Prodigious substrate specificity of AAC(6′)-APH(2″), an aminoglycoside antibiotic resistance determinant in enterococci and staphylococci

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Background: High-level gentamicin resistance in enterococci and staphylococci is conferred by AAC(6′)-APH(2″), an enzyme with 6′-*N*-acetyltransferase and 2″-*O*-phosphotransferase activities. The presence of this enzyme in pathogenic gram-positive bacteria prevents the successful use of gentamicin C and most other aminoglycosides as therapeutic agents.

Results: In an effort to understand the mechanism of aminoglycoside modification, we expressed AAC(6′)-APH(2″) in *Bacillus subtilis*. The purified enzyme is monomeric with a molecular mass of 57 kDa and displays both the expected aminoglycoside *N*-acetyltransferase and *O*-phosphotransferase activities. Structure–function analysis with various aminoglycosides substrates reveals an enzyme with broad specificity in both enzymatic activities, accounting for AAC(6′)-APH(2″)'s dramatic negative impact on clinical aminoglycoside therapy. Both lividomycin A and paromomycin, aminoglycosides lacking a 6′-amino group, were acetylated by AAC(6′)-APH(2″). The infrared spectrum of the product of paromomycin acetylation yielded a signal consistent with *O*-acetylation. Mass spectral and nuclear magnetic resonance analysis of the products of neomycin phosphorylation indicated that phosphoryl transfer occurred primarily at the 3′-OH of the 6-aminohexose ring A, and that some diphosphorylated material was also present with phosphates at the 3′-OH and the 3‴-OH of ring D, both unprecedented observations for this enzyme. Furthermore, the phosphorylation site of lividomycin A was determined to be the 5″-OH of the pentose ring C.

Conclusions: The bifunctional AAC(6′)-APH(2″) has the capacity to inactivate virtually all clinically important aminoglycosides through *N*- and *O*-acetylation and phosphorylation of hydroxyl groups. The extremely broad substrate specificity of this enzyme will impact on future development of aminoglycosides and presents a significant challenge for antibiotic design.

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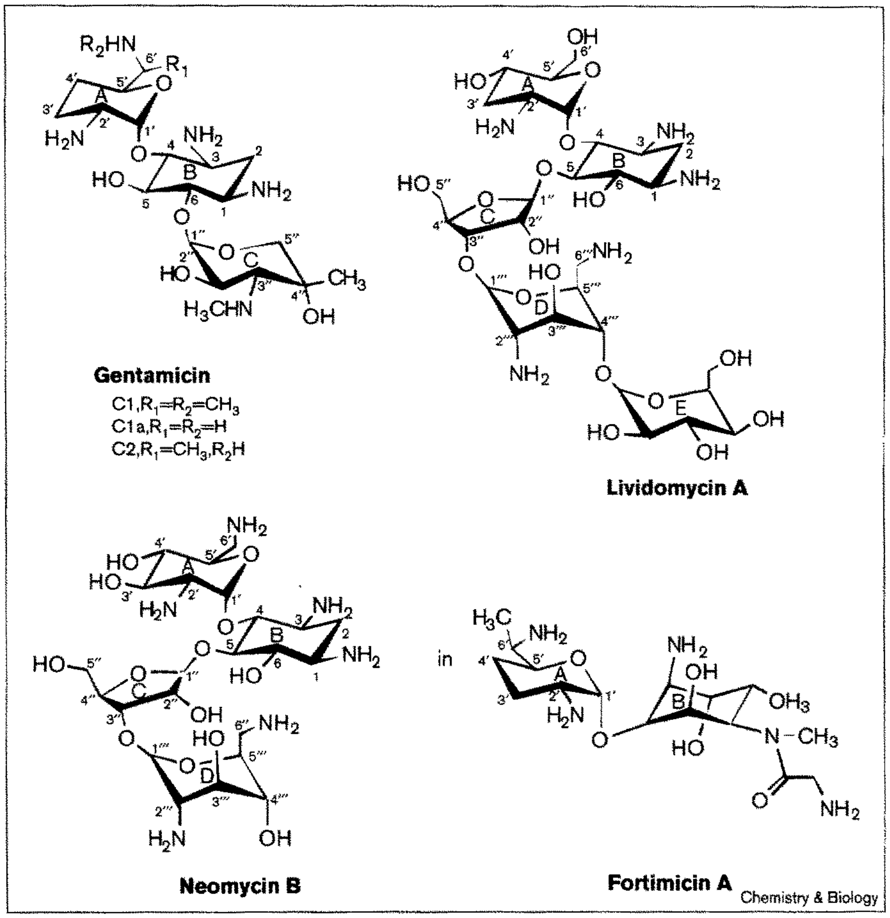
Introduction

Aminoglycoside antibiotics are important bactericidal agents used for the treatment of many bacterial infections [1]. The aminoglycosides are generally comprised of a central 2-deoxystreptamine aminocyclitol ring to which amino sugars are linked either at positions 4 and 6, such as gentamicin and kanamycin, or at position 4 and 5, such as neomycin and lividomycin (Figure 1). These cationic molecules exert their antibacterial effects by binding to the 30S ribosomal subunit and thereby disrupting bacterial translation [2]. The bactericidal action and broad antibacterial specificity of these drugs has rendered them highly useful for the treatment of nosocomial (hospital acquired) infections.

Bacterial resistance to aminoglycosides is a growing problem [3–5]. The primary route of clinical resistance is the acquisition of genes encoding enzymes that covalently modify the drugs by *O*-phosphorylation, *N*-acetylation and *O*-adenylation [6,7]. The wide-spread dissemination of bacteria harboring these resistance genes in healthcare institutions has compromised the usefulness of aminoglycosides, many of which are consequently no longer clinically effective.

The gentamicin C complex is comprised of three closely related aminoglycoside components, C1, C1a and C2 (Figure 1), and is extensively used in the clinic [1]. In particular, it is required for use in combination with a penicillin to achieve a synergistic killing effect for the treatment of serious enterococcal infections [8]. Enterococci can harbor several distinct aminoglycoside-modifying enzymes [9], but one protein with two enzymatic activities, 6′-*N*-acetyltransferase and 2″-*O*-phosphotransferase (AAC(6′)-APH(2″)), has been implicated in high-level gentamicin C resistance (minimal inhibitory concentrations [MICs] > 2,000 g/ml) [10]. The gene that encodes

Figure 1



Structures of representative aminoglycoside antibiotics.

this bifunctional enzyme is generally found on transposable elements (e.g. Tn*5281* in *Enterococcus faecalis* [11]) and these elements are frequently carried on R plasmids but can also be detected integrated into the chromosome [12,13]. The mobile nature of this gene has permitted intergenus transfer and the *aac(6′)-aph(2″)* gene can be found in staphylococci as well, where it also results in high-level gentamicin C resistance, (e.g. Tn*4001* in *Staphylococcus aureus* [14] and Tn*4031* in *Staphylococcus epidermidis* [15]).

The complete *aac(6′)-aph(2″)* gene has been cloned and sequenced from isolates of *E. faecalis* [16] and *S. aureus* [17]. The protein is predicted to have a molecular mass of 56,993 Da, in good agreement with the experimentally determined sizes of enzymes previously purified from *S. aureus* and *S. epidermidis* [18]. Construction of 5′-truncated *aac(6′)-aph(2″)* genes has revealed that the AAC activity resides in the amino-terminal portion of the enzyme and that the APH activity is localized to the carboxyl terminus [16]. This is in agreement with amino-acid alignments that demonstrate sequence homology to AAC(6′) enzymes in the amino terminus and to APH enzymes in the carboxyl terminus; these observations have led to the suggestion that the bifunctional enzyme has arisen from a gene-fusion event [6,16]. The kinetic mechanism of both the AAC and APH activities have been determined using enzyme partially purified from *S. aureus* [19], and, recently, the enzyme has been expressed and purified from *Escherichia coli* and the predicted regiospecificity of both phosphoryl (2″-OH) and acetyl (6′-NH_2) transfer confirmed using kanamycin [20]. Moreover, bifunctionalization (6′-acetyl and 2″-phosphoryl) of kanamycin was also detected in this system, although it is not known if this results in higher MICs. The regiospecificity of phosphoryl transfer to the neomycin class of aminoglycosides that have pentoses in the C ring (Figure 1) has not been determined. We report overexpression of the AAC(6′)-APH(2″) bifunctional enzyme from *Bacillus subtilis* along with characterization of substrate specificity and regiospecificity of phosphoryl transfer.

Results

Expression of AAC(6′)-APH(2″) in *B. subtilis*

When we expressed AAC(6′)-APH(2″) in *E. coli* using several gene constructs, we consistently found a mixture of products that consisted of the intact 57 kDa enzyme and a 36 kDa protein with only APH(2″) activity, which

Table 1

Purification of AAC(6′)-APH(2″) from a 10 l culture of *B. subtilis* 1A752/pBF-14.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (n-fold)
Cell lysate	1950	APH* –	APH –	APH –	APH –
		AAC 47.3	AAC 0.024	AAC 100	AAC 0
Q Sepharose	629	APH 22.4	APH 0.036	APH 100	APH –
		AAC 27.8	AAC 0.044	AAC 59	AAC 1.9
Sephadex G100	255	APH 26.3	APH 0.103	APH 100	APH 4.3
		AAC 24.8	AAC 0.098	AAC 53	AAC 4.0
Gentamicin-agarose	71.8	APH 15.8	APH 0.22	APH 70	APH 9.2
		AAC 13.0	AAC 0.181	AAC 28	AAC 7.5

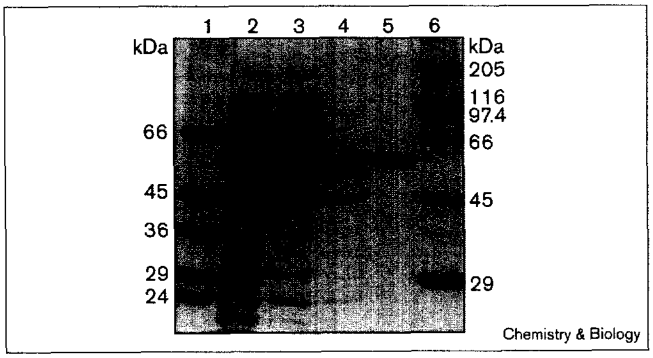
1 U = 1 μmole/min. *High levels of nonspecific ATPase activity precluded accurate detection of APH activity. APH activity was monitored by pyruvate kinase/lactate dehydrogenase coupled assay and therefore the overall increase in specific activity was low due to contaminating ATPases that prevented accurate assay in crude fractions.

was not the result of protease activity but rather appears to be due to an alternate translation initiation event (D.M.D. and G.D.W., unpublished observations). The poor yields of intact AAC(6′)-APH(2″) when expressed in *E. coli* led us to explore expression in *B. subtilis*. The gene encoding AAC(6′)-APH(2″) was amplified from pSF815A [16] and cloned into plasmid pRB374 [21] to give plasmid pBF-14, which places the bifunctional gene under transcriptional control of the constitutive *B. subtilis vegII* promoter. This construct gave excellent expression of AAC(6′)-APH(2″) in *B. subtilis* 1A752 and a three-step purification provided intact enzyme with a yield of greater than 70 mg per 10 l of cell culture (Table 1, Figure 2).

Substrate specificity of AAC(6′) activity

As expected, the purified enzyme had a very broad substrate range (Tables 2 and 3). Aminoglycosides with a free 6′-amino group were all acetylated with comparable efficiency (10^4–10^5 M^{-1} s^{-1}) as was fortimicin, an unusual property of the AAC(6′) class of enzymes. These values are similar to other aminoglycoside acetyltransferases such AAC(6′)-Ii from *E. faecium* [22]. Unexpectedly, the 6′-hydroxy aminoglycoside antibiotics lividomycin A and paromomycin were not inhibitors of the 6′-*N*-acetylase activity as they have been shown to be for AAC(6′)-Ii [22]. Instead, they were substrates for the acetyltransferase with a K_m comparable to other aminoglycosides, but with a diminished k_{cat} (Table 2). This suggested either transfer to an alternate amino group on the molecule or *O*-acetyltransfer to position 6′. Enzymatically acetylated paromomycin and lividomycin A were highly susceptible to mild base hydrolysis supporting the *O*-acetyltransfer hypothesis (Figure 3a). Purification of acetyl-paromomycin and subsequent analysis by infrared spectrometry identified a prominent signal at 1719 cm^{-1}, consistent with formation of the 6′-acetyl ester (Figure 3b).

Figure 2



Purification of AAC(6′)-APH(2″) from *B. subtilis* 1A752/pBF-14. Sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS–PAGE) on an 11% gel of the progress of the purification of AAC(6′)-APH(2″) was stained with Coomassie Blue R-250. Lysis and purification were performed according to the procedure in the Materials and methods section. Lane 1, molecular mass markers; lane 2, cell lysate (S1); lane 3, Q Sepharose; lane 4, Sephadex G100; lane 5, Gentamicin C-agarose; lane 6, molecular mass markers.

Substrate specificity and regiospecificity of APH(2″) activity

Unlike the AAC(6′) activity of the bifunctional enzyme, there was more deviation in the specificity of the APH activity with K_m varying from 2–200 μM and k_{cat}/K_m values from 10^3–10^5 M^{-1} s^{-1} (Table 3). These values are similar to those found with the broad specificity aminoglycoside kinase APH(3′)-IIIa [23] but are lower than those found for other APH(3′)s in which k_{cat}/K_m approach diffusion-controlled levels [24]. The broad scope of aminoglycosides that are substrates for the APH(2″) surpasses the APH(3′)s and other aminoglycoside kinases, however. The regiospecificity of phosphoryl transfer to kanamycin A has been determined using nuclear magnetic resonance (NMR) methods to be at the 2″-OH in ring C [20]. The regiospecificity of Pi transfer for the 4,5-disubstituted class of aminoglycosides such as neomycin, in which ring C is a pentose, was unknown, however. Large-scale inactivations of neomycin C revealed two products that were identified as mono-phosphoryl neomycin (m/z 695.4) and diphosphoryl neomycin (m/z 775.3) using electrospray mass

Table 2

Kinetic parameters for AAC(6′) activity.

Substrate*	K_m (μM)	k_{cat} (s^{-1})	K_i (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Kanamycin A	1.4 ± 0.5	0.31 ± 0.05	50.2 ± 22.3	2.21×10^5
Kanamycin B	7.5 ± 5.0	1.3 ± 0.5	23.1 ± 15.1	1.7×10^5
Tobramycin	2.3 ± 0.9	0.68 ± 0.1	50.0 ± 20.1	3.0×10^5
Dibekacin	2.4 ± 1.8	1.5 ± 0.4	43.8 ± 27.3	6.2×10^5
Amikacin	20.9 ± 5.3	2.11 ± 0.35	23.7 ± 7.8	1.01×10^5
Gentamicin C†	4.6 ± 1.5	0.85 ± 0.15	76.6 ± 39.7	1.85×10^5
Gentamicin B	1.1 ± 0.2	0.3 ± 0.01		2.73×10^5
Isepamicin	18.7 ± 9.0	1.66 ± 0.50	88.3 ± 52.7	8.87×10^4
Netilmicin	3.2 ± 0.5	0.87 ± 0.04		2.72×10^5
Sisomicin	6.5 ± 2.1	2.92 ± 0.31		4.5×10^5
Neomycin B	4.2 ± 0.8	1.53 ± 0.09		3.64×10^5
Ribostamicin	6.1 ± 2.5	2.06 ± 0.47	54.7 ± 31.4	3.38×10^5
Butirosin A	25.8 ± 2.7	1.27 ± 0.06		4.92×10^4
Neamine	8.4 ± 5.4	3.0 ± 1.3	15.0 ± 9.4	3.6×10^5
Fortimicin	2.2 ± 0.4	0.19 ± 0.01		8.64×10^4
Lividomycin A	10.1 ± 2.8	0.020 ± 0.001		2.0×10^3
Paromomycin	17.5 ± 5.0	0.032 ± 0.02		1.8×10^3
Acetyl CoA‡	5.9 ± 1.4	0.73 ± 0.04		1.24×10^5

*Acetyl CoA concentration was held at 0.1 mM for aminoglycoside assays. †Gentamicin C is a complex of gentamicin C1, C1a and C2. ‡Nonvarying substrate was kanamycin A (0.1 mM).

spectrometry. ^{1}H, ^{13}C and ^{31}P-NMR spectra of product neomycin monophosphate were consistent with phosphorylation on the 3′OH of ring A as we have shown previously for APH(3′)-IIIa [25].

In order to determine the sites of phosphorylation in neomycin bisphosphate a combination of one- and two-dimensional NMR techniques were used. The ^{1}H chemical shifts and coupling constants are reported in Tables 4 and 5, whereas the ^{31}P NMR data are shown in Table 6. The NMR data for neomycin are also included in Tables 1 and 2 for comparison. The analysis began by recording the ^{1}H and ^{31}P NMR spectra. When the ^{1}H NMR spectrum (Figure 4) was examined the majority of signals appeared between 2.90 and 4.40 ppm. There were also two groups of resolved signals. The first group was located in the 5.20–5.80 ppm region and consisted of three doublets arising from the anomeric protons. The other group of

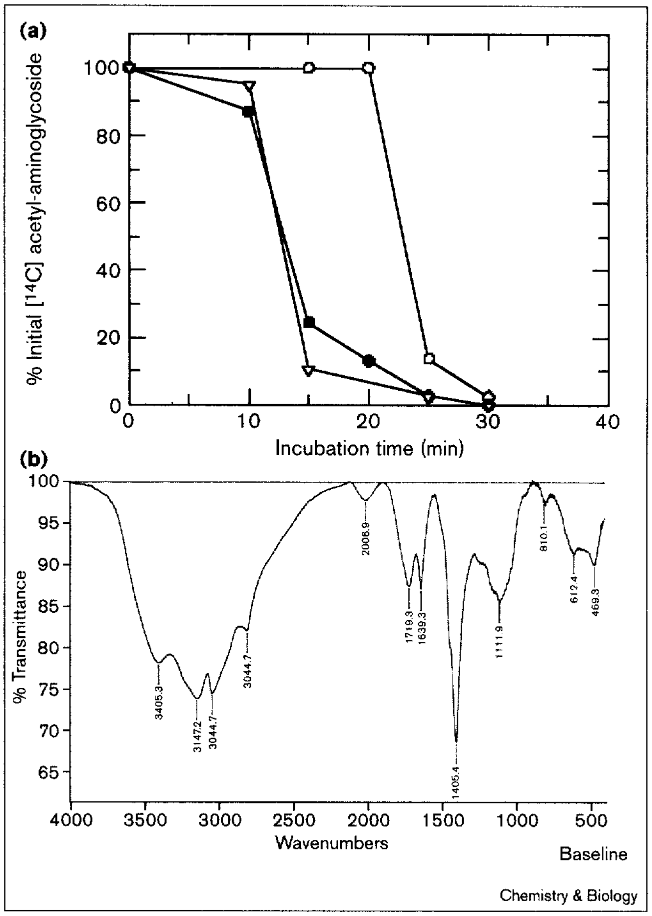
Table 3

Kinetic parameters for APH(2″) activity.

Substrate*	K_m (μM)	k_{cat} (s^{-1})	K_i (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Kanamycin A	7.0 ± 0.9	0.41 ± 0.02		5.84×10^4
Kanamycin B	9.3 ± 2.2	1.07 ± 0.16	65.4 ± 19.6	1.15×10^5
Tobramycin	4.8 ± 1.1	0.33 ± 0.02		6.9×10^4
Dibekacin	3.6 ± 0.8	0.34 ± 0.02		9.4×10^4
Amikacin	158 ± 33	0.16 ± 0.01		1.0×10^3
Gentamicin C†	3.4 ± 0.5	0.23 ± 0.01		6.8×10^4
Gentamicin B	38.9 ± 8.5	0.65 ± 0.09	173 ± 49	1.67×10^4
Isepamicin	197 ± 44.0	0.59 ± 0.04		3.00×10^3
Netilmicin	36.4 ± 5.7	0.21 ± 0.01		5.77×10^3
Sisomicin	12.2 ± 1.1	0.4 ± 0.01		3.28×10^4
Neomycin B	1.9 ± 0.2	0.19 ± 0.01		1.0×10^5
Paromomycin	3.4 ± 1.5	0.49 ± 0.1	77.5 ± 36.1	1.44×10^5
Lividomycin A	32.9 ± 8.1	0.22 ± 0.02		6.7×10^3
Ribostamycin	2.0 ± 0.8	0.25 ± 0.04	176 ± 108	3.71×10^5
Butirosin A	11.6 ± 1.4	0.43 ± 0.09		3.71×10^4
Neamine	9.6 ± 3.1	0.17 ± 0.01		1.8×10^4
ATP‡	24.1 ± 6.1	0.17 ± 0.02		7.1×10^3

*ATP concentration was held at 1 mM for aminoglycoside assays. †Gentamicin C is a complex of gentamicin C1, C1a and C2. ‡Nonvarying substrate was kanamycin A (0.1 mM).

Figure 3



Evidence for O-acetylation of paromomycin and lividomycin. **(a)** Sensitivity of [^{14}C]-acetylated aminoglycosides to alkaline treatment. The amount of label remaining on the enzymatically acetylated aminoglycosides was determined by use of the phosphocellulose-binding assay as described in the Materials and methods section. ○ acetyl-kanamycin A; ● acetyl-lividomycin A; ▽ acetyl-paromomycin. **(b)** Infrared spectrum of purified acetyl-paromomycin.

Table 4

^{1}H Chemical shifts of neomycin (1) and neomycin bisphosphate (2) in D_2O.

Proton	Chemical shift (ppm)	
	1	2
Ring A		
1	5.824	5.773
2	3.242	3.289
3	3.831	4.226
4	3.309	3.463
5	3.873	3.961
6	3.318	3.280
6′	3.089	3.130
Ring B		
1	3.149	3.080
2e	2.188	2.090
2a	1.636	1.411
3	3.149	3.000
4	3.76*	3.538
5	3.76*	3.723
6	3.546	3.462
Ring C		
1	5.268	5.277
2	4.288	4.252
3	4.379	4.354
4	4.080	4.073
5	3.772	3.812
5′	3.598	3.630
Ring D		
1	5.136	5.211
2	3.421	3.603
3	4.096	4.303
4	3.678	3.825
5	4.180	4.243
6	3.274	3.291
6′	3.221	3.243

*Estimated chemical shifts owing to second-order effects resulting from signal overlap of ring B protons 4 and 5.

peaks appeared in the low frequency end of the spectrum at 2.090 and 1.411 ppm. These multiplets were assigned to the C-2 equatorial and axial methylene protons, respectively, on the aminocyclitol ring (ring B). The differentiation of the aminocyclitol methylene protons was based on the magnitude of the vicinal 3-bond coupling constants and the general trend of lower frequency chemical shifts for axial protons in cyclohexanes.

Two signals were observed in the ^{31}P spectrum at 4.407 and 3.482 ppm. Each phosphorus signal underwent selective irradiation and the ^{31}P-decoupled ^{1}H NMR spectrum was acquired. Irradiation of the phosphorus at 3.482 ppm resulted in the doublet of triplets at 4.303 ppm in the ^{1}H spectrum to collapse into a triplet. Similarly, when the 4.407 ppm phosphorus signal was decoupled the proton multiplet at 4.226 ppm was reduced to a doublet of doublets. These spin-decoupling experiments identified the protons that were adjacent to the phosphate groups by removing the 3-bond ^{1}H-^{31}P coupling (Table 6). The next step was to assign these protons to specific positions in the neomycin structure.

The assignment of the main group of multiplets between 2.90 and 4.40 ppm initially involved following the correlations between spin-coupled protons in the COSY spectrum starting from the anomeric protons. Only the protons from the ribose ring C were sufficiently resolved to allow a complete assignment from the COSY spectrum (Table 4). The sequence of correlations in the COSY spectrum starting from the 5.277 ppm anomeric proton revealed a six proton spin system that could only arise from the ribose ring. The critical outcome of this analysis was that the protons affected by the ^{31}P-decoupling experiments were not from ring C. Only a partial assignment of the protons

Table 5

^{1}H–^{1}H Coupling constants for neomycin (1) and neomycin bisphosphate (2) in D$_2$O.

Proton	Coupling constant (Hz)	
	1	2
^3J		
Ring A		
1,2	3.9	3.9
2,3	10.8	10.0
3,4	9.2	8.8
4,5	10.2	9.8
5,6	3.3	3.8
5,6′	7.5	6.8
Ring B		
1,2e	4.2	4.2
1,2a	12.5	12.4
1,6	10.2*	10.4
3,2e	4.2	4.1
3,2a	12.5	12.2
3,4	10.2*	9.4
4,5	*	9.8
5,6	*	9.2
Ring C		
1,2	2.2	2.4
2,3	4.8	5.0
3,4	6.9	6.9
4,5	3.0	2.9
4,5′	5.1	4.9
Ring D		
1,2	1.8	1.8
2,3	3.2	3.2
3,4	3.2	3.2
4,5	1.5	1.5
5,6	6.8	6.8
5,6′	3.9	4.3
^2J		
Ring A		
6,6′	−13.5	−13.5
Ring B		
2e,2a	−12.5	−12.4
Ring C		
5,5′	−12.4	−12.4
Ring D		
6,6′	−13.7	−13.6
^4J		
Ring D		
2,4	1.2	1.5

*Coupling constants were estimated or not measured owing to second order effects as a result of the overlapped of the ring B protons 4 and 5.

Table 6

^{31}P Chemical shifts and ^{31}P–^{1}H coupling constants of neomycin bisphosphate (2) in D$_2$O.

Phosphorous	Chemical shift (ppm)	Coupling constant (Hz)	
Ring A		$^3J_{P,H}$	
3-P	4.407	H-3,P	7.1
Ring-D			
3-P	3.482	H-3,P	9.6

from carbohydrate rings A and D could be determined from the COSY spectrum because signal overlap made it difficult to unambiguously follow the connectivity between adjacent protons on these rings.

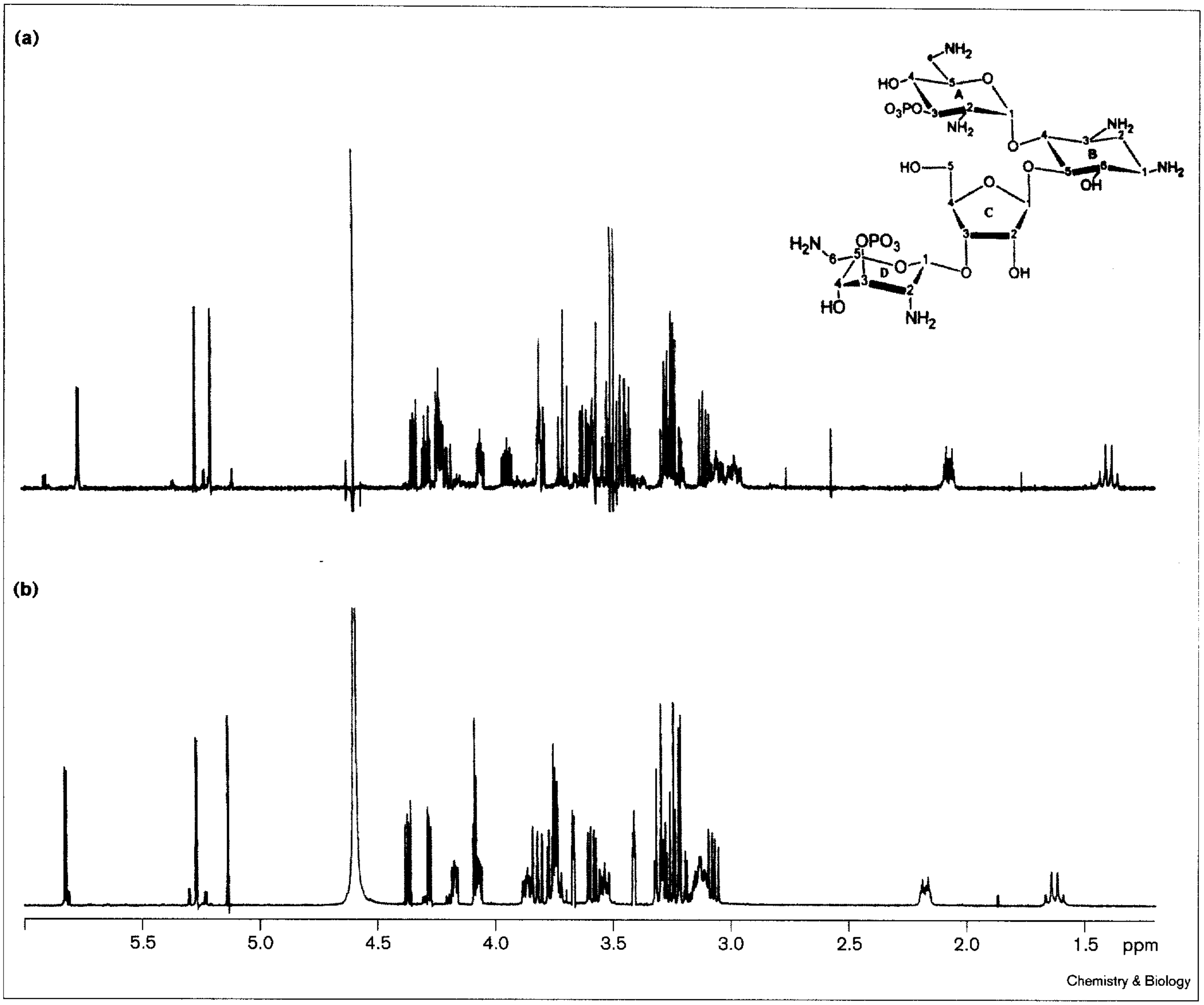
The aminocyclitol protons were readily located in the COSY spectrum by following the connectivity pathway starting from the C-2 methylene protons. The assignment of the aminocyclitol protons remained uncertain, however, because of the nearly identical multiplicity and chemical shifts of the protons on carbons 1 and 3 of the B ring. As the phosphorus-coupled protons were not part of the aminocyclitol spin system the analysis to this point indicated that phosphorylation must have occurred only in the A and D pyranose rings.

The differentiation of the pyranose ring A and D protons relied on the detection of a nuclear Overhauser enhancement (NOE) between the anomeric protons and the protons on the ether-linked carbons in the neighbouring rings. A two-dimensional NOE experiment was performed using the transverse-ROESY pulse sequence [26]. In the ROESY spectrum the anomeric proton at 5.773 ppm displayed a cross-peak with the B ring proton at 3.538 ppm. This result confirmed that the 5.773 ppm doublet was the ring A anomeric proton, whereas the 3.538 ppm signal was the aminocyclitol C-4 proton. Establishing the assignment of the ring B H-4 proton allowed the chemical shifts of the remaining B ring protons to be assigned.

The anomeric proton at 5.211 ppm displayed a strong ROESY cross-peak with the ribose ring C H-2 and H-3 protons at 4.252 and 4.353 ppm, respectively. Thus the signal at 5.211 ppm was from the ring D anomeric proton. A re-examination of the COSY data resulted in the assignment of the ring D H-2, H-3 and H-4 protons (Table 4). The identification of the phosphorus-coupled proton at 4.303 ppm as the D ring H-3 confirmed one of the sites of phosphorylation as the hydroxyl group on position 3 of neomycin ring D (position $3'''$).

In order to locate the other site of phosphorylation and resolve a number of overlapped ^{1}H signals the selective one-dimensional TOCSY pulse sequence was used [27,28]. When this pulse sequence was used to selectively excite the A ring anomeric proton, a ^{1}H spectrum was produced that contained all the A ring protons and therefore allowed the assignment of their chemical shifts (Table 4). The only signals that remained overlapped were the A ring H-2 and H-6 protons. These signals could be resolved by repeating the selective TOCSY experiment by irradiating either the H-1 or H-5 protons and using a shorter spin lock time to limit the extent of coherence transfer through the spin system. The most significant result of the selective

Figure 4



Comparison of the 500 MHz H NMR spectra of **(a)** neomycin and **(b)** neomycin bisphosphate in D_2O at 30°C.

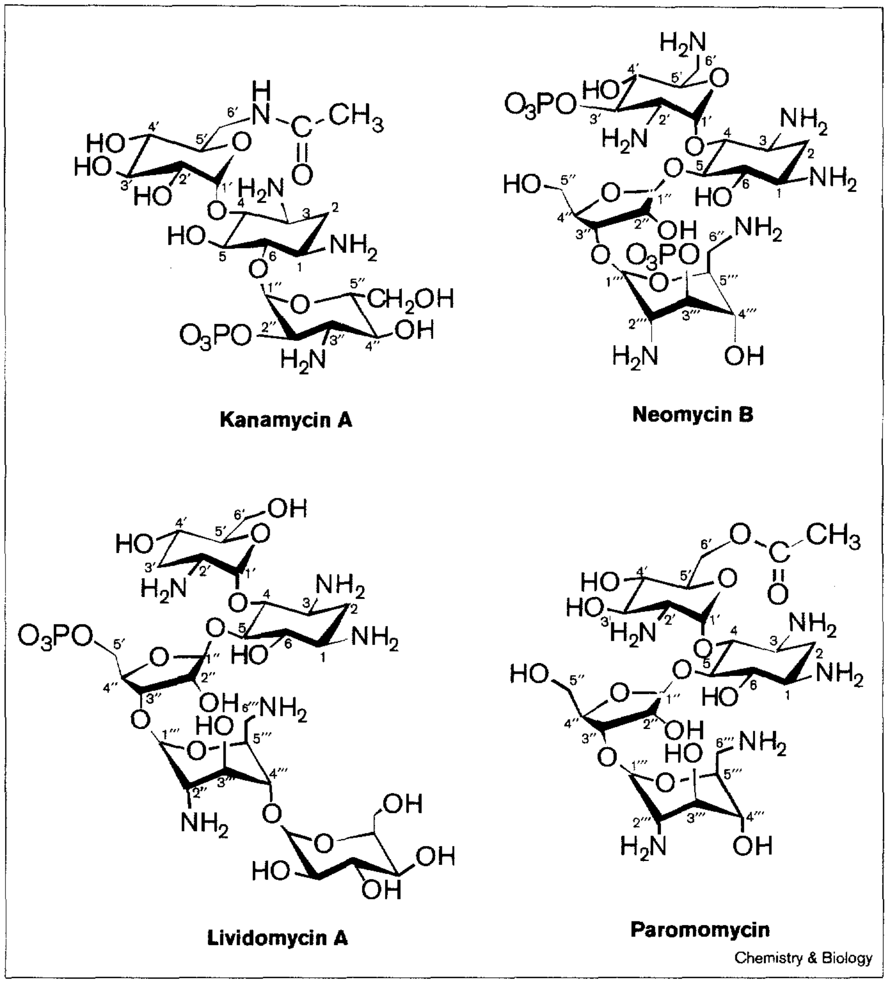
TOCSY experiments was that the phosphorus-coupled ^1H at 4.226 ppm was found to be part of the A ring spin system and could be assigned to position 3 of ring A, which is position 3′ in the aminoglycoside literature. A similar series of selective TOCSY experiments were performed in order to complete the chemical shift assignments of the D ring C-5 and C-6 protons (Table 4).

The unexpected identification of the sites of phosphorylation of neomycin as position 3′ and 3‴ prompted us to examine phosphorylation of the 3′-deoxy aminoglycoside lividomycin A. NMR analysis of the product of AAC(6′)-APH(2″) catalyzed phosphorylation of this compound revealed phosphorylation at the 5″-OH of the pentose ring as we have shown for APH(3′)-IIIa [25]. Thus the APH(2″) domain has 3′, 5″, 2″ and 3‴ phosphoryl transfer capacity (Figure 5).

Discussion

Clinical aminoglycoside resistance in enterococci and staphylococci is the result of acquisition of modifying enzymes found on mobile genetic elements. Chief among these resistance proteins is AAC(6′)-APH(2″) [7]. This bifunctional enzyme is devastatingly effective at conferring resistance to virtually all aminoglycosides that incorporate a 2-deoxystreptamine ring including the clinically important antibiotics gentamicin C, tobramycin, netilmicin and amikacin. The molecular basis for antibiotic resistance is the covalent modification of the drugs. Specifically, AAC(6′)-APH(2″) harbors both acetyl CoA-dependent

Figure 5



Kanamycin A

Neomycin B

Lividomycin A

Paromomycin

Chemistry & Biology

Products of AAC(6′)-APH(2″)-catalyzed acetylation and phosphorylation of aminoglycosides.

N-acetyltransferase and ATP-dependent *O*-phosphotransferase activities [16]. These results suggest that the gene has arisen by a fusion of *aac* and *aph* genes. Amino-acid sequence alignment revealed homology to other aminoglycoside *N*-acetyltransferases in the amino-terminal region and aminoglycoside kinases in the carboxy-terminal region, a designation that was confirmed by the construction of amino- and carboxy-terminal truncated enzymes [16]. As a first step in delineating the molecular mechanism of this important enzyme, we required a good source of pure AAC(6′)-APH(2″). Several attempts were made to prepare an *E. coli* overexpressing strain, but none of these proved fruitful (D.M.D. and G.D.W., unpublished observations), which led us to explore expression in *B. subtilis*. Placement of the bifunctional gene under control of the constitutive *vegII* promoter gave dramatically better yields of enzyme when compared with any of the *E. coli* strains and did not give any appreciable truncated product, which was a problem in *E. coli*. The reason for the improved yields in *B. subtilis* is unknown, but might reflect a superior ability of *B. subtilis* to adjust to a foreign mRNA structure, which results in poor expression or ribosome stalling in *E. coli*.

A three-step purification protocol gave excellent yields of pure 57 kDa bifunctional enzyme with both AAC and APH activities. Steady-state kinetic analysis confirmed the broad substrate range of the enzyme, which was predicted by the *in vivo* aminoglycoside resistance profile. The AAC activity shows remarkable substrate promiscuity. On the basis of these resistance profiles, the site of *N*-acetylation was predicted to be exclusively the primary amino group at C-6′ and this has recently been unambiguously established using NMR [20]. Unlike other known AAC(6′) enzymes, AAC(6′)-APH(2″) confers resistance to fortimicin through *N*-acetylation. All aminoglycosides with a free amino group at C6′ are excellent substrates with k_{cat}/K_m of the order of 10^5 M^{-1} s^{-1}. Aminoglycoside K_m values vary from roughly 1–26 μM and k_{cat} values from 0.2–3 s^{-1}, but k_{cat}/K_m is largely invariant over the 15 6′-amino-aminoglycosides analyzed (maximum $\Delta k_{cat}/K_m$ = 12.6). We were surprised to find that the 6′-hydroxy aminoglycosides paromomycin and lividomycin A are substrates for AAC(6′)-APH(2″) (Table 2). In comparison, these compounds are competitive inhibitors of kanamycin for *E. faecium* AAC(6′)-Ii [22]. The acetyl group is readily removed under basic conditions suggesting *O*-acetyltransfer is occurring and this activity was confirmed by infrared analysis of acetyl-paromomycin.

This unprecedented activity adds to the prodigious substrate range of the enzyme and serves as a cautionary note for future aminoglycoside development as amino or hydroxyl substitution is generally regarded as critical for biological activity. This is supported by recent NMR studies of paromomycin bound to the A site of the *E. coli* 16S rRNA, which have demonstrated at least one contact between the 6′-OH to the phosphate group of A1493 [29]. Substitution of the AAC(6′) susceptible amino group at this position by a hydroxyl group will therefore not escape the acetyl-transfer activity of the AAC(6′)-APH(2″) enzyme.

The kinase activity of the AAC(6′)-APH(2″) enzyme is highly tolerant of many different substitutions of the 6-amino pyranose ring (ring A). The presence or absence of hydroxyl groups at C3′ and C4′, derivatization of C2′ with OH or NH_2, methylation at C6′ or unsaturation at C4′-C5′ have only minor effects on specificity. Similarly, substitution of the 2-deoxystreptamine ring at C6 or at C5 with hexose or pentoses respectively, is tolerated. A dramatic effect is observed, however, with substituents at N1 of the 2-deoxystreptamine ring. Amikacin (N1-4-amino-2-hydroxy butyryl-kanamycin A) shows a 22-fold increase in K_m and a 2.5-fold decrease in k_{cat} over kanamycin A, and butirosin (N1-4-amino-2-hydroxy butyryl-ribostamicin) has a 5.8-fold higher K_m than ribostamicin. Similarly, netilmicin (N1-ethylsisomicin) has a threefold increase in K_m and twofold decrease in k_{cat} over the parent compound sisomicin, and isepamicin (N1-4-amino-2-hydroxybutyryl-N3″-acetylgentamicin B) has a fivefold increase in K_m over gentamicin B. Thus N1 substitution is detrimental to the APH activity.

Phosphorylation has been shown to occur at C2″ of the 3-amino hexose ring (ring C) of 4,6-disubstituted 2-deoxystreptamine aminoglycosides by heteronuclear NMR [20]. Consistent with this regiospecificity is the observation that compounds with a variety of substitutions at C5″ (H, CH_2OH) and C4″ (H, axial OH, equatorial CH_3) are tolerated as is *N*-methylation (gentamicins) or *N*-acetylation (isepamicin) of N3″. The 4,5-disubstituted 2-deoxystreptamine aminoglycosides, which have a pentose in ring C, are also efficiently phosphorylated. Aminoglycosides composed of two rings (neamine), three rings (ribostamycin and butirosin) and four rings (neomycin and paromomycin) are comparably phosphorylated, but the five-ring drug, lividomycin, shows a 15-fold greater K_m. Analysis of the products of neomycin phosphorylation revealed that the APH(2″) enzyme can also transfer a phosphate group to position 3′ of ring A and 3‴ of ring D. On the other hand, lividomycin is phosphorylated on position 5″ of the pentose ring. The K_m difference for this compound compared with the other 4,5-disubstituted aminoglycosides might therefore reflect either the influence of the E ring on substrate binding or a decrease in reactivity of the 5″-OH. Of course, these are not necessarily mutually exclusive. The important implication of these studies is that the APH(2″) activity is relatively impervious to the structure of the aminoglycoside, and therefore this enzyme is a highly potent drug resistance determinant.

Significance

The remarkable potential for both *N*- and *O*-acetyl transfer by the AAC(6′) activity coupled with the virtual indiscriminate capacity for phosphorylation of rings A, C and D of aminoglycosides by the APH(2″) domain of AAC(6′)-APH(2″) were unexpected and have sweeping implications. First, the broad specificities of both the acetyltransferase and kinase activities of AAC(6′)-APH(2″) give rise to an enzyme that has been exquisitely evolved to counter virtually any 2-deoxystreptamine aminoglycoside antibiotic. Second, the distinct co-substrate requirements and mechanisms of the AAC and APH domains mean that the identification of a single inhibitor molecule that would have the capacity to attenuate AAC(6′)-APH(2″) activity and therefore potentiate aminoglycoside activity is remote. The broad specificity of both activities, however, implies significant capacity to recognize diverse structures. It should therefore be possible to identify distinct inhibitory compounds for both domains that together could restore biological activity of clinically important aminoglycosides such as gentamicin C.

Materials and methods

Chemicals

Isepamicin, sisomicin and netilimicin were gifts of G. Miller, Schering Plough Research Institute and gentamicin B and fortimicin were gifts of J. Davies, University of British Columbia. Neamine was the gift of Shahriar Mobashery, Wayne State University. All other aminoglycosides, 4,4′-dithiodipyridine and pyruvate kinase-lactate dehydrogenase mix were from Sigma. [γ-^{32}P]-ATP was from Dupont-NEN. All restriction endonucleases and Vent DNA polymerase were from New England Biolabs. ATP and acetyl CoA were from Boehringer Manheim. AffiGel 15 was from BioRad, and all other chromatography resins were from Pharmacia.

Overexpression of AAC(6′)-APH(2″) in B. subtilis

The *aac(6′)-aph(2″)* gene was amplified by PCR using Vent DNA polymerase from plasmid pSF815A [16] (gift of G. Eliopoulos, New England Deaconess Hospital, Boston, MA) using the DNA primers P1 (5′-CCA GGT ACC *CAT ATG* AAT ATA GTT GAA AAT GAA) and P2 (5′-CCA *AAG CTT* GGA TCC TCA ATC TTT ATA AGT CCT TTT) that incorporate unique *Nde*I and *Hind*III restriction sites (denoted in italics), respectively. Plasmid pBF-9 was constructed by ligation of the 1.5 kb bifunctional digested with *Nde*I and *Hind*III with pKK223-3** digested with the same enzymes. Plasmid pKK223-3** was constructed by destruction of an internal *Nde*I site in pKK223-3* [30]. The construct pBF-9 places the bifunctional gene under control of the Tac promoter and contains the Shine-Delgarno sequence from gene 10 of bacteriophage T7. Plasmid pBF-14 was constructed by ligation of the

1.5 kb *XbaI* to *BamHI* fragment from pBF-9 with plasmid pRB374 [21] (gift from Reinhold Brückner of Mikrobielle Genetik, Universität Tübingen, Tübingen, Germany). This placed the gene under the transcriptional control of the constitutive vegetative promoter, *vegII*, and the translational control of the ribosome-binding site from gene10 of bacteriophage T7. This construct was used to transform the protease deficient strain *B. subtilis* 1A752 (*apr, bglT/bglS(Δ)EV, eglS(Δ)102, his, npr*) (obtained from D.R. Zeigler, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH) by electroporation using a Bio Rad Gene Pulser apparatus equipped with a pulse controller by minor modification of the method of Kusaoke *et al.* [31].

Preparation of gentamicin C-agarose affinity column

Affigel 15 (*N*-hydroxy succinimide activated agarose, BioRad) was washed with two volumes of isopropanol and six volumes of water. Gentamicin C complex (250 mg/ml) was then added in one volume of 100 mM NaHCO$_3$ pH 8.3 and the slurry was gently rocked at 4°C for 2 h and then incubated at room temperature for 2 h. The resin was filtered, placed in 1 M ethanolamine pH 8.3 to cap unreacted sites, and rocked at room temperature for 90 min. The suspension was filtered, washed with ten volumes of water, four volumes of 1.5 M (NH$_4$)SO$_4$, followed by another ten volumes of water and resuspended in 50 mM HEPES pH 7.5, 1 mM EDTA and kept at 4°C. The resin was stored in 5 mM NaN$_3$ when not in use.

Purification of AAC(6′)-APH(2″) from B. subtilis 1A752/pBF-14

A 25 ml overnight culture of *B. subtilis* 1A752/pBF-14 in Penassay broth (Difco antibiotic medium #3) served as inoculum for 10 l of Penassay broth supplemented with 200 µg/ml of gentamicin C. AAC(6′)-APH(2″) was purified from *B. subtilis* 1A752/pBF-14. The culture was grown at 37°C until the end of logarithmic phase (OD$_{600}$ = 1.6). The cells were collected by centrifugation at 5000 × g for 10 min and resuspended in 40 ml of lysis buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride and 0.1 mM dithiothreitol). Cell lysis was achieved by two consecutive passes through a French pressure cell at 20,000 psi. The cell debris was removed by centrifugation at 10,000 × g for 20 min and the lysate was applied to a Q Sepharose FF anion exchange column (bed vol. 200 ml). The matrix was washed with several column volumes of buffer A (50 mM HEPES pH 7.5, 1 mM EDTA) and AAC(6′)-APH(2″) was eluted with a linear gradient with buffer B (50 mM HEPES pH 7.5, 1 mM EDTA and 1 M NaCl). Fractions were assayed for both APH and AAC enzymatic activities (described below). Active fractions eluted between 350 mM and 500 mM NaCl. These were pooled and concentrated to a final volume of 5 ml over an Amicon PM30 ultrafiltration membrane. The concentrated sample was applied to a Sephadex G100 gel filtration column (bed vol. 560 ml) and eluted with buffer A + 200 mM NaCl. Active fractions were pooled, separated into two aliquots and dialyzed against buffer A.

Both aliquots were separately loaded onto a gentamicin C-agarose affinity column (bed vol. 50 ml) and washed with three column volumes of buffer A. The bifunctional enzyme was eluted from the column with a linear gradient of buffer B at a flow rate of 0.5 ml/min. The fractions containing AAC(6′)-APH(2″) were pooled, concentrated over an Amicon PM30 membrane, and dialyzed against 25 mM HEPES pH 7.5. Pure bifunctional enzyme retained activity for at least 2 months at 4°C.

Protein concentrations were determined using the Bradford method [32].

Enzyme assays

Routine assay for aminoglycoside phosphorylation during enzyme purification was performed using a phosphocellulose-binding assay as previously described [33] with kanamycin A and [γ-^{32}P]-ATP. For more detailed analyses using purified enzymes, phosphorylation of aminoglycoside substrates by AAC(6′)-APH(2″) was monitored using a pyruvate kinase/lactate dehydrogenase coupled assay system, which links the release of ADP to the oxidation of NADH to NAD$^+$, and the resulting decrease in absorbance at 340 nm was monitored spectrophotometrically using a Cary 3E UV–visible spectrophotometer [23]. Reactions contained 950 µl of assay buffer (50 mM HEPES pH 8.0, 10 mM MgCl$_2$, 40 mM KCl, 0.5 mg/ml NADH, 2.5 mM phosphoenolpyruvate and 1 mM ATP). A volume of 25 µl of aminoglycoside solution was added and the mixtures were preincubated at 36°C for 5 min. The reactions were initiated by the addition of 25 µl of enzyme solution (typically 0.8 mg/ml stock solution). Fractions throughout the enzyme purification were assayed with 100 µM kanamycin A and were corrected for the presence of contaminating ATPases when required by a control reaction lacking the aminoglycoside substrate.

All substrates for the kinetic assays were titrated using the coupled assay. Initial rates were obtained directly from the progress curves and then analyzed without external weighting by nonlinear least squares fit to equation 1 or equation 2 for reactions where substrate inhibition was observed, using the Grafit 3.0 software [34]. Kinetic constants are reported ± the standard error obtained from the fit of the data.

$$v = V_{max}S/(K_m + S) \qquad (1)$$

$$v = V_{max}S/(K_m + S + S^2/K_i) \qquad (2)$$

The acetylation of aminoglycoside substrates was monitored by coupling the acetylation of the aminoglycoside to the cleavage of 4,4′-dithiodipyridine which gives an increase in absorbance monitored at 324 nm (ε_m 19,800 M^{-1}cm^{-1}) [35]. Reactions contained 800 µl of assay buffer (50 mM HEPES pH 7.0, 1 mM EDTA, 2 mM 4,4′-dithiodipyridine, 80 µM acetyl CoA and 25 µl of aminoglycoside solution). The solutions were preincubated at 37°C for 5 min and then the reactions were initiated by the addition of 25 µl of enzyme solution (0.8 µg/ml). Initial rates were analyzed as described above.

Determination of regiospecificity of phosphoryl transfer by NMR

All spectra were recorded on a Bruker Avance DRX-500 NMR spectrometer. Proton spectra were acquired at 500.13 MHz using a 5 mm broadband inverse probe with triple axis gradient capability. Spectra were obtained in 120 scans in 32K data points over a 3.894 kHz spectral width (4.207 s acquisition time). Sample temperature was maintained at 30°C by a Bruker Eurotherm variable temperature unit. The free induction decay (FID) was processed using Gaussian multiplication (line broadening: −1.5 Hz, Gaussian broadening: 0.2). The FID was also zero-filled to 64K before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the absolute value mode using the pulse sequence 90°–t$_1$–45°– acq and included pulsed field gradients for coherence selection. The data were acquired in 48 scans for each of the 256 FIDs that contained 2K data points in the F2 dimension over a 3.205 kHz spectral width. The ^{1}H 90 pulse width was 6.6 µs. A 1.0 s relaxation delay was used between acquisitions. Zero-filling in the F1 dimension produced a 1K × 1K data matrix with a digital resolution of 3.130 Hz/point in both dimensions. During two-dimensional Fourier transformation a sine-bell squared window function was applied to both dimensions. The transformed data were then symmetrized.

Phosphorus-31 NMR spectra were recorded at 202.456 MHz using the 5 mm broadband inverse probe with triple axis gradient capability. The spectra were acquired over a 48.544 kHz spectral width in 32K data points (0.338 s acquisition time). The ^{31}P pulse width was 3.0 µs (30 flip angle). A relaxation delay of 0.5 s was used. The FID was processed using exponential multiplication (line broadening: 4.0 Hz) and zero-filled to 64K before Fourier transformation.

The transverse ROESY two-dimensional spectra were acquired in the phase sensitive mode. The data were acquired in 96 scans for each of the 128 FIDs that contained 2K data points in the F2 dimension over a 3.931 kHz spectral width. The ^{1}H 90 pulse width was 6.6 µs while the 90 pulse width for the spin locking pulse was 104.0 µs. A 1.0 s relaxation delay was used between acquisitions. A mixing time of 800.0 ms was used. Linear prediction to 256 data points followed by zero-filling in the F1 dimension produced a 1K × 1K data matrix with a digital resolution of 3.130 Hz/point in both dimensions. During the real two-dimensional Fourier transformation a sine-bell squared window function shifted by $\pi/2$ was applied to both dimensions. The transformed data were not symmetrized. The ROESY two-dimensional spectrum was plotted displaying negative contours only.

Selective one-dimensional TOCSY ^{1}H NMR spectra were recorded over a 4.006 KHz spectral width in 32K data points (4.089 s acquisition time). Gaussian shaped pulses were defined by 256 data points with the pulse being truncated at 1% of the maximum pulse amplitude. The 90 Gaussian pulse width was 25.5 µs. This pulse was followed by a 40 µs fixed delay and then by the standard TOCSY MLEV-17 spin lock pulse sequence. The 90 spin lock pulse width was 27.0 µs. A 1.0 s relaxation delay was used. Spin lock periods of either 40.0 or 120.0 ms were used and this was followed by a z-filter which contained 5 variable delay times ranging from 4.0 to 18.0 ms. The transmitter offset was adjusted to the frequency of the ^{1}H being selectively excited. Thirty-two scans were acquired for each of the delays in the z-filter resulting in a total of 160 scans. The FID was processed using Gaussian multiplication (line broadening: –1.5 Hz; Gaussian broadening: 0.2) and was zero-filled to 64K before Fourier transformation.

The compounds used in this study were dissolved in 99.996% D_2O (Isotec, Inc.) to a concentration of approximately 3.0 mg/ml for neomycin-bisphosphate and 15.0 mg/ml for neomycin. Chemical shifts are reported in ppm relative to TMS using the HDO signals at 4.60 ppm as internal reference for the ^{1}H NMR spectra Phosphorus-31 NMR spectra were referenced using external 85% phosphoric acid in D_2O.

Base hydrolysis of acetylated aminoglycosides

Reactions contained 100 µM aminoglycoside, 36.4 µM [^{14}C]-acetyl CoA (1.2×10^5 cpm/nmol), AAC(6')-APH(") (2.6 pmol) and HEPES HCl buffer, pH 7.5 in a final volume of 10 µl. Following completion of the acetyl transfer reaction, 10 µl of 80 mM NaOH was added and the reaction mixtures incubated at 80°C (final pH 11–12). Samples were neutralized with the addition of 10 µl of 1 M HEPES pH 7.5 and analyzed by application of the solution on Whatman P-81 phosphocellulose paper. The papers were washed three times in water, dried, and the amount of radioactivity bound determined by scintillation counting.

Thin layer chromatography, purification and infrared spectroscopy

O-acetylated paromomycin was produced by incubating 5 mg of the aminoglycoside (150 µM), 150 µM acetyl Coenzyme A, 300 nM AAC(6')-APH(2") in 50 ml of 50 mM HEPES pH 7.5 for 4 h. The modified aminoglycoside product was partially purified by batch anion exchange chromatography on AG50W-X8 resin with a 1% NH_4OH step gradient. The eluted product was concentrated by lyophilization and resuspended in 1 ml of water. Samples were then separated on Whatman PLK-5 glass-backed silica plates using a 5:2 methanol:ammonium hydroxide mobile phase. The R_f of the product was 0.23 whereas that of paromomycin was 0.35. Silica was scraped off the plate and the acetylated aminoglycoside was extracted with 15 ml of methanol under mildly acidic conditions. Samples were neutralized by the dropwise addition of NaOH followed by lyophilization. The dried product was ground into a KBr pellet and analyzed by infrared spectroscopy using a BioRad FTS-40 Fourier Transform Infrared Spectrometer.

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