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High-level amikacin resistance in *Pseudomonas aeruginosa* associated with a 3′-phosphotransferase with high affinity for amikacin☆

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Abstract

This work describes the characterization of the phosphotransferase enzymatic activity responsible for amikacin resistance in two clinical *Pseudomona aeruginosa* strains, isolated from a hospital that used amikacin as first-line aminoglycoside. Amikacin-resistant *P. aeruginosa* PA40 and PA43 (MIC: 128 mg/l) were shown to have APH activity with a substrate profile similar to that of APH(3′)-VI. The enzyme from *P. aeruginosa* PA40 was purified to $>70\%$ homogeneity. The K_m of amikacin for this enzyme was 1.4 μM, the V_{max}/K_m ratio for amikacin was higher than for the other aminoglycosides tested and PCR and DNA sequencing ruled out the presence of *aph(3′)-IIps*. Amikacin resistance in this strain was, therefore, associated with APH(3′)-VI and the high affinity of this enzyme for amikacin could explain the high-level resistance that we observed.

Keywords: *Pseudomonas aeruginosa*; Amikacin resistance; APH(3′)-VI; Purification; Kinetics analyses

1. Introduction

The most important mechanism of high-level bacterial resistance to aminoglycosides (AGs) in clinical isolates is enzymatic modification of these drugs. AG-modifying enzymes are classified on the basis of the chemical reaction catalyzed (*N*-acetylation, *O*-nucleotidylation and *O*-phosphorylation) and the specific site of modification on the antibiotic. Aminoglycoside 3′-phosphotransferases [APH(3′)s] catalyze phosphorylation of the hydroxyl group at the 3′ position of the aminohexose I of kanamycin and structurally related antibiotics.

So far, seven different isozymic forms of APH(3′) have been described and can be distinguished on the basis of their in vitro substrate range [1]. Types I, II, III, VI and VII have been detected in human pathogens and types IV and V were present in aminoglycoside-producing microorganisms. Amikacin (AMK) modification in vitro has been described with enzyme preparations containing APH(3′)-III [2], APH(3′)-VII [3,4] or APH(3′)-II [5]. However, these isozymes do not

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confer AMK resistance in bacterial hosts because of poor affinity for this substrate. Nevertheless, the enzyme APH(3′)-VI, which had been described only in *Acinetobacter baumannii* [6–8], *Serratia marcescens* and *Klebsiella pneumoniae* [9] when this work was initiated, confers high-level AMK resistance. APH(3′)-VI was recently also reported in *Pseudomonas aeruginosa* [10,11]. Since APH(3′)-VI had not been studied enzymologically, it was not known whether its kinetic parameters could account for the AMK resistance that it confers.

We have studied two high-level AMK-resistant *P. aeruginosa* clinical strains, which harbour an APH(3′) enzyme with a substrate profile very similar to the type VI enzyme, previously described in other Gram-negative bacilli. We purified this APH(3′) enzyme and carried out kinetic studies to compare it with other APH(3′) enzymes that do not confer AMK resistance in spite of AMK modification that can be demonstrated in vitro. We ruled out the presence of *aph(3′)-IIps*, a chromosomal gene encoding an APH(3′) enzyme that was recently reported in strains of *P. aeruginosa* [12].

2. Methods

2.1. Bacterial strains

The two *P. aeruginosa* strains used in this investigation (*P. aeruginosa* PA40 and PA43) (obtained from G. Miller, Schering-Plough) were originally isolated from patients at the Veterans Administration Hospital of Denver (CO), at a time when antibiotic policy prescribed the use of AMK as the first-line AG for treatment of Gram-negative infections. *P. aeruginosa* 27853 was obtained from American Type Culture Collection (Manassas, VA) and was used as a positive control for the presence of chromosomally-encoded APH(3′)-IIb [12].

2.2. Antibiotics and susceptibility testing

The antibiotics used were: kanamycin (KAN), amikacin (AMK) and lividomycin (LIV) (Bristol-Myers Squibb Co., Princeton, NJ); netilmicin (NET), SCH-21561, SCH-21562 and gentamicin (GEN) C complex and B (Schering-Plough Research, Kenilworth, NJ); tobramycin (TOB) (Eli Lilly and Co., Indianapolis, IN); butirosin (BUT) and paromomycin (PAR) (Parke-Davis and Co., Ann Arbor, MI), ribostamycin (RIB) (Meiji Seika Kaisha, Ltd., Tokyo, Japan); and streptomycin (STR) (Sigma, St. Louis, MO).

MICs were determined on Mueller–Hinton agar plates (Difco, Detroit, MI) by the NCCLS standard agar dilution method [13]. The bacterial inocula were made by preparing appropriate dilutions of overnight broth cultures of organisms in fresh Mueller–Hinton broth (Difco) and these were deposited onto antibiotic-containing plates with a Steers replicator device, yielding a final inoculum of $\approx 10^4$ CFU per spot. Plates were examined for growth after 18–24 h incubation at 37°C. *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 24213 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

2.3. Preparation of sonic extracts

Cells were grown in brain heart infusion (BHI) broth (Difco) for 24 h at 37°C. Cultures were diluted in fresh BHI broth medium (1:50) and the cells were grown for 4 h at 37°C and harvested by centrifugation. Pellets were washed twice with 0.15 M NaCl and then resuspended in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer pH 8.0. Cells were broken by a series of three 30-s sonic disruptions in an ice bath. The disrupted materials were centrifuged at $12\,000 \times g$ for 15 min at 4°C and the supernatants (sonic extracts) were aspirated and kept on ice.

2.4. Detection of AG-modifying enzymes

Aminoglycoside-modifying enzymes were assayed by the phosphocellulose paper binding procedure, as previously described [14] and modified [5]. Radioactive compounds were purchased from Amersham.

Phosphotransferase activity was measured in a solution containing 0.1 M cacodylate buffer (pH 7.0), 7.5 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT) and 0.824 mM [γ-^{32}P]-ATP ($5–10 \times 10^{12}$ cpm/mol). Acetylating activity was measured in a solution containing 0.1 M Tris–maleate (pH 5.7), 10 mM $MgCl_2$, 2.5 mM DTT and 0.825 mM [1-^{14}C] acetyl coenzyme A. Adenyltransferase activity was assayed in a solution containing 0.1 M Tris–HCl (pH 7.8), 7.5 mM $MgCl_2$, 2.5 mM DTT and 0.33 mM [1-^{14}C]ATP. In the three enzymatic determinations for initial screening, the AG (substrate) concentrations used were 167 and 16.7 μM. The incubation conditions were 20 min at 30°C (phosphotransferase) or at 37°C (acetyltransferase and adenyltransferase).

2.5. Purification of APH(3′) enzyme from *P. aeruginosa PA40*

An overnight culture of *P. aeruginosa* PA40 in BHI broth was diluted in fresh broth (1:50) and was grown at 37°C with continuous shaking for 4 h. The total volume obtained was 20 l. Cells were harvested by centrifugation, washed twice with 0.15 M NaCl and centrifuged again. The final pellet was suspended in 0.1 M HBG buffer (0.1 M HEPES buffer, pH 8.0, containing 10% glycerol and 10 mM β-mercaptoethanol) and sonicated on ice. The sonic extract was centrifuged at

18 000 × *g* for 20 min at 4°C and the supernatant was subjected to streptomycin sulphate precipitation to remove nucleic acids. The supernatant was dialyzed against 0.1 M HBG buffer. The dialysate was concentrated in an Amicon micro-concentration system (Amicon Corp., Lexington, MA) and the sample was then loaded onto a Sephadex G75 chromatography column. Elution from the Sephadex G75 column was performed with the 0.1 M HBG buffer at 4°C and fractions of 1 ml were collected. Those with AG phosphotransferase activity were pooled and then applied to a kanamycin-CH-Sepharose-4B affinity chromatography column, that was prepared according to the recommendation of the manufacturer (Pharmacia). The sample was eluted with a linear gradient of NaCl (0.1–1 M) in 0.1 M HBG buffer and fractions with AG phosphotransferase activity were pooled and dialyzed against 0.1 M HBG buffer. The degree of purification achieved in each step was assessed by comparing the specific activity (cpm/min × mg/protein).

To determine the purity of the final AG phosphotransferase preparation, samples were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide, 2.7% bisacrylamide) with the method of Laemmli [15]. Protein concentration was determined by the method of Lowry et al. [16].

2.6. Enzyme kinetics measurements

To determine kinetics for the purified enzyme preparation, the standard phosphotransferase assay was modified to obtain initial rates by spotting samples at 15, 30, 45, 60, 90, 120 and 150 s.

Kinetics constants for the phosphotransferase from *P. aeruginosa* PA40 were determined by fixing the concentration of ATP at 0.4 mM and varying the substrate (AG) concentration. K_m and V_{max} values were obtained by using the Lineweaver–Burk double reciprocal plot. K_m and V_{max} values for ATP were determined by using a constant 0.1 mM concentration of AMK as the substrate. Negative controls contained no enzyme. All assays were performed at least in duplicate.

2.7. Southern hybridization

For Southern hybridization, total DNA from *P. aeruginosa* strains PA40 and PA43 was isolated [17] and digested in separate reactions with *Bam*HI, *Eco*RI and *Hin*dIII. Digested DNAs were separated by electrophoresis in 0.8% agarose in 1 × TAE [18]. DNAs in the gel were transferred to Zeta-Probe GT nylon membrane (BioRad, Hercules, CA) in alkali, according to the recommendations of the manufacturer. A 0.8-kb PCR fragment, including the entire coding region for the *aphA-6* gene of *A. baumannii* strain BM2580 (kindly provided by Patrice Courvalin), was conjugated with a thermostable alkaline phosphatase using a kit (AlkPhos Direct labelling and detection kit, Amersham, Arlington Heights, IL). Prehybridization and hybridization were carried out at 60°C for 30 min and 18 h, respectively, in the hybridization solution provided with the kit. After hybridization, washes were as follows. Two 15-min washes at 60°C were in 2 M urea/0.1% SDS/50 mM sodium phosphate, pH 7.0/150 mM NaCl/10 mM $MgCl_2$; these were followed by two 10-min washes in 50 mM Tris–HCl, pH 10.0/100 mM NaCl/2 mM $MgCl_2$ at room temperature. At this point, the membrane was exposed to CDP-Star™ (Amersham) for 5 min. The drained membrane was wrapped in plastic wrap and exposed to BioMax MS X-ray film (Kodak, Rochester, NY).

2.8. PCR and automated DNA sequencing

For amplification of the gene encoding APH(3′)-VI, PCR reactions contained the following primers: APH(3′)-VI-1, 5′-GACGACGACAAGGATATGGAATTGCCCAATATTATT-3′, and APH(3′)-VI-2, 5′-GGAACAAGACCCGTTCAATTCAATTCATCAAGTTT-3′. Reactions were carried out in a PTC-100 thermal cycler (MJ Research, Watertown, MA) and included 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 0.001% (w/v) gelatin, 0.2 mM dNTPs and 100 ng total DNA from *P. aeruginosa* PA40 (and also, in parallel experiments, with DNA from PA43), in a total reaction volume of 50 μl. After a 5-min denaturation step at 95°C, the reactions continued with 35 cycles of 1 min at 94°C, 1 min at 44°C and 2 min at 72°C. This was followed by a final extension step at 72°C for 5 min. PCR products were visualized by agarose gel electrophoresis and fragments were purified from low-melt agarose using a Wizard PCR prep kit (Promega, Madison, WI).

Primers for amplification of *aph(3′)-IIps* gene were: APHIIb-1 (5′-ATG CAT GAT GCA GCC ACC TCC AT-3′) and APHIIb-4 (5′-CCT ACT CTA GAA GAA CTC GTC CA-3′). As a positive control, these primers were used to amplify the corresponding 767 bp fragment from the *aph(3′)-IIps* gene of *P. aeruginosa* 27853 [12]. In addition, as a general control for *P. aeruginosa*, amplification of a 431-bp fragment of the chromosomally encoded single-copy gene, *radA*, from this organism was used. For this, the following primers were employed: Rad-1f, 5′-TGA TGG TNA TNG ACT CGA TCC-3′ and Rad-2R, 5′-ATN GCC GAA GGC TTG CTC ACT TC-3′ (Howell et al. *P. aeruginosa* oxidative stress operon, unpublished; Genbank Accession No. U89384). PCR was carried out as above, but the annealing temperatures of reactions were at 45 and 54°C.

Purified PCR fragments were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), sequenced

using a dRhodamine terminator cycle sequencing kit (ABI/Perkin-Elmer, Foster City, CA) and analyzed on a Genetic 310 Analyzer (ABI/Perkin-Elmer).

3. Results

3.1. Strains and antibiotic susceptibility pattern

The clinical strains *P. aeruginosa* PA40 and PA43 were speciated by oxidase test, pigment production and the biochemical pattern shown by the API 20 NF system (BioMerieux, La Balme Les Grottes, France). The AG MICs for *P. aeruginosa* PA40 and PA43 are shown in Table 1. Both strains were highly resistant to amikacin (128 mg/l).

Table 1
Aminoglycoside susceptibility pattern of *P. aeruginosa* strains

Aminoglycoside	MIC (mg/l)	
	P. aeruginosa (PA40)	*P. aeruginosa* (PA43)
Amikacin	128	128
Gentamicin	>32	>32
Tobramycin	16	8
Netilmicin	64	32
Kanamycin	512	512
SCH-21561[a]	64	128
SCH-21562[a]	>512	>512
Butirosin	>512	>512
Lividomycin	512	512

[a] Data supplied by G. Miller. Microtiter MIC was determined with Mueller–Hinton broth with calcium and magnesium supplementation. SCH-21561: 2′-*N*-ethyl-netilmicin; SCH-21562: 6′-ethyl-netilmicin.

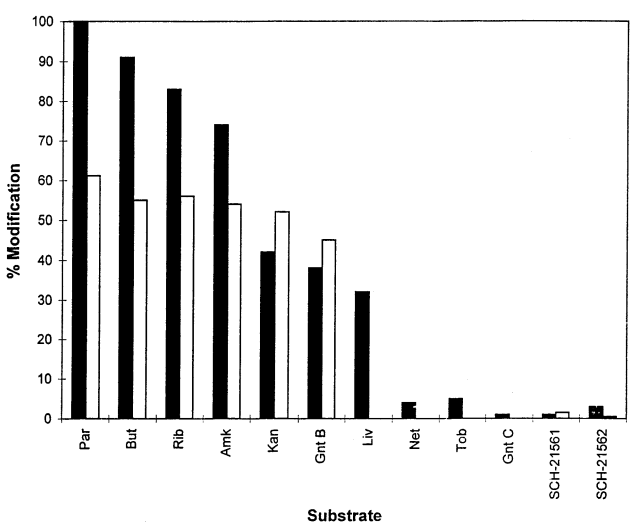

Fig. 1. Substrate profile of phosphotransferase enzymatic activity from crude sonic extract of *P. aeruginosa* PA40 using two aminoglycoside concentrations: 167 μM (■) and 16.7 μM (□). Phosphorylation is expressed relative to paromomycin (167 μM), defined as 100%. Abbreviations: PAR, paromomycin; BUT, butirosin; RIB, ribostamycin; AMK, amikacin; KAN, kanamycin A; GEN, gentamicin B; LIV, lividomycin A; NET, netilmicin; SCH-21561, 2′-*N* ethyl netilmicin; SCH-21562, 6′-*N* ethyl netilmicin; TOB, tobramycin. The substrate profile of *P. aeruginosa* PA43 was almost identical.

3.2. AG-modifying enzymes

Sonic extracts from *P. aeruginosa* PA40 and PA43 were assayed to detect the presence of AG-modifying enzymatic activities and the results were very similar in both strains. They had phosphotransferase and acetyltransferase enzymatic activities, but no nucleotidyltransferase activity was observed. The substrate profile of the acetyltransferase did not include amikacin and was compatible with an AG 2′-acetyltransferase. At the lower concentration tested (16.7 μM), modification of lividomycin was up to 40% that of netilmicin and ≈60% that of paromomycin, whereas modification of amikacin was undetectable.

Fig. 1 shows the substrate profile of the phosphotransferase enzymatic activity from *P. aeruginosa* PA40. For this initial screening, two different substrate concentrations were used (167 and 16.7 μM) for each AG, in order to detect possible substrate inhibition. Results are expressed with respect to the modification of 167 μM of PAR (100%). Substrate modification higher than 40% was observed with 16.7 μM of PAR, BUT, RIB, AMK, KAN and GEN B. AMK phosphorylation was even higher than that observed for KAN and very close to that of PAR, BUT and RIB. Substrate inhibition was detected with GEN B and KAN. Lividomycin modification was low (28%) when a high substrate concentration (167 μM) was used and it could not be detected with 16.7 μM. The substrate profile from the sonic extract of *P. aeruginosa* PA40 (and from *P. aeruginosa* PA43) was similar to that of an APH(3′)-VI enzyme, with high-level modification of AMK even at low AMK concentrations [6].

3.3. Purification of the APH(3′) activity from P. aeruginosa PA40

Elution from the Sephadex G75 column gave a 10.4-fold purification of the APH(3′) enzyme. A further 129-fold purification was obtained by Kanamycin-Sepharose-4B activated affinity chromatography, to a purity of ≈70%. Acetyltransferase activity, that had been observed with KAN in the sonic extract from PA40, was not detected in the purified preparation.

The molecular weight of the APH(3′) enzyme estimated by SDS-PAGE was ≈29 kDa.

3.4. Kinetics analyses of APH(3′) from P. aeruginosa PA40

The K_m, V_{max} and the ratio V_{max}/K_m were determined for several substrates with the partially purified

Table 2
Kinetics parameters of the partially purified APH(3′) activity from *P. aeruginosa* PA40

Aminoglycoside	K_m (μM)	V_{max}[a]	V_{max}/K_m
Amikacin	1.4	21.2	15.17
Paromomycin	3.8	24.9	6.44
Ribostamycin	1.2	16.8	14.02
Butirosin	1.6	13.2	8.20
Kanamycin	2–3.5[b]	N.D.[c]	N.D.
ATP	155.0	28.6	0.18

[a] Total of nmoles of substrate phosphorylated per minute per miligram of protein.
[b] Apparent K_m; determination was complicated by strong substrate inhibition.
[c] Not done.

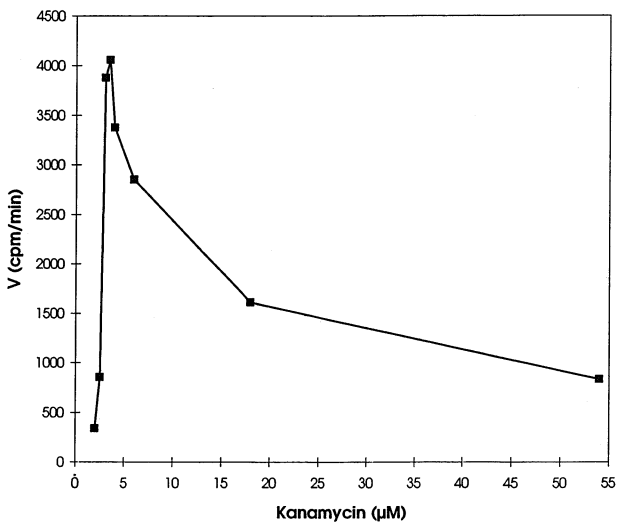

Fig. 2. Effect of kanamycin concentration (μM) on the enzymatic activity (cpm/min) of the purified APH (3′) enzyme from *P. aeruginosa* PA40.

APH(3′) enzyme from *P. aeruginosa* PA40 and are shown in Table 2. The K_m for AMK was in the same range (1–2 μM) as those of RIB and BUT and even lower than that of PAR. The V_{max}/K_m ratio of AMK was higher than that observed for the other AGs.

APH(3′) activity displayed substrate inhibition for each AG substrate tested, although the lowest concentration at which inhibition was observed differed between substrates. The strongest substrate inhibition was detected when KAN was used as substrate. Fig. 2 shows the relation between the enzymatic activity (cpm/min) and the KAN concentration (μM). An increase of 1.5 μM in KAN concentration (from 2 to 3.5 μM) produced a 12-fold increase in enzymatic activity. Strong substrate inhibition was observed for KAN concentrations over 3.5 μM. This inhibition prevented determination of kinetics parameters for KAN, but it was estimated that the K_m for this substrate must be in the range of 2–3.5 μM. This was subsequently confirmed by a coupled spectrofluorometric assay [19]. Strong substrate inhibition was also observed with GEN B for substrate concentrations over 6 μM; this prevented the determination of the kinetics parameters for this AG substrate.

Kinetics determinations carried out with lividomycin showed a different pattern, with very low affinity of the enzyme for this substrate. Only with lividomycin concentrations over 133 μM was even low enzymatic activity observed and a modest 4-fold increase was detected when its concentration reached 416 μM.

Amplification with primers specific for *aph(3′)-IIps* was negative for strains PA40 and PA43, while a positive result was obtained for *P. aeruginosa* 27853; PCR with primers for the single copy *radA* gene produced the expected 431-bp DNA fragment for all three pseudomonad strains.

3.5. PCR and Southern hybridization

PCR from genomic DNA of *P. aeruginosa* PA40 or PA43 yielded an $\approx$800 bp fragment, which was the same size as the PCR product including the entire coding region for the *aphA-6* gene of *A. baumannii* strain BM2580. When this latter fragment was used as the probe, it detected strong hybridization to both the PCR product from PA40 and the control from *A. baumannii* strain BM2580; in addition, a band of hybridization was also observed with the total DNA of PA40 digested with three different restriction enzymes.

3.6. DNA sequence analyses

DNA sequence analysis of the cloned PCR fragments from *A. baumannii* strain BM2580 and from PA40 yielded essentially the same nucleotide sequence as published [8], with no changes observed that affected the predicted amino acid sequences for the respective proteins (data not shown).

4. Discussion

The two high-level AMK-resistant *P. aeruginosa* strains in this study were isolated in 1984 from a hospital th at employed AMK as its primary AG [10]. Both strains had an identical susceptibility profile for all antibiotics tested. In the same hospital, Jacoby et al. [20] described the appearance of Gram-negative pathogens with AMK resistance due to a new ANT(4′)-II enzyme. The appearance of novel AMK resistance due to an APH(3′) enzyme in enteric Gram-negative bacilli was seen in 1984 at a hospital with high AMK usage [9].

Although most hospitals have not experienced an increase in AMK resistance with primary amikacin usage [21–23], several hospitals have reported an increase in AMK resistance related to AMK use [24–26].

Both AMK-resistant *P. aeruginosa* strains in this report had an identical susceptibility profile for all antibiotics tested. Therefore, the APH activity from only one strain was purified and characterized. The substrate profile shown by this phosphotransferase enzyme was compatible with an APH(3′) enzyme with a high affinity for AMK. It was very similar to the substrate profile of the APH(3')-VI enzyme documented in *A. baumannii* [6] and described in amikacin-resistant *S. marcescens* and *K. pneumoniae* strains [9] At the time *P. aeruginosa* strains PA40 and PA43 were isolated (in 1984), there had been no other reports of APH(3′)-VI-like enzymes in pseudomonads. The gene for this enzyme has since been found to reside on a transposon (Tn*1528*) in a *Providencia stuartii* strain; the dissemination of this gene via transposition can occur in *E. coli*, *Proteus mirabilis*, *Morganella morganii*, *P. stuartii* and *P. aeruginosa* [10]. On the other hand, in one survey [11] of *Pseudomonas* spp. in the US from 1987 to 1988, no more than 4% of isolates appeared to contain APH(3′)-VI. In the current study, results from DNA hybridization, PCR amplification and DNA sequence analysis showed that the APH in *P. aeruginosa* PA40 is identical to the APH(3′)-VI from *A. baumannii* BM2580. Furthermore, we ruled out the presence of *aph(3′)-IIps* in total cellular DNA, so the recently reported APH(3′)-IIb enzyme [12] could not have contaminated our purified enzyme preparation.

Most studies with initial characterization of AG-modifying activities use a single high AG concentration to obtain the substrate profile. The use of both a high and a low concentration (167 and 16.7 μM) for each aminoglycoside substrate in the screening phase of this study allowed us to detect substrate inhibition (with GEN B and KAN) and provided evidence suggesting that the affinity of the enzyme was poor for one of the substrates (lividomycin) against which activity was demonstrated. Thus, although the utilization of just a single high concentration may in some cases permit comparison of maximal activities among the various substrates, the activities at lower substrate concentrations that are likely to be closer to the concentrations that the enzyme would confront in vivo are probably more relevant biologically. Moreover, the use of just the higher concentration might miss the significance of activity against a substrate for which there is substrate inhibition, such as GEN BUT or KAN, whereas testing also with a lower substrate concentration could detect it. The substrate inhibition which was also observed with the other substrates in kinetics studies of the partially purified enzyme was not detected with the two substrate concentrations that we used for screening, since it was of little consequence at those concentrations. The use of more than one substrate concentration appeared beneficial in preliminary studies, which suggested that low affinity of AG 3′-phosphotransferase-II for AMK [5,27] and low affinity of AG 2″-nucleotidyltransferase for NET [28] could explain why modification of AMK or NET by these respective enzymes in vitro was not reflected in resistance conferred in vivo. The use of more than one substrate concentration in the preliminary studies of an enzyme has also been recommended by other authors [29,30].

The purification process provided a partially purified APH(3′) enzyme ($>70\%$ purity). The Kan-Sepharose 4B affinity chromatography allowed the separation of the two AG-modifying activities present in the crude extract. Affinity chromatography has also been the most important step of purification of other AG-modifying enzymes [5,28].

Substrate inhibition was observed with all of the AG substrates, but it appeared at different concentrations for the various substrates. This inhibition was greater with GEN B and with KAN at concentrations above 6 and 3.5 μM, respectively. Such inhibition was also detected in the assays with sonic extracts. Substrate inhibition has been described for GEN and TOB with ANT (2″) [29,30] and for KAN and GEN with AAC(3)-V [31]. Using unpurified enzymes, Bongaerts and Vliegenthart [28] detected substrate inhibition for neomycin with ANT(4′)(4″) and for KAN, GEN and TOB with APH(2″).

Kinetics parameters determined for AMK with the partially purified APH(3′) from *P. aeruginosa* PA40, likely explain the observed high-level AMK resistance. The K_m of the enzyme for AMK was very low (1.4 μM), especially when compared with that (720 μM) determined for the enzyme APH(3′)-II [5], which normally does not confer AMK resistance. The high affinity for AMK of the APH(3′) enzyme from *P. aeruginosa* probably accounts for the high level of AMK resistance. The relationships between the MIC levels compared with the kinetics parameters, such as the K_m [32], the catalytic efficiency (V_{max}/K_m) [29] or both [28], have been documented previously.

The appearance of a 3′ phosphotransferase that inactivates AMK efficiently raises concern about the potential proliferation and dissemination of such resistance. As long as AMK remains one of the most reliable antibiotics to be used in severe *P. aeruginosa* infections, the presence of strains with high-level AMK resistance should be carefully monitored in clinical microbiology laboratories.

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