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Dissecting the cosubstrate structure requirements of the *Staphylococcus aureus* aminoglycoside resistance enzyme ANT(4′)

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ABSTRACT

Aminoglycosides are important antibiotics used against a wide range of pathogens. As a mechanism of defense, bacteria have evolved enzymes able to inactivate these drugs by regio-selectively adding a variety of functionalities (acetyl, phospho, and nucelotidyl groups) to their scaffolds. The aminoglycoside nucleotidyltransferase ANT(4′) is one of the most prevalent and unique modifying-enzymes. Here, by TLC, HRMS, and colorimetric assays, we demonstrate that the resistance enzyme ANT(4′) from *Staphylococcus aureus* is highly substrate and cosubstrate promiscuous. We show that deoxy-ribonucleotide triphosphates (dNTPs) are better cosubstrates than NTPs. We demonstrate that the position of the triphosphate group (5′ and not 3′) on the ribose/deoxyribose ring is important for recognition by ANT(4′), and that NTPs with larger substituents at the 3′-position of the ribose ring are not cosubstrates for ANT(4′). We confirm that for all aminoglycosides tested, the respective nucleotidylated products are completely inactive. These results provide valuable insights into the development of strategies to combat the ever-growing bacterial resistance problem.

1. Introduction

Aminoglycosides are broad-spectrum antibiotics used in the treatment of serious bacterial infections (Fig. 1A) [1]. The most common mechanism of resistance to this class of drugs is their inactivation by covalent chemical modifications by the aminoglycoside-modifying enzymes (AMEs) phospho- (APHs), acetyl- (AACs), and nucleotidyltransferases (ANTs) (Fig. 1B) [2]. Although they constitute the smallest family of AMEs, the ANTs are of great clinical relevance as they are widely distributed in both Gram-negative and Gram-positive drug-resistant pathogens.

The four classes of ANTs identified so far (ANT(2″), ANT(3″), ANT(6), and ANT(4′)) have been divided according to the regiospecificity of their site of modification on the parent aminoglycosides that they inactivate. ANT(2″) enzymes have been found in various Gram-negative bacteria including *Klebsiella pneumoniae* [3], *Escherichia coli* [4,5], and *Pseudomonas aeruginosa* [6], whereas an ANT(3″) has been identified as part of a bifunctional enzyme ANT(3″)-Ii/AAC(6′)-IId from *Serratia marcescens* [7]. ANT(3″) was shown to adenylate not only the 3″-position of streptomycin, but also the 9′-hydroxyl group of spectinomycin. A streptomycin adenylyltransferase from *E. coli* was also found to adenylate the 3″- and 6′-positions of streptomycin [8]. Recently, an ANT(6)-Ib was discovered in a streptomycin-resistant *Campylobacter fetus* subsp. *fetus* [9]. One of the most prevalent and unique nucleotidyltransferase enzymes, ANT(4′), has been isolated from both Gram-positive and Gram-negative bacteria. The ANT(4′) from the Gram-positive *Staphylococcus epidermidis* was found to adenylate the equatorial 4″-hydroxyl of dibekacin, which lacks a 4′-hydroxyl target, but not the equivalent hydroxyl of sisomicin or gentamicin, also lacking a 4′-hydroxyl moiety [10]. However, the ANT(4′) from the Gram-negative *P. aeruginosa* failed to modify the 4″-hydroxyl of both dibekacin and gentamicin [11,12]. The most studied *Staphylococcus aureus* ANT(4′) [13,14] has also been found to adenylate a variety of aminoglycosides including dibekacin [15], and to bind a number of nucleotide triphosphate cosubstrates [16].

In light of its clinical significance and its already partially established substrate and cosubstrate profile [15,16], we decided to perform an in-depth functional analysis of the promiscuous *S. aureus* ANT(4′). More specifically, we aimed to dissect the cosubstrate structure requirements of this enzyme. A detailed knowledge of the properties of this enzyme will help us better understand the mechanisms by which Nature deactivates the currently used aminoglycoside antibiotics. It will also be useful for guiding the design of the next generation of drugs as well as the development of novel

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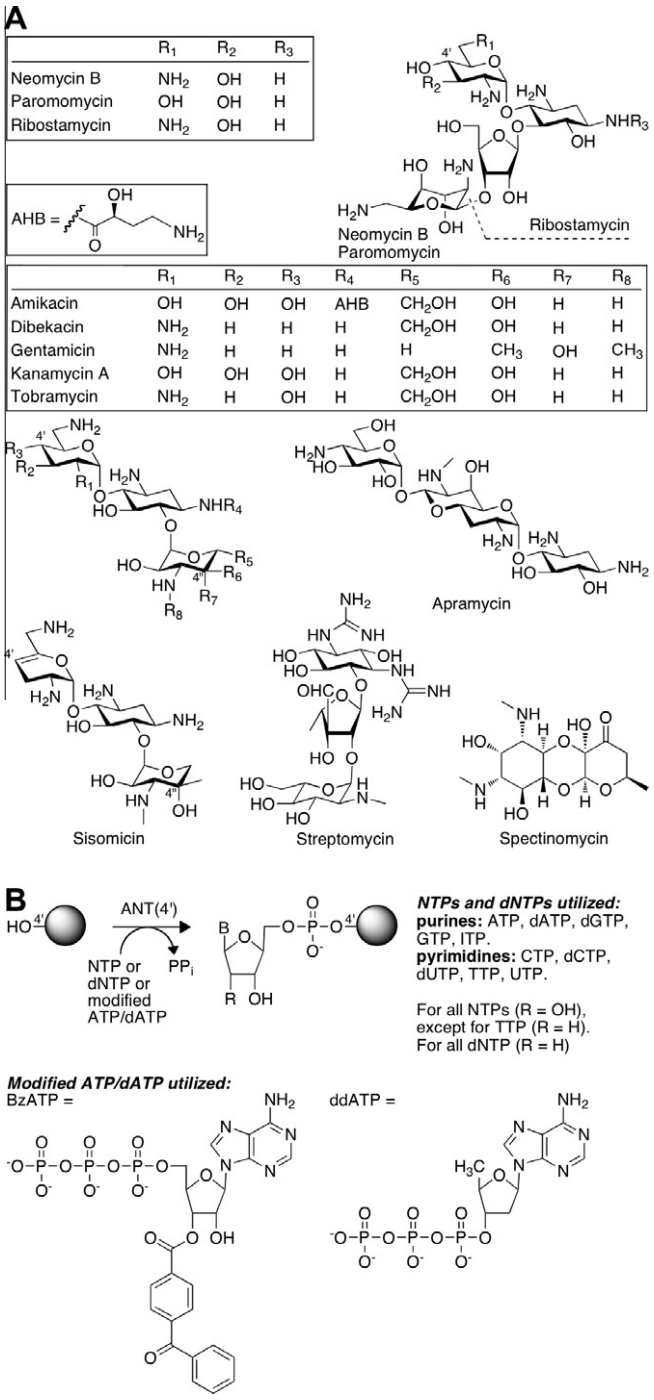

Fig. 1. (A) Structures of the antibiotics discussed in this study. (B) A schematic of the reaction catalyzed by ANT(4′) with the nucleotide cosubstrates tested in this study.

strategies to circumvent the ever-growing resistance problem. Herein, we report the kinetic characterization of the ANT(4′) enzyme and the confirmation of 4′- and 4″-nucleotidylated product formation. Additionally, we investigate the biological activity of the compounds generated and briefly address the mechanism of the ANT(4′) enzyme by ATP-[^{32}P] PP_i exchange assays.

2. Materials and methods

2.1. Materials and instrumentation

Chemically competent *E. coli* BL21 (DE3) cells were from Invitrogen. The pANT(4′)-pET28b plasmid was a gift from Prof. Juan L. Asensio (Instituto de Química Bio-orgánica General (CSIC), Spain) [13]. The molybdate/malachite green reagent (Cat. No. POMG-HTS) was from Bioassay Systems USA. The inorganic pyrophosphatase (Cat. No. I1643), aminoglycosides, and triphosphate derivatives were from Sigma–Aldrich. [^{32}P] PP_i was from Perkin Elmer. Kinetic UV–vis assays were carried out on a multimode SpectraMax M5 plate reader using 96-well plates. High-resolution mass spectrometry (HRMS) analyses were done using a Waters spectrometer model 3100 mass detector under atmospheric pressure ionization.

2.2. Overproduction and purification of ANT(4′)

The pANT(4′)-pET28b plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression and purification. One liter of Luria–Bertani (kanamycin (50 μg/mL)) broth was inoculated with an overnight culture (10 mL) and grown (37 °C, 200 rpm) to OD_{600} of ~0.5 before induction with IPTG (final concentration, 500 μM). The induced culture was grown for 16 h (30 °C, 200 rpm). The cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C). The cell pellets were resuspended in lysis buffer [400 mM NaCl, 25 mM Tris–HCl (pH 8.0), 10% (v/v) glycerol] and lysed (1 pass at 10,000–15,000 psi, Avestin EmulsiFlex-C3 high-pressure homogenizer). The insoluble cell debris was removed by centrifugation (16,000 rpm, 45 min, 4 °C). Imidazole (final concentration, 2 mM) was added to the supernatant and incubated with 0.75 mL of Ni–NTA agarose resin (2 h, 4 °C). The resin was loaded onto a column and eluted with lysis buffer in a stepwise imidazole gradient (10 mL of 2 (1×) and 5 (1×) mM followed by 5 mL of 20 (1×), 40 (1×), 60 (1×), 200 (2×), and 500 (2×) mM imidazole). The fractions containing the pure protein (determined by SDS–PAGE) were pooled, dialyzed [50 mM HEPES (pH 8.0), 10% (v/v) glycerol] at 4 °C, and concentrated using Amicon Ultra PL-10 (Supplementary Fig. S1). ANT(4′) was stored at 4 °C.

2.3. Generation of a standard curve for P_i concentrations using the molybdate/malachite green reagent

To establish a standard curve of P_i concentrations, NaH_2PO_4 (9.8, 19.5, 39.1, 78.1, 156.3, 312.5, 625 μM, 1.25, 2.5, 5, and 10 mM) in reaction buffer [Tris–HCl (pH 7.5) (50 mM), $MgCl_2$ (10 mM), DTT (1 mM), KCl (50 mM)] (160 μL) was reacted with molybdate/malachite green reagent (40 μL). After 15 min of color development, the liberated P_i concentration was measured by absorbance at 600 nm. The linear portion of the plot was best fit to $y = 2.206x$, where $x = [P_i]$ (in mM) and y = absorbance at 600 nm (Supplementary Fig. S2).

2.4. Determination of aminoglycoside and cosubstrate specificity of ANT(4′) and determination of kinetic parameters by colorimetric assays

The nucleotidyltransferase activity of ANT(4′) was monitored by colorimetric assays. To determine the substrate and cosubstrate specificity of ANT(4′), reactions (160 μL) containing Tris–HCl (pH 7.5) (50 mM), $MgCl_2$ (10 mM), DTT (1 mM), KCl (50 mM), inorganic pyrophosphatase (0.2 U/mL), aminoglycoside (0.2 mM), and NTP (1 mM) were performed at 25 °C. The reactions were initiated by addition of ANT(4′) (0.25 μM), incubated for 2, 4, 6, 8, and 10 min, and terminated by quenching with the molybdate/malachite green reagent (40 μL). After 15 min of color development, the liberated P_i concentration was measured by absorbance at 600 nm.

For the determination of K_m and k_{cat} for the aminoglycoside substrates (Supplementary Figs. S3–4), reactions were performed as described above with a constant concentration of ATP (1 mM) and varying concentrations of aminoglycosides (0, 1, 5, 10, 25,

80, and 200 μM for kanamycin A; 0, 1, 5, 10, 25, and 100 μM for amikacin, paromomycin, neomycin B, ribostamycin, and tobramycin; 0, 10, 25, 75, 125, 200, and 300 μM for gentamicin; 0, 25, 75, 125, 200, 300, and 400 μM for sisomicin). The time points utilized for kanamycin A were 0, 2, 4, 6, 8, and 10 min, whereas for all other aminoglycosides, time points of 0, 1, 2, 3, 4, and 5 min were utilized. For tobramycin, five additional time points at intervals of 20 or 30 s were used at lower concentrations of the aminoglycoside. The experiments were carried out in triplicate for each substrate concentration with a negative control (no ANT(4′)).

For the determination of K_m and k_{cat} for the NTP and dNTP cosubstrates (Supplementary Figs. S5–6), reactions were performed as described above with at least a 2-fold excess of kanamycin A (5 mM for 2 and 1 mM NTP and dNTP trials and 1 mM for every other NTP and dNTP concentrations) and varying concentrations of NTPs and dNTPs (0, 25, 100, 200, 500 μM, and 1 mM for UTP and TTP; 0, 100, 200, 500 μM, as well as 1 and 2 mM for ATP; 0, 25, 50, 75, 100, 200, 500 μM, and 1 mM for GTP; 0, 25, 50, 100, 150, 200, 500 μM, as well as 1 and 2 mM for ITP; 0, 25, 50, 100, 150, 200, and 500 μM for CTP; 0, 50, 100, 350, 500, 750 μM, and 1.5 mM for dATP; 0, 50, 100, 400, 500, and 750 μM for dCTP; 0, 100, 200, 350, 500, and 750 μM for dGTP; 0, 100, 200, 300, 350, 500, and 750 μM for dUTP). The time points utilized for all NTPs and dNTPs were 0, 1, 2, 3, 4, and 5 min. The experiments were carried out from 2 to 4 times for each cosubstrate concentration with a negative control (no ANT(4′)). The K_m and k_{cat} values for all NTPs and dNTPs (except CTP) were determined by a Michaelis–Menten curve fit of the data using Kaleidagraph 3.6 curve fitting software. The K_m and k_{cat} values for CTP were determined by Lineweaver–Burk analysis, as high enough concentrations to obtain a Michaelis–Menten curve were not attainable due to high background signal.

2.5. ATP-[^{32}P]PP$_i$ exchange assays

To verify if there is a fast reversible exchange in the active site of the enzyme that would result in the formation of [^{32}P]ATP, ATP-[^{32}P]PP$_i$ reactions (100 μL) containing Tris–HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), KCl (50 mM), TCEP (pH 7.0) (5 mM), ATP (5 mM), aminoglycoside (100 μM), and [^{32}P]PP$_i$ (1 mM, 84.12 Ci/mM) were run at 25 °C. The reactions were started by addition of ANT(4′) (0.1 μM) and incubated for up to 30 min prior to quenching with charcoal suspension (500 μL) [1.6% (w/v) activated charcoal, 4.5% (w/v) $Na_4P_2O_7$, and 3.5% (v/v) perchloric acid in H_2O]. The charcoal was pelleted by centrifugation before being washed twice with the wash solution (500 μL) [4.5% (w/v) $Na_4P_2O_7$ and 3.5% (v/v) perchloric acid in H_2O], resuspended in H_2O (500 μL), and counted by liquid scintillation. The experiments were done in duplicate for each substrate concentration with a negative control (no ANT(4′)). No exchange was observed by this assay for all aminoglycosides tested, which is consistent with the previously reported dramatically slower kinetics in the reverse direction for this enzyme when tested with AMP-kanamycin A as starting material [15].

2.6. TLC visualization and HRMS analysis of ANT(4′) reactions

Reaction mixtures (25 μL) containing Tris–HCl (pH 7.5) (50 mM), $MgCl_2$ (10 mM), DTT (1 mM), KCl (50 mM), aminoglycoside (3.2 mM), NTP (6 mM), and ANT(4′) (14.4 μM) were incubated at rt overnight and loaded onto a TLC plate (Silica gel F254 250 mm thickness). Negative control reaction mixtures contained all reagents, but no ANT(4′) enzyme. Visualization was performed using a cerium-molybdate stain. The eluent systems utilized and R_f values observed are reported in Table S1. Representative examples of TLCs are depicted in Fig. 2. In the case of gentamicin, the starting material was partially consumed, but no new spots were observed on the TLC when reacted with UTP or TTP.

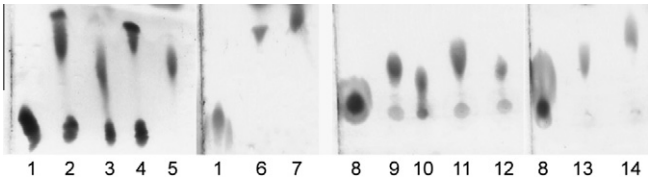

Fig. 2. TLC showing the formation of 4′-AMP-kanamycin A (lane 2), 4′-GMP-kanamycin A (lane 3), 4′-IMP-kanamycin A (lane 4), 4′-CMP-kanamycin A (lane 5), 4′-UMP-kanamycin A (lane 6), 4′-dTMP-kanamycin A (lane 7), 4′-AMP-amikacin (lane 9), 4′-GMP-amikacin (lane 10), 4′-IMP-amikacin (lane 11), 4′-CMP-amikacin (lane 12), 4′-UMP-amikacin (lane 13), and 4′-dTMP-amikacin (lane 14) from the parent drugs kanamycin A (lane 1) and amikacin (lane 8) by action of ANT(4′).

The masses of novel aminoglycoside-NMPs were determined by HRMS. To prepare the samples for MS analyses, methanol (25 μL) was added to the reaction mixtures described above. The precipitated protein was removed by centrifugation (14,000 rpm, rt, 10 min), and the supernatant was freeze-dried overnight. The aminoglycoside-NMP derivatives were dissolved in a minimum volume of H_2O prior to HRMS analysis. Fig. 3 displays representative examples of HRMS for some of the kanamycin A derivatives generated.

2.7. Antibacterial activity screen by disk diffusion and bioTLC assays

The reaction conditions described for TLC visualization were used for antibacterial assays. For the disk diffusion assays (Supplementary Fig. S7), aliquots of the total reaction mixtures (10 μL) were loaded onto sterile disks placed on LB agar plates. Overlays of soft LB agar (0.75 g of agar per 100 mL of LB broth) (6 mL) containing *Bacillus subtilis* (60 μL of an overnight culture) were poured onto the plates. After >10 h of incubation at 30 °C, plates were examined for clear zones of inhibition. All novel NMP-aminoglycosides were found to be inactive against *B. subtilis*. However, for neomycin B reacted with GTP and ATP, very small zones of inhibition could be observed. To test if the activity came from unreacted parent neomycin B or from the novel GMP-neomycin B and AMP-neomycin B, bioTLC assays were performed as previously described [17]. Briefly, portions of the reaction mixtures (10 μL) were loaded onto a TLC plate and allowed to migrate using 3:2/MeOH:NH_4OH. The plate was air-dried for 2–3 h prior to being overlayed with soft LB agar (12 mL) infused with *B. subtilis* (120 μL of an overnight culture). The *B. subtilis* overlay was grown at 30 °C until clear zones of inhibited growth were observed (overnight). The R_f values of the starting material and products on the stained TLCs corresponded to the R_f values of the zones of inhibition on the overlay. The bioTLCs confirmed that the halos of inhibition seen in the disk diffusion assays for nucleotidylated neomycin B were solely caused by unreacted parent drug.

3. Results and discussion

3.1. Heterologous expression and purification of ANT(4′)

The 33.3-kDa ANT(4′) from *S. aureus* was overexpressed in *E. coli* as a NHis$_6$-tagged protein in order to establish its aminoglycoside substrate and nucleotide triphosphate cosubstrate profile *in vitro*. Purification to homogeneity of the soluble protein was achieved by Ni(II)–NTA affinity chromatography to yield about 7 mg of ANT(4′) per liter of culture.

3.2. Substrate and cosubstrate specificity of ANT(4′)

To determine the substrate specificity of ANT(4′), a variety of commercially available aminoglycosides possessing or lacking a 4′-hydroxyl functionality were utilized. To monitor product

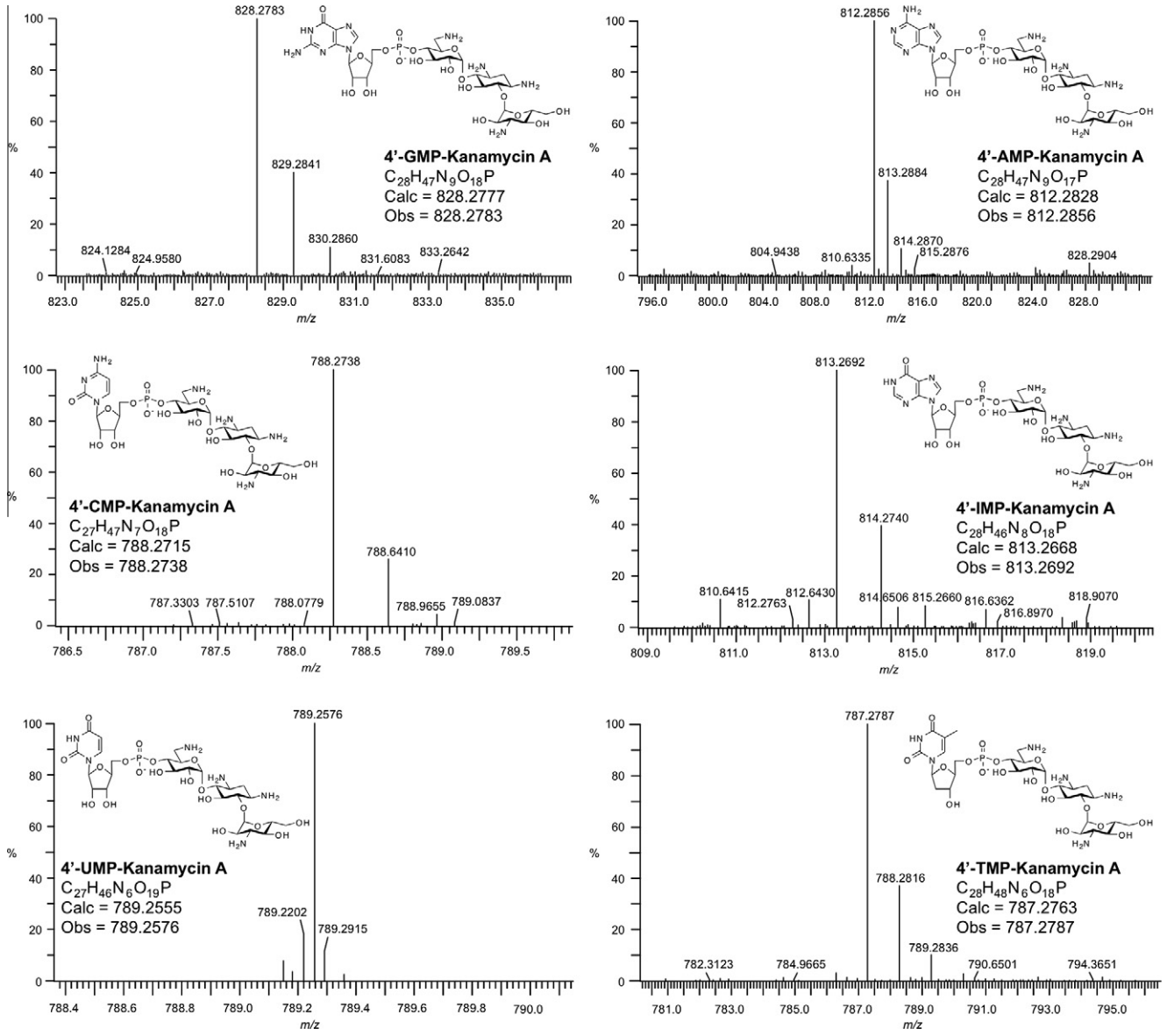

Fig. 3. Representative high-resolution mass spectra of 4′-nucleotidylated aminoglycosides (kanamycin A derivatives are shown).

formation in the nucleotidylation reactions, we chose two complementary methods. Simple TLC assays allowed us to readily and clearly detect product formation (Fig. 2 and Supplementary Table S1). HRMS analyses then confirmed the identity of the novel nucleotidylated aminoglycosides (Fig. 3). As expected, all aminoglycosides containing a 4′-hydroxyl in their structures were found to be substrates of ANT(4′). Not surprisingly, spectinomycin and apramycin, lacking both a 4′- and a 4″-hydroxyl group were shown to not be substrates of ANT(4′). Interestingly, we observed that streptomycin possessing a 4″-hydroxyl moiety, did not act as a substrate of ANT(4′). However, sisomicin and gentamicin, also lacking a 4′-hydroxyl moiety, but possessing a 4″-hydroxyl group were both found to be modified by ANT(4′).

Initial cosubstrate binding studies by Asensio and co-workers indicated that the triphosphate moiety of NTPs was sufficient for binding to ANT(4′) [16]. In order to dissect the cosubstrate structure requirements for ANT(4′) activity further to gain insight for future design of inhibitors of ANT(4′), a number of NTPs, dNTPs, and nucleotides modified at the 3′-position of the ribose ring were utilized. ANT(4′) displayed very broad cosubstrate promiscuity. All NTPs and dNTPs utilized were found to be cosubstrates for the enzyme, indicating that: (1) the base (purine or pyrimidine) on the ribose/deoxyribose ring does not play a major role in binding to the ANT(4′) enzyme, and (2) the 3′-position of the ribose ring can tolerate a proton or a hydroxyl group. However, the 3′-position of the ribose ring cannot tolerate large substituents as demonstrated by the fact that an adenosine triphosphate analog modified at the 3′-position with a 4-benzoylbenzoyl moiety, BzATP, was found not to be a cosubstrate of ANT(4′). In addition, by showing that 2′,5′-dideoxy-3′-triphosphate (ddATP) is not a cosubstrate for the enzyme, we confirmed that the 3′-position of the nucleotide cannot accommodate large moieties and that the location of the triphosphate at the 5′-position of the ribose/deoxyribose ring is important for activity.

3.3. Kinetic characterization of ANT(4′)

With the initial results confirming the activity of ANT(4′) against the majority of aminoglycosides, NTPs, and dNTPs tested (Fig. 1), we decided to determine the kinetic parameters for this enzyme (Table 1 and Supplementary Figs. S3–6). Inspired by the

Table 1
Kinetic parameters for ANT(4′).

Aminoglycoside	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
Aminoglycosides kinetic parameters[a]			
Amikacin	5.71 ± 0.73	111 ± 4	19
Apramycin[d]	–	–	–
Gentamicin[c]	171 ± 59	136 ± 23	0.09
Kanamycin A	6.13 ± 0.69	135 ± 4	22
Neomycin B	4.36 ± 0.85	137 ± 7	31
Paromomycin	5.51 ± 0.99	130 ± 8	24
Ribostamycin	5.60 ± 1.83	215 ± 19	38
Sisomicin[c]	59.8 ± 15.3	39 ± 3	0.65
Spectinomycin[d]	–	–	–
Streptomycin[d]	–	–	–
Tobramycin	1.48 ± 0.46	285 ± 20	193
NTPs and dNTPs kinetic parameters[b] NTP			
ATP	1370 ± 140	290 ± 16	0.21
BzATP[e]	–	–	–
CTP	423 ± 21	94 ± 5	0.22
dATP	769 ± 208	302 ± 38	0.39
ddATP[e]	–	–	–
dCTP	736 ± 287	193 ± 43	0.26
dGTP	710 ± 250	243 ± 50	0.34
dUTP	292 ± 36	96 ± 5	0.33
GTP	1160 ± 340	270 ± 50	0.23
ITP	500 ± 87	122 ± 8	0.24
TTP	350 ± 80	100 ± 9	0.29
UTP	2410 ± 140	325 ± 14	0.13

[a] Determined using 1 mM ATP.
[b] Determined using up to 5 mM kanamycin A.
[c] These aminoglycosides lack a 4′-hydroxyl, but contain a 4″-hydroxyl group.
[d] These aminoglycosides were found to not be substrates for ANT(4′).
[e] These nucleotides were found to not be cosubstrates for ANT(4′).

work of Wright and co-workers on the adenylyltransferase LinB [18] and our previous success with monitoring adenylation reactions by colorimetric assay [19], we chose the malachite green reagent to measure the P_i concentration following rapid complete degradation by inorganic pyrophosphatase of the PP_i released during NMP-aminoglycoside formation by action of ANT(4′). It has been recently demonstrated by NMR spectroscopy that triphosphate (TP) can serve as a cosubstrate for ANT(4′). However, using our colorimetric assay, we could not test TP as pyrophosphatase hydrolyzes this compound [20]. As previously observed with ANT(4′) by a rather cumbersome assay relying on the sequential action of four enzymes [15], we observed relatively small differences between the rates of catalysis for different antibiotics. We established that the nucleotidyl transfers at the 4″-position for gentamicin [k_{cat}/K_m 1.6 $\mu M^{-1}min^{-1}$] and sisomicin [k_{cat}/K_m 1.3 $\mu M^{-1}min^{-1}$] are achieved ~30 to 300-fold less efficiently than for the transfer at the 4′-position on the other aminoglycosides tested. With a K_m of 1.48 ± 0.46 μM and a k_{cat} of 285 ± 20 min^{-1}, we found tobramycin to be the best substrate of ANT(4′). Previous studies reported that purine nucleotide triphosphates (ATP, GTP) may act as more efficient cosubstrates for nucleotidylation of aminoglycosides than the pyrimidines nucleotide triphosphates (TTP, UTP, CTP) [10]. In contrast, we observed little difference in the rate of catalysis among the cosubstrates (additional dATP, dCTP, dGTP, dUTP, ITP and TTP) tested. Interestingly, all dNTPs and TTP, which does not have a hydroxyl group at the 2′-position of the ribose ring, were found to be about twice as efficient cosubstrates for ANT(4′) as their NTP counterparts.

3.4. The irreversibility of the ANT(4′) mechanism

ANTs have been proposed to follow a Theorell-Chance kinetic mechanism in which the release of the nucleotidylated aminoglycosides is the rate-limiting step. The aminoglycoside substrates bind the enzyme before the cosubstrates do for ANT(4′) [15], whereas ATP binds before the aminoglycosides for ANT(3″) [7], ANT(2″) [21], and SMATase [22]. To probe potential reversibility of the NMP attachment to the aminoglycosides, we performed ATP-[^{32}P] PP_i exchange assays (see Supplementary data). No formation of [^{32}P]ATP was observed with any of the aminoglycosides tested, which is consistent with the previously reported significantly slower kinetics in the reverse direction for this enzyme when tested with AMP-kanamycin A as starting material [15].

3.5. Confirmation that nucleotidylation always results in completely inactive compounds

Nature has evolved three different types of aminoglycoside-modifying enzymes (AACs, APHs, and ANTs) to inactivate the aminoglycoside antibiotics currently used in the clinic. We previously demonstrated that acylation of aminoglycosides does not always result in a complete inactivation of the parent drug [17]. We also demonstrated that by taking advantage of the substrate/cosubstrate promiscuity of acyltransferases, novel novobiocin [23] and acetylcholine [24,25] analogs could be produced. One would expect that addition of a variety of nucleotides to aminoglycoside scaffolds would result in a complete inactivation of the parent drugs. To test if all nucleotidylations totally inactivate the parent compounds tested, we qualitatively assessed the biological activity of our newly generated nucleotidylated aminoglycosides by antibacterial disk diffusion assays (Supplementary Fig. S7). As expected, all parent drugs (P) showed clear inhibition of the growth of *B. subtilis*, whereas all nucleotidylated compounds displayed no antibacterial activity. For nucleotidylated products that appeared to be potentially weakly active by disk diffusion assays, bioTLCs [17] were performed and confirmed that the weak antibacterial activity arose from unreacted starting parent aminoglycosides.

4. Conclusions

In summary, we have presented evidence that ANT(4′) can transfer a large number of nucleotides to a broad variety of aminoglycosides including sisomicin and gentamicin, which do not possess a 4′-hydroxyl moiety. However, nucleotides with larger substituents at the 3′-position of the ribose ring were found not to be cosubstrates for ANT(4′). We have also demonstrated that: (1) dNTPs are better cosubstrates than NTPs, (2) the position of the triphosphate moiety (5′ and not 3′) on the ribose/deoxyribose ring is important for recognition by ANT(4′), and (3) large moieties are not tolerated at the 3′-position of the ribose ring. In addition to assessing the substrate and cosubstrate profile of this enzyme, we confirmed that there was no significant reversibility of nucleotide transfer as no PP_i exchange on the ATP was observed. As expected, we also note that all 4′-nucleotidylations tested in this study result in the complete biological inactivation of the parent drugs. We take the valuable knowledge gained from this work as a guide for establishing strategies for the development of novel aminoglycoside antibiotics in the future towards the goal of developing new ways to outsmart bacteria and combat resistance. With this in mind, we are now considering the identification of inhibitors of this resistance enzyme to be used in conjunction with currently existing drugs. Aminoglycosides lacking 4′- and/or 4″-hydroxyl groups could also present a solution to alleviate the resistance problem associated with this enzyme. Efforts in these directions are currently underway in our group.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.119.

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