oligonucleotide primers, IIA-PCR-D and IIA-PCR-R (Table 1). We performed a total of three PCRs, using 19, 22, and 25 cycles, respectively. Following this, the PCR products in the three tubes were combined and fragments generated by PCR were separated on a 1% agarose gel and purified with Wizard SV Gel and the PCR Clean-Up system (Promega). Purified DNA fragments were digested with the NdeI and HindIII restriction endonucleases, repurified with Wizard SV Gel and the PCR Clean-Up system, and cloned between the NdeI and HindIII sites of the pHF022 vector. Purified plasmid DNA of the pHF022 vector containing the

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[∇] Published ahead of print on 9 February 2010.

TABLE 1. Primers used in this study

Primer	Sequence ^a
oHF014.....	GCGGATATCCCCGTAGAAAAGATCAAAGG
oHF016.....	GTATCTGCAGAGCGCTGGCATTGACCCTG
oHF021.....	GTTTCTGCAGCTAAATACATTCAAATATGTATCC
oHF022.....	CTTTTCGATATCGTCTGACGCTCAGTGGAAACG
IlaPCR-D.....	CTGGA AAAAGGAGATATACCATATG
IlaPCR-R.....	GAGTGC GGCCGAAGCTT

^a Underlined are sites for the following restriction endonucleases: EcoRV (GATATC), PstI (CTGCAG), NdeI (CATATG), and HindIII (AAGCTT).

mutagenized gene for APH(2'')-IIa was used to transform electrocompetent *E. coli* NEB 5-alpha cells (NEB catalog no. C2989K). After overnight incubation in LB broth supplemented with 100 µg/ml of ampicillin (selection marker for the pHF022 vector), the bacterial culture was diluted 500-fold into fresh LB broth supplemented with amikacin or isepamicin at various concentrations. Bacteria were incubated overnight at 37°C, and plasmid DNA was isolated from bacterial cultures that had grown at the highest concentration of each antibiotic. These DNA samples were used to transform chemically competent *E. coli* JM83 cells. Transformed cells were plated on LB agar supplemented with 100 µg/ml of ampicillin, and plates were incubated overnight at 37°C. Resulting individual colonies were picked for testing susceptibility to amikacin and isepamicin and subsequent DNA sequencing of the gene for the APH(2'')-IIa phosphotransferase. Since only *E. coli* double mutants with increased resistance to amikacin and isepamicin were isolated, *E. coli* strains containing single mutations in the *aph(2'')-IIa* gene were constructed by swapping the fragments of the double mutant and wild-type genes cloned between the NdeI and HindIII sites of the pHF022 vector. Separation of the individual mutations was possible because of the existence of the unique BstEII restriction endonuclease site between the triplets encoding H92 and N268 and D196 and N268 in the double mutant *aph(2'')-IIa* genes. The R92H single mutant enzyme was constructed by combining the 649-bp NdeI-BstEII fragment of the gene for the R92H/D268N double mutant enzyme (this fragment encodes the R92H substitution but not the D268N substitution) with the 405-bp BstEII-HindIII fragment of the wild-type gene. The D268N single mutant enzyme was constructed by combining the BstEII-HindIII fragment of the gene for the R92H/D268N double mutant enzyme (this fragment encodes only the D268N substitution) with the NdeI-BstEII fragment of the wild-type gene. Finally, the N196D single mutant enzyme was constructed by combining the NdeI-BstEII fragment of the gene for the N196D/D268N double mutant enzyme (this fragment encodes only the N196D substitution) with the BstEII-HindIII fragment of the wild-type gene.

Antibiotic susceptibility testing. Antibiotic susceptibility profiles of parental *E. coli* JM83, *E. coli* JM83 harboring the pHF022 vector, and *E. coli* JM83 harboring the pHF022 vector with cloned genes for the wild-type and mutant APH(2'')-IIa enzymes were determined by the broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (7). MIC testing was performed in Mueller-Hinton II broth (Becton Dickinson and Company) with a bacterial inoculum of 5×10^5 CFU/ml, and results were recorded after incubation of microtiter plates for 20 h at 35°C. Reported MIC values are the averages of at least three independent experiments.

Purification of the mutant APH(2'')-IIa enzymes. For inducible expression of the wild-type and mutant APH(2'') phosphotransferases, their genes were excised from the pHF022 vector by the restriction endonucleases NdeI and HindIII and recloned into the NdeI and HindIII sites of the pET22b expression vector. Ligation mixtures were used to transform *E. coli* BL21(DE3), and transformants were selected on the LB agar supplemented with 100 µg/ml of ampicillin. For large-scale protein purification, 5 ml of overnight cultures of *E. coli* BL21(DE3) expressing APH(2'')-IIa and the R92H/D268N and N196D/D268N mutant derivatives was inoculated into 500 ml of minimal medium supplemented with 100 µg/ml of ampicillin. Bacteria were incubated at 37°C with agitation until the optical density at 600 nm (OD_{600}) reached ~0.4 and then induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 15°C overnight. Bacterial cells were pelleted by centrifugation ($3,000 \times g$ for 20 min at 10°C), resuspended in 60 ml of buffer A (25 mM HEPES, pH 7.5, 1 mM EDTA, and 0.2 mM dithiothreitol [DTT]), and disrupted by sonication at 4°C. The lysates were centrifuged ($20,000 \times g$ for 30 min at 10°C), and the supernatants were supplemented with 1.5% streptomycin sulfate to precipitate nucleic acids. After 0.5 h of incubation, supernatants were centrifuged ($20,000 \times g$ for 30 min at 10°C) and subsequently dialyzed against 25 mM HEPES, pH 7.5, 200 mM NaCl. Wild-type APH(2'')-IIa and the N196D/D268N mutant derivative were purified by gentamicin-Affigel 10 (Bio-Rad) affinity chromatography. Enzymes were eluted with a

NaCl gradient (0 to 1 M) in 25 mM HEPES, pH 7.5. Fractions were examined by measuring enzyme activity and subsequently by SDS-PAGE. Fractions containing pure enzyme were pooled, concentrated, dialyzed against 25 mM HEPES, pH 7.5, 0.5 mM DTT, and stored at -80°C. The R92H/D268N mutant enzyme was purified similarly except that a kanamycin-Affigel 10 affinity column was used for protein purification with a 0 to 2 M NaCl gradient in 25 mM HEPES, pH 7.5, for protein elution.

Enzyme kinetics. Phosphorylation of aminoglycosides by APH(2'')-IIa and mutant derivatives was monitored with a continuous spectrophotometric assay in the presence of ATP or GTP as the second substrate (17). Kinetic assays were performed in a total volume of 250 µl containing 100 mM HEPES, pH 7.5, 10 mM MgCl₂, 20 mM KCl, 2 mM phosphoenolpyruvate, 140 µM NADH, 15 units/ml pyruvate kinase, and 20 units/ml lactate dehydrogenase. Kinetic parameters for aminoglycoside antibiotics were determined using a fixed concentration of ATP (200 µM) and various concentrations of aminoglycosides. Kinetic parameters for ATP and GTP were determined using kanamycin A at a fixed concentration (10 µM) and ATP or GTP at various concentrations. The reactions were initiated by adding enzyme (5 to 10 nM final concentration). For determination of k_{cat} , K_m , and k_{cat}/K_m for aminoglycoside antibiotics, ATP and GTP data were fit nonlinearly with the Michaelis-Menten equation (equation 1) using the program Prism 5 (GraphPad Software, Inc.).

$$v = \frac{V_{max} [A]}{K_a + [A]} \quad (1)$$

where V_{max} represents the maximum velocity, $[A]$ is the concentration of the variable substrate, and K_a is the Michaelis constant.

RESULTS AND DISCUSSION

Susceptibility profile of *E. coli* JM83 producing APH(2'')-IIa and mutant derivatives. The only *E. coli* strain isolated that exhibited increased resistance to amikacin and isepamicin contained double amino acid substitutions in APH(2'')-IIa; no resistant *E. coli* strain contained single amino acid substitutions. The R92H/D268N and N196D/D268N double mutant enzymes produce an 8-fold increase in the MICs for both amikacin and isepamicin but not for any other 4,6-disubstituted antibiotics (kanamycin A, kanamycin B, gentamicin, tobramycin, dibekacin, netilmicin, sisomicin, or G418) against the parental *E. coli* strain (Table 2). Mutants containing single amino acid substitutions were constructed to confirm that these single mutants do not result in higher levels of aminoglycoside resistance. Antibiotic susceptibility testing demonstrated that the single R92H, N196D, or D268N substitution in the APH(2'')-IIa enzyme does not significantly change the susceptibility of *E. coli* JM83 to any of the aminoglycoside antibiotics tested (Table 2).

The level of resistance to aminoglycoside antibiotics is influenced by many factors, including the amount of modifying enzyme produced (this, in turn, depends on the gene copy number, promoter strength, and enzyme stability) and the rate of penetration of the antibiotic into the bacterial cell. The mutant APH(2'')-IIa enzymes described in this study elevate MIC values of amikacin and isepamicin from 2 to 16 µg/ml when the enzymes are expressed in an *E. coli* JM83 background. APH(2'')-IIa originated in enterococci, whose facultative anaerobic metabolism significantly lowers the rate of uptake of all aminoglycosides, thus producing intrinsic resistance to these antibiotics at MIC levels ranging from 4 to 256 µg/ml. According to CLSI recommendations, enterococci with gentamicin MICs of ≤ 500 µg/ml are still considered susceptible, as gentamicin can successfully be used against such isolates in combination with cell-wall-active antibiotics such as β-lactams and vancomycin. Acquisition by enterococci of aminoglyco-

TABLE 2. Antimicrobial susceptibilities of *E. coli* JM83 producing APH(2'')-IIa and its mutant derivatives

Enzyme	MIC ($\mu\text{g/ml}$) of drug ^a :									
	AMK	ISE	KANA	KANB	GEN	TOB	DBK	NET	SIS	G418
APH(2'')-IIa	2	2	256	64	16	64	64	8	8	512
N196D/D268N	16	16	256	64	16	64	64	8	4	512
R92H/D268N	16	16	256	64	16	64	64	8	8	512
R92H	2	4	256	64	16	64	64	16	8	512
N196D	2	2	256	64	16	64	64	8	8	512
D268N	2	2	256	64	16	32	32	4	4	512
Control ^b	2	2	2	1	0.25	1	0.5	0.25	0.25	2

^a AMK, amikacin; ISE, isepamicin; KANA, kanamycin A; KANB, kanamycin B; GEN, gentamicin; TOB, tobramycin; DBK, dibekacin; NET, netilmicin; SIS, sisomicin.

^b *E. coli* JM83 harboring the pHF022 vector.

side-modifying enzymes results in a significant increase in resistance to aminoglycoside antibiotics. Thus, the clinical isolate *Enterococcus faecium* SF11770, in which the gene encoding the APH(2'')-IIa enzyme was originally identified, produces resistance to amikacin and isepamicin at 512 $\mu\text{g/ml}$ while the MICs of other aminoglycoside antibiotics are higher than 2,000 $\mu\text{g/ml}$. Thus, one would expect that the appearance of R92H/D268N or N196D/D268N mutant enzymes in clinical enterococcal isolates would produce even higher levels of resistance to amikacin and isepamicin.

Biochemical characterization of the R92H/D268N and N196D/D268N mutant enzymes. Both double mutant enzymes were purified to homogeneity by single-step affinity chromatography. The kinetic parameters for APH(2'')-IIa and the N196D/D268N and R92H/D268N mutant derivatives are presented in Table 3. The wild-type enzyme and the double mutant derivatives can utilize both ATP and GTP as phosphate donors, although ATP is a slightly better substrate than GTP. APH(2'')-IIa turns over ATP 4.6-fold faster than GTP and has a 4.3-fold lower K_m value for ATP than for GTP. The N196D/D268N mutant enzyme has very similar k_{cat} values for the two nucleoside triphosphates (NTPs) while the R92H/D268N mutant has almost identical K_m values for ATP and GTP but turns over ATP two times more efficiently than GTP. It is expected that the wild-type and both double mutant enzymes would experience saturation with either of these substrates *in vivo*, as the concentration of ATP and GTP inside the cell is well above their K_m values (estimated concentrations of ATP and GTP in *E. coli* are 3,560 and 1,160 μM , respectively) (3). In agreement with the antibiotic susceptibility profiles, APH(2'')-IIa and both mutant derivatives efficiently phosphorylate kanamycin A, to-

bramycin, and dibekacin with k_{cat}/K_m values above $1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. All three enzymes are characterized by very low ($\leq 2.1 \mu\text{M}$) K_m values for these aminoglycosides, implying that the enzyme affinities for kanamycin A, tobramycin, and dibekacin are very high. High binding affinities would allow the enzymes to bind and phosphorylate incoming antibiotics when their concentration in the cells is still very low, below the levels resulting in irreversible impairment of protein synthesis. The catalytic efficiencies of APH(2'')-IIa for isepamicin and amikacin are significantly lower (k_{cat}/K_m values of 4.4×10^5 and $1.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively) than for all other 4,6-disubstituted aminoglycosides tested. These lower catalytic efficiencies are the result of both higher K_m values (62.9 μM for amikacin and 13.5 μM for isepamicin) and lower turnover numbers for these two antibiotics. The catalytic efficiencies of the N196D/D268N and R92H/D268N mutant enzymes are increased 7- to 8-fold for amikacin and around 5-fold for isepamicin in comparison to the wild-type enzyme. Increase in the catalytic efficiency of mutant enzymes for amikacin is the result of a moderate decrease in K_m values accompanied by an increase in turnover numbers. The improved catalytic efficiency of these enzymes for isepamicin results entirely from a significant decrease in K_m values. In contrast to amikacin, the rate of turnover of isepamicin is slightly decreased for both mutant phosphotransferases.

Substrate binding in the APH(2'')-IIa mutant enzymes. Comparison of the structures of the aminoglycosides used for this study indicates that unlike other antibiotics, amikacin and isepamicin contain a bulky functionality at position 1 of the 2-deoxystreptamine ring (Fig. 1). These functionalities [(*S*)-4-amino-2-hydroxybutyryl in amikacin and (*S*)-3-amino-2-hy-

TABLE 3. Kinetic parameters of APH(2'')-IIa and its mutant derivatives

Substrate ^b	APH(2'')-IIa ^a			N196D/D268N mutant			R92H/D268N mutant		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{ s}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{ s}^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{ s}^{-1}$)
KANA	45.1 \pm 0.4	2.1 \pm 0.1	(2.2 \pm 0.3) $\times 10^7$	13.8 \pm 0.5	<1	>1.4 $\times 10^7$	20.5 \pm 0.5	<1	>2.0 $\times 10^7$
TOB	26.1 \pm 0.5	1.5 \pm 0.2	(1.7 \pm 0.4) $\times 10^7$	16.6 \pm 0.6	<1	>1.7 $\times 10^7$	26.7 \pm 1.5	<1	>2.7 $\times 10^7$
DBK	17.1 \pm 0.1	1.2 \pm 0.1	(1.4 \pm 0.3) $\times 10^7$	16.9 \pm 0.5	<1	>1.7 $\times 10^7$	27.5 \pm 1.2	1.2 \pm 0.2	(2.3 \pm 0.4) $\times 10^7$
ISE	5.7 \pm 0.1	13.5 \pm 1.1	(4.4 \pm 0.6) $\times 10^5$	4.1 \pm 0.1	2.0 \pm 0.2	(2.0 \pm 0.2) $\times 10^6$	2.8 \pm 0.2	1.2 \pm 0.3	(2.3 \pm 0.6) $\times 10^6$
AMK	8.3 \pm 0.5	62.9 \pm 8.3	(1.3 \pm 0.3) $\times 10^5$	13.8 \pm 0.3	13.5 \pm 0.8	(1.0 \pm 0.5) $\times 10^6$	22.3 \pm 0.4	23.7 \pm 1.2	(9.4 \pm 0.5) $\times 10^5$
ATP	43.3 \pm 1.5	16.3 \pm 1.6	(2.7 \pm 0.4) $\times 10^6$	12.1 \pm 0.3	5.6 \pm 0.5	(2.2 \pm 0.2) $\times 10^6$	21.4 \pm 0.5	29.5 \pm 2.7	(7.2 \pm 0.7) $\times 10^5$
GTP	9.4 \pm 0.1	70.0 \pm 2.0	(1.3 \pm 0.1) $\times 10^5$	10.6 \pm 0.3	16.2 \pm 1.5	(6.5 \pm 0.6) $\times 10^5$	8.9 \pm 0.2	28.2 \pm 2.7	(3.2 \pm 0.3) $\times 10^5$

^a Data for the APH(2'')-IIa enzyme were reported previously (14, 15).

^b KANA, kanamycin A; TOB, tobramycin; DBK, dibekacin; ISE, isepamicin; AMK, amikacin.

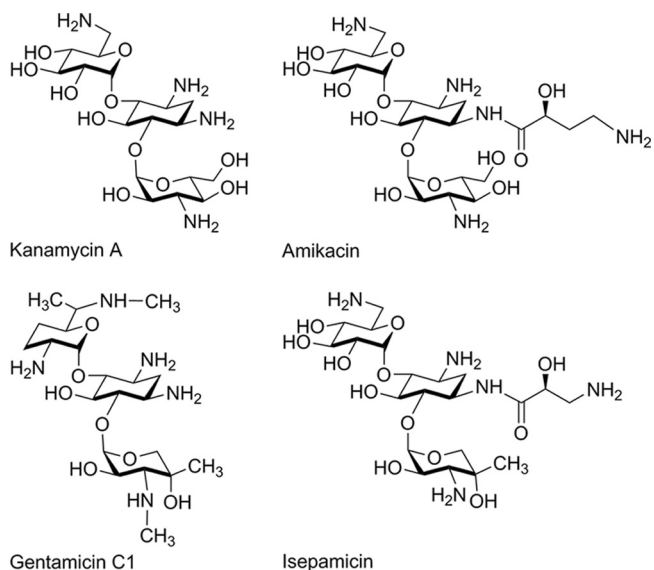


FIG. 1. Structures of some aminoglycoside antibiotics used in this study.

droxypropionyl in isepamicin] are likely to reduce affinity of the APH(2'')-IIa for these antibiotics, resulting in high K_m values. This assumption is supported by the 31-fold-elevated K_m of the enzyme for amikacin in comparison to that for kanamycin A, even though these aminoglycosides are structurally identical except for the functionality at position 1 of the 2-deoxystreptamine ring [an amino group in kanamycin A versus (*S*)-4-amino-2-hydroxybutyryl in amikacin]. Alignment of

the amino acid sequences of the APH(2'') enzymes indicates that R92 is not a conserved amino acid residue in these phosphotransferases (Fig. 2). In contrast, only two amino acid residues are found in position 196 [residue numbering for APH(2'')-IIa]: APH(2'')-Ia and APH(2'')-IIa have asparagine at this position, while the two other enzymes have aspartate (Fig. 2). Thus, the N196D substitution in APH(2'')-IIa results in a negatively charged aspartate residue as found in the APH(2'')-IIIa and -IVa phosphotransferases. Negatively charged residues [aspartate in APH(2'')-IIa or glutamate in APH(2'')-Ia and -IVa] are conserved at position 268. Substitution of asparagine for aspartate at this position produces no significant loss of resistance to aminoglycoside antibiotics, an indication that a negatively charged residue at position 268 is not critical for the ability of the APH(2'') enzymes to produce a resistance phenotype. As the sizes of aspartate and asparagine residues are very similar, it remains to be elucidated if size conservation at this position is important for manifestation of resistance.

Recently, we reported the X-ray structures of the binary complex of APH(2'')-IIa with gentamicin and of the ternary complex with AMPPCP and the inhibitor streptomycin (22). These structures allow us to evaluate the precise location of the mutations within the enzyme's three-dimensional structure. Two of the mutations (N196D and D268N) are within the substrate binding cleft located between the core subdomain and the helical subdomain (Fig. 3a). In the binary gentamicin complex, the side chain of N196 accepts a hydrogen bond from the N1 amine on the central 2-deoxystreptamine ring of the substrate. D268 is on the opposite side of the binding site on the long bent helix ($\alpha 9$) which forms the wall of the cleft. In the gentamicin complex D268 makes no interactions with the sub-

APH(2'')-Ia	MEYRYDDNATNVKAMKYLIEHYFDNFVKVDSIEIIGSGYDSVAYLVNNEYIFKTKFSTNKKKGYAK	65
APH(2'')-IIa	-----MVNLD AEIYEHLNKQIKINELRYLSSGDDSDTFLCNEQYVVKVPRKRSVRISQKR	55
APH(2'')-IIIa	-----MKQNKLYHTTMITMQFPDISIQSVESLGEGRNYAILVNGDWVFRFPKQGGADELNK	58
APH(2'')-IVa	-----MRTYTFDQVEKAI EQLYPDFTINTIEISGEGNDCIAYEINRDFIFKFPKHSRGSNTLNFN	59
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APH(2'')-Ia	EKAIYNFLNTNLETNVKIPNIEYSYIS---DELSILGYKEIKGTFLTPEIYSTMSSEEQNLKRD	127
APH(2'')-IIa	EFELYRFLNCKL-SYQIPAVVYQ-----SDRFNIMKYIKGEIITYEQYHKLSEKEDALAYD	112
APH(2'')-IIIa	EIQLLPLLVG--CVKVNIPQYVYIGKRS--DGNPFVGYRKYVQQLIGEDGMAVFPDADKRLALQ	119
APH(2'')-IVa	EVNILKRIHN--KLPLPIPEVVFTGMPSETYQMSFAGFTKIKGVPLTPLLNNLPKQSQNAAKD	122
	* : : ** : : : * : : : : : :	
APH(2'')-Ia	IASFLRQMGLDYTD---ISECTIDNKQNVLEEYILLRETIYND--LTDIEKDYIESFMER-LN	185
APH(2'')-IIa	EATFLKELHSIEIDCSVSLFSDALVNNKKDKFLQDKLLISILEKEQLLTDEMLEHIETIYENILN	177
APH(2'')-IIIa	LAEFMNELSAFPVET-AISAGVPVTNLKNIKILLSEAVEDQVFP--LDESLRDYLTRFQSYM	181
APH(2'')-IVa	LARFLSELHSINISG---FKSNLVLDFREKINEDNKKIKKLLSRE--LKGPMKKVDDFYRDILE	182
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APH(2'')-Ia	ATTVFEGKKCLCHNDFSCNHLLLDGN---RLTGIIDFGDSGIIDEYCDFIYLLEDSEEEIGTNF	247
APH(2'')-IIa	NAVLFKYTPCLVHNDFSANNMIFR--NN---RLFGVIDFGDFNVGDPDNDFLCLLDCSTDDFGKEF	238
APH(2'')-IIIa	HPVYTRYTPRLIHGDLSPDHFLTNLNSRQTPLTGIIDFGDAASDPDYDYVYLLEDCEG---LF	242
APH(2'')-IVa	NEIYFKYYPCLIHNDFSSDHILFDTEKNT--ICGIIDFGDAASDPDNDFISLMED-DEEYGMF	244
	. * * * * : : : : : : * * * * : * * * : * :	
APH(2'')-Ia	GEDILRMYGNIIEKAKAYQDIVEEYPIETIVYGIKNIKQEFIENGRKEIYKRTYK-----	305
APH(2'')-IIa	GRKVLKYYQHKAPVAERKAELNDVYWSIDQIIYGYERKDREMLIKGVSELQTAEMFIF---	299
APH(2'')-IIIa	TRQVMAYRGEVDLDTIRKVSFLVTFDQVSYLLEGLRARDQDWISEGLELLEEDKANNFGANSA	306
APH(2'')-IVa	VSKILNHYKHKDIPVLEKYRMKEKYWSFEKIIYGYEYGMWYEEGLNEIRSIKIK-----	301
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FIG. 2. Multiple amino acid sequence alignment of the four APH(2'') enzymes. The amino acid residues R92, N196, and D268 are highlighted in gray. Stars indicate fully conserved residues, double dots indicate strongly conserved residues, and single dots indicate weakly conserved residues.

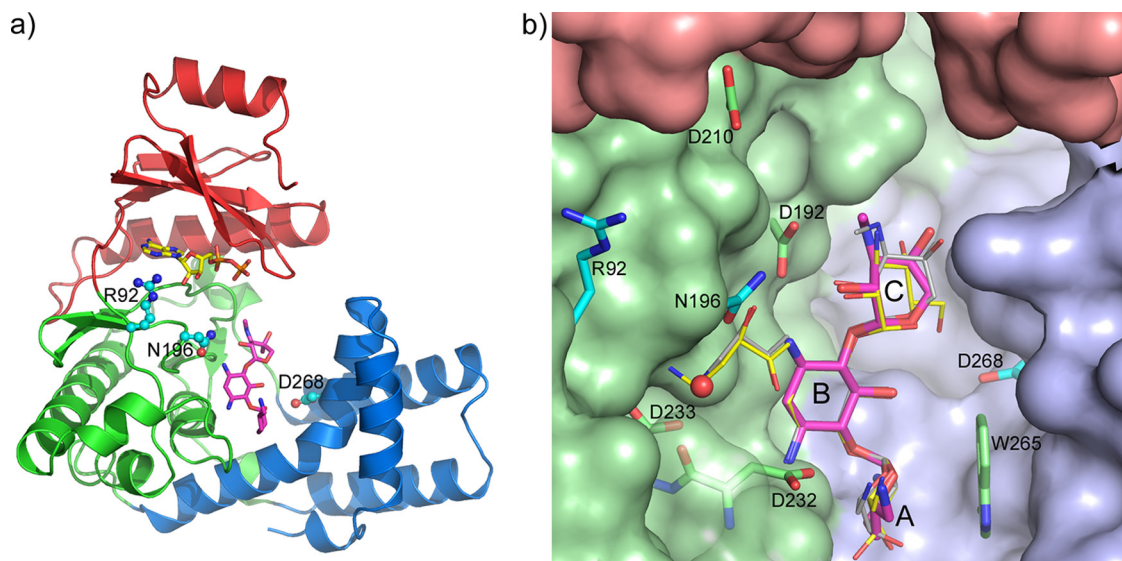


FIG. 3. (a) Cartoon representation of the wild-type APH(2'')-IIa structure showing the three structural domains in red (N-terminal domain), green (core subdomain), and blue (helical subdomain). The nucleotide binding site is indicated by the ADP molecule in yellow sticks (between the N-terminal domain and core subdomain), and the substrate binding site is indicated by the gentamicin in magenta sticks (between the core and helical subdomains). The location of the three mutations (R92, N196, and D268) in the two double mutants (R92H/D268N and N196D/D268N) are indicated by cyan ball-and-stick models. (b) Close-up view of the aminoglycoside binding site in wild-type APH(2'')-IIa, represented as a molecular surface with structural domains colored in the same scheme as in panel a. The side chains for R92, N196, and D268 are shown as cyan sticks, with some of the other important residues shown as green sticks. Gentamicin as observed in wild-type APH(2'')-IIa is given as thick magenta sticks, and the three rings are labeled A, B, and C (the central B ring is the 2-deoxystreptamine ring). Amikacin (thin yellow sticks) and isepamicin (thin gray sticks) are modeled into the binding site such that the three rings of each aminoglycoside overlie the corresponding rings in the gentamicin molecule.

strate. The third mutation, R92, is at the C-terminal end of the linker joining the N-terminal domain and the core subdomain (Fig. 3a). This linker connects to a loop which passes close to the substrate binding site before folding into the subdomain. Modeling of amikacin and isepamicin into the APH(2'')-IIa substrate binding sites (Fig. 3b) allows us to speculate as to the decreased activity toward these substrates in the wild-type enzyme and as to how the point mutations may give rise to increased activity toward these two aminoglycosides. The 2-deoxystreptamine ring (the B ring in Fig. 3b) and the aminoglycan substituent at position 4 (the A ring in Fig. 3b) of gentamicin appear to interact with a highly specific binding platform provided by D228, S230, D232, and D233 from the core subdomain and D262 and W265 from helix α 9 (22). Clearly, were amikacin and isepamicin to bind to wild-type APH(2'')-IIa in the same manner as that of gentamicin, there would be a severe steric clash between the N196 side chain and the bulky hydroxyalkylamide functionalities on the antibiotics (Fig. 3b). The observation that wild-type APH(2'')-IIa is able to turn over these two substrates indicates that they are able to bind in a productive manner, which implies that either the hydroxyalkylamide functionalities or the N196 side chain needs to move away. The difference in K_m between amikacin (62.9 μ M) with an (*S*)-4-amino-2-hydroxybutyryl group and isepamicin (13.5 μ M) with a shorter (*S*)-3-amino-2-hydroxypropionyl tail suggests that this steric effect is less significant when the size of the functionality is decreased by one carbon atom.

In the wild-type APH(2'')-IIa structure, the amide nitrogen of N196 is approximately 3.6 Å from the side chain of the catalytic aspartate D192, which is also hydrogen bonded

to the N1 amide of the 2-deoxystreptamine ring. This region of the binding site is highly negatively charged, with at least three other aspartate residues in close proximity (the 2-deoxystreptamine ring is surrounded by at least three other aspartate residues, D210, D232, and D233). Mutation of residue 196 to aspartate would greatly destabilize this position, and if amikacin or isepamicin were to enter the binding site, this might result in the side chain and/or the entire peptide flipping away from its current position, thus allowing for the binding of the hydroxyalkylamide groups.

The presence of the R92 side chain close to residue 196 is also intriguing. Although it does not form any specific interactions in the wild-type structure, small changes in the conformation of the arginine side chain would bring the guanidinium group within hydrogen bonding distance of the carbonyl oxygen atoms of residues 195 and 196, which may help stabilize the protein in the vicinity of residue 196. Upon mutation of this residue to histidine, which is significantly shorter than arginine, these putative hydrogen bonding interactions would be lost and the hydroxyalkylamide tail groups of an incoming amikacin or isepamicin may be sufficient to force the N196 side chain out of the way. However, this does not explain the role of residue 268, which is the common point of mutation in both of the double mutants. It is difficult to understand why loss of the negative charge at this position, upon going from aspartate to asparagine, has such a profound effect upon enzyme activity in conjunction with either the N196D or the R92H mutation. One intriguing possibility is that amikacin and isepamicin may bind in a completely different manner, essentially a 180° vertical rotation relative to their orientation in Fig. 3b, such that the A

and C aminoglycan rings remain in the same location (with the 2''-OH remaining oriented for efficient attack on the γ -phosphate of the ATP). The 2-deoxystreptamine ring and the hydroxyalkylamide tail would now point into the pocket occupied by residue 268. However, given the narrow shape of the binding cleft and the highly specific A-B ring binding platform, such a rotation of the substrate is rather unlikely and the specific role of the D268N mutation must await further structural studies with these mutants.

Summary. Aminoglycoside antibiotics in combination with a cell wall agent (β -lactam or glycopeptide) remain the therapy of choice for enterococcal endovascular infections (1). A major impediment for successful use of this combination of drugs is the production of aminoglycoside-modifying enzymes. The APH(2'') phosphotransferases, APH(2'')-Ia, -IIa, -IIIa, and -IVa, are of special concern as they produce resistance to gentamicin, an aminoglycoside most commonly used for treatment of enterococcal endocarditis. The aminoglycoside antibiotics amikacin and isepamicin have been suggested as potential alternatives to gentamicin against strains producing APH(2'')-IIa, -IIIa, or -IVa enzymes (4, 6, 18). Kinetics studies with purified enzymes demonstrated that amikacin and isepamicin are not phosphorylated by APH(2'')-IIIa and are poor substrates for APH(2'')-IIa and -IVa (2, 16, 17). Our present study has demonstrated that APH(2'')-IIa is able to produce an 8-fold-increased level of resistance to these clinically important antibiotics in *E. coli* as a result of two amino acid substitutions. It is reasonable to expect that expression of such mutant enzymes in clinical enterococcal isolates notorious for poor aminoglycoside uptake would result in very high levels of resistance to amikacin and isepamicin that would prevent their clinical use. Over the last several decades hundreds of mutant β -lactamases with extended spectra of activity have emerged in various Gram-negative pathogens. These mutant enzymes contain from one to several amino acid substitutions. Our study indicates that mutant APH(2'')-IIa enzymes with improved catalytic efficiency against aminoglycosides that are poor substrates could be generated as a result of only two amino acid substitutions. This raises the question of why such mutants have not yet been reported in clinical enterococcal isolates. It is possible that if such mutant enzymes are already in circulation in the clinical setting, they easily can be overlooked because of the current methodology of aminoglycoside resistance testing in clinical laboratories, where enterococci are screened typically for high-level resistance to gentamicin and streptomycin but not to other aminoglycosides such as amikacin and isepamicin. Another reason that clinical mutants have not been reported may be that the epidemiology of aminoglycoside-modifying enzymes has not been studied nearly as extensively as the epidemiology of β -lactamases. Also, when the identities of aminoglycoside-modifying enzymes are reported in the literature, it is typically confirmed by PCR-based amplification of their genes without subsequent cloning and detailed MIC testing. Our results showing the potential for only two mutations in the *aph(2'')-IIa* gene to result in significantly higher amikacin and isepamicin resistance have implications for the evolution of aminoglycoside resistance not only in Gram-positive organisms but Gram-negative bacteria as well, since *aph(2'')-IIa* has been reported in both enterococci and *E. coli*. Design of new aminoglycosides or inhibitors of aminoglycoside-modifying en-

zymes should take into account the potential for these enzymes to evolve.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (RO1 AI057393).

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