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Recognition of Aminoglycoside Antibiotics by Enterococcal-Staphylococcal Aminoglycoside 3′-Phosphotransferase Type IIIa: Role of Substrate Amino Groups

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The interactions of the aminoglycoside 3′-phosphotransferase IIIa with aminoglycoside antibiotics lacking specific amino groups were examined by steady-state kinetic analyses. The results demonstrate that an amino group on C-1 and either an amino or a hydroxyl group at the 2′ and 6′ positions are important for detoxification of aminoglycosides by this enzyme.

Aminoglycoside antibiotics are clinically important bactericidal drugs which are useful in the treatment of many nosocomial infections (4). They are especially important against enterococcal infections, for which they are used synergistically with a β-lactam antibiotic (7, 8). The only current alternative treatment for enterococcal infections is vancomycin, but the emergence of resistance to this drug is a growing clinical problem. Therefore, given the current therapeutic reality, enterococcal resistance to aminoglycosides has taken on a more ominous importance. The aminoglycoside phosphotransferases (APH) constitute a family of enzymes widespread in both gram-positive and gram-negative bacteria which confer resistance to a wide spectrum of aminoglycosides (3). These intracellular enzymes transfer the γ-phosphate from ATP to a hydroxyl group on the drug, a process which effectively obviates the antibiotic action of these cationic molecules.

The APH(3′) subfamily of enzymes is composed of at least seven different isozymes, which show roughly 40% amino acid sequence similarity, and all share the capacity to phosphorylate a variety of aminoglycosides (10). The enzyme APH(3′)-IIIa is found in enterococci and staphylococci and confers resistance to a broad range of drugs including kanamycin, amikacin, neomycin, and lividomycin (5, 14). Inactivation of lividomycin, a 3′-deoxyaminoglycoside antibiotic, has been shown to be due to phosphorylation at the 5″-OH of the pentose ring (13). Overexpression of the enzyme in *Escherichia coli* has permitted detailed study of the mechanism and specificity of APH(3′)-IIIa (5).

While APH(3′)-IIIa will phosphorylate many aminoglycosides, the central 2-deoxystreptamine aminocyclitol ring is essential for catalysis (5). Thus, neamine (Fig. 1) represents the minimal substrate which will be phosphorylated by APH(3′)-IIIa, but the role of individual substrate amino groups in catalysis is not known. The recent synthesis of several aminoglycosides lacking amino groups at specific sites (9) has permitted the evaluation of their role in APH(3′)-IIIa-catalyzed phosphotransfer. The structure of the compounds tested is shown in Fig. 1. The steady-state kinetic parameters were determined with purified overexpressed APH(3′)-IIIa using a continuous coupled assay as previously described (5), and the results are summarized in Table 1.

Removal of the amino groups from the 2-deoxystreptamine ring has significant implications for catalysis. First, loss of the C-1 amino group (compound 1) results in a decrease in specificity as evidenced by a fivefold lowering of k_{cat}/K_m. This is consistent with our observation that amikacin, a compound in which the C-1 amino group has been substituted by an (*S*)-4-amino-2-hydroxybutyrylamide group, is also a poor substrate for APH(3′)-IIIa ($k_{cat}/K_m = 0.1 \times 10^5$ [5]). However, removal of the amino group linked to C-3 (compound 2) has no effect on catalysis. Therefore, of the two aminocylitol amino groups, only the C-1 amino group plays an important role in APH(3′)-IIIa recognition.

Loss of the amino groups in the 6-aminohexose ring proved highly significant. Removal of the amino group from C-2′ (compound 3) resulted in a 10-fold loss of specificity, while loss of the 6′-amino group had an even more dramatic effect with a 30-fold decrease in k_{cat}/K_m. The importance of the N-6 amino group was emphasized with the kanamycin analog compound 5 for which specificity dropped 150-fold. In both compounds, most of the effect is borne by the K_m. The kinetic mechanism of APH(3′)-IIIa is a Theorell-Chance ordered mechanism where ATP binds first and then the aminoglycoside (6). In such a mechanism, the aminoglycoside K_m is a ratio of the first-order rate of release of ADP from the enzyme (k_{cat} in this case) and the second-order rate of binding of the drug to the active site. Since k_{cat} is generally not grossly affected with these compounds (Table 1), K_m is therefore a good indicator of the affinity of the enzyme for the drug, and thus the 6′-amino group plays a critical role in substrate binding.

The effect of removing the amino group from C-3″ (compound 6) was modest, consistent with our observation that neamine represents the minimal, and essential, aminoglycoside recognition unit.

The pK_a values for neomycin amino groups have been determined by ^{15}N-nuclear magnetic resonance, and N-1, N-2′, and N-6′ have pK_a values of 8.04, 7.55, and 8.60, respectively (1). Therefore, at physiologic pH these amino groups should be largely protonated. Since loss of these groups results in a decrease in specificity, it is possible that they provide specific ionic interactions with the enzyme. However, kanamycin A and kanamycin B, which differ only in that they have a hydroxyl or an amino group, respectively, at position 2′, are equally good substrates (5); therefore, interaction with a heteroatom in the

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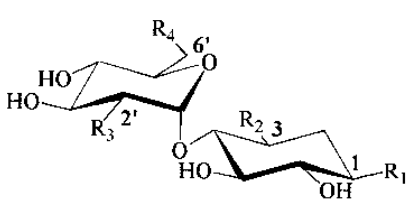
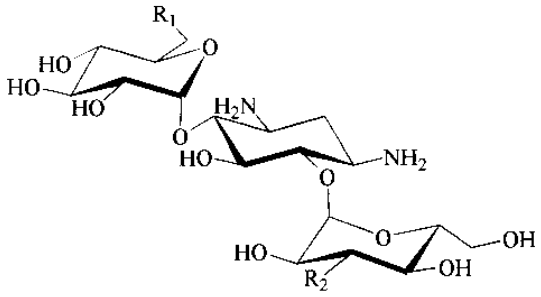


Neamine R_1 = $R_2 = R_3 = R_4 = NH_3^+$

Compound **1** $R_1 = H$, $R_2 = R_3 = R_4 = NH_3^+$
Compound **2** $R_2 = H$, $R_1 = R_3 = R_4 = NH_3^+$
Compound **3** $R_3 = H$, $R_1 = R_2 = R_4 = NH_3^+$
Compound **4** $R_4 = H$, $R_1 = R_2 = R_3 = NH_3^+$



Kanamycin A R_1 = $R_2 = NH_3^+$
Compound **5** $R_1 = H$, $R_2 = NH_3^+$
Compound **6** $R_2 = H$, $R_1 = NH_3^+$

FIG. 1. Structures of aminoglycosides examined in this study.

enzyme active site at this position is more likely due to hydrogen bonding.

Similarly, compounds such as paromomycin and lividomycin A, which replace the 6′-amino group with a hydroxyl, are excellent APH(3′)-IIIa substrates (5). In these cases also, hydrogen bonding rather than charged pair interactions would be predicted to be involved in substrate binding. Removal of the NH_2 group from C-6′ has a greater effect on k_{cat}/K_m than loss of the N-2′ (Table 1), and this difference may lie in the strength of the hydrogen bonding interaction(s). We have recently determined the three-dimensional structures of the aminoglycosides amikacin and butirosin bound to an APH(3′)-IIIa · Cr-ATP complex using nuclear magnetic resonance (2). The structures which emerged from this study demonstrate that these compounds bind to the enzyme in significantly different conformations. Importantly, the positions of the 6′-amino groups do not overlap. Therefore, the comparison of the butirosin-like compounds such as paramomycin and lividomycin A bearing 6′-hydroxyls with the 6′-amino compound kanamycin (which has a similar structure as amikacin) may not hold. Thus, we cannot rule out an ion-pairing interaction with the 6′-NH_3^+ of neamine and kanamycin with the active site residues lining the APH(3′)-IIIa active site. Regardless of the precise nature of the electrostatic interactions, a heteroatom linked to C-6′ is preferred for efficient aminoglycoside detoxification by APH (3′)-IIIa.

TABLE 1. Steady-state kinetic parameters for the synthetic aminoglycoside substrates

Substrate	K_m (μM)	k_{cat} (s^{-1})	K_i[a] (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$) × 10^{-5}
Neamine	20.0 ± 2.8	2.00 ± 0.15	2.00 ± 0.4	1.0
Compound 1	51.8 ± 6.0	1.03 ± 0.05	ND[b]	0.2
Compound 2	7.1 ± 2.5	1.16 ± 0.11	ND	1.6
Compound 3	35.9 ± 5.7	0.39 ± 0.01	ND	0.11
Compound 4	81.8 ± 20.7	0.278 ± 0.038	ND	0.034
Kanamycin A	12.6 ± 2.6	1.76 ± 0.06	6.3 ± 1.6	1.4
Compound 5[c]	1,000 ± 580	0.93 ± 0.41	ND	0.0093
Compound 6[d]	21.3 ± 3.0	1.52 ± 0.11	0.56 ± 0.16	0.71

[a] K_i is due to substrate inhibition resulting from binding of unphosphorylated aminoglycoside to the ADP · APH(3′)-IIIa complex (6).
[b] ND, no substrate inhibition detected.
[c] Compound 6 in reference 9.
[d] Compound 7 in reference 9.

These results are also important in that they are significantly different from those obtained with the gram-negative enzymes APH(3′)-Ia (12) and APH(3′)-IIa (11). These enzymes show 49 and 47% amino acid sequence similarity to APH(3′)-IIIa, respectively, but the effects that compounds 1, 2, 3, 4, 5, and 6 had on specificity with these enzymes were orders of magnitude more severe than with APH(3′)-IIIa (9), and these effects were primarily on k_{cat} rather than K_m. These differences are consistent with the fact that APH(3′)-Ia and IIa show generally a higher specificity for aminoglycosides, as evidenced by 10- to 100-fold higher k_{cat}/K_m (11, 12). In addition, the kinetic mechanism of APH(3′)-IIIa is Theorell-Chance (6), a form of an ordered BiBi mechanism, while the kinetic mechanism of APH (3′)-Ia is random-equilibrium BiBi (12). Taken together, these results demonstrate that the APH(3′) enzymes, despite a significant amino acid sequence homology, are surprisingly mechanistically different. Specifically, compared with the gram-negative enzymes, it appears that APH(3′)-IIIa has traded off a decrease in catalytic efficiency for the capacity to phosphorylate a wide spectrum of aminoglycosides. These differences in mechanism and substrate specificity need to be reconciled for future development of broad-specificity APH inhibitors.

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