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Revisiting the Nucleotide and Aminoglycoside Substrate Specificity of the Bifunctional Aminoglycoside Acetyltransferase(6′)-Ie/Aminoglycoside Phosphotransferase(2″)-Ia Enzyme*

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Hilary Frase, Marta Toth, and Sergei B. Vakulenko[1]

From the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Background: The bifunctional AAC(6′)-Ie/APH(2″)-Ia enzyme was reported to phosphorylate all classes of aminoglycoside antibiotics using ATP.
Results: GTP, and not ATP, is the cosubstrate of the enzyme. 4,5-disubstituted and atypical aminoglycosides are not substrates.
Conclusion: The enzyme is a narrow spectrum GTP-dependent kinase that phosphorylates 4,6-disubstituted aminoglycosides exclusively.
Significance: Knowledge of enzyme activity is essential for developing novel antibiotics and conducting effective antimicrobial therapy.

The bifunctional aminoglycoside-modifying enzyme aminoglycoside acetyltransferase(6′)-Ie/aminoglycoside phosphotransferase(2″)-Ia, or AAC(6′)-Ie/APH(2″)-Ia, is the major source of aminoglycoside resistance in Gram-positive bacterial pathogens. In previous studies, using ATP as the cosubstrate, it was reported that the APH(2″)-Ia domain of this enzyme is unique among aminoglycoside phosphotransferases, having the ability to inactivate an unusually broad spectrum of aminoglycosides, including 4,6- and 4,5-disubstituted and atypical. We recently demonstrated that GTP, and not ATP, is the preferred cosubstrate of this enzyme. We now show, using competition assays between ATP and GTP, that GTP is the exclusive phosphate donor at intracellular nucleotide levels. In light of these findings, we reevaluated the substrate profile of the phosphotransferase domain of this clinically important enzyme. Steady-state kinetic characterization using the phosphate donor GTP demonstrates that AAC(6′)-Ie/APH(2″)-Ia phosphorylates 4,6-disubstituted aminoglycosides with high efficiency (k_{cat}/K_m = 10^5-10^7 M^{-1} s^{-1}). Despite this proficiency, no resistance is conferred to some of these antibiotics by the enzyme *in vivo*. We now show that phosphorylation of 4,5-disubstituted and atypical aminoglycosides are negligible and thus these antibiotics are not substrates. Instead, these aminoglycosides tend to stimulate an intrinsic GTPase activity of the enzyme. Taken together, our data show that the bifunctional enzyme efficiently phosphorylates only 4,6-disubstituted antibiotics; however, phosphorylation does not necessarily result in bacterial resistance. Hence, the APH(2″)-Ia domain of the bifunctional AAC(6′)-Ie/APH(2″)-Ia enzyme is a *bona fide* GTP-dependent kinase with a narrow substrate profile, including only 4,6-disubstituted aminoglycosides.

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[1] To whom correspondence should be addressed: 417 Nieuwland Science Hall, Dept. of Chemistry and Biochemistry, Notre Dame, IN 46556. Tel.: 574-631-2935; Fax: 574-631-6652; E-mail: svakulen@nd.edu.

Aminoglycosides are broad spectrum antibiotics used to treat a wide variety of bacterial infections caused by both Gram-negative and Gram-positive pathogens (1). Based on their structure, they are broadly classified into three major classes: 4,6-disubstituted, 4,5-disubstituted, and atypical (Fig. 1). The most important mechanism of bacterial resistance to this class of antibiotics is enzymatic modification, which greatly diminishes the affinity of aminoglycosides for the bacterial 30 S ribosomal subunit, their target *in vivo* (2, 3). Three families of aminoglycoside-modifying enzymes are known: aminoglycoside *O*-nucleotidyltransferases, aminoglycoside *N*-acetyltransferases, and aminoglycoside *O*-phosphotransferases. Most of these enzymes are monofunctional; however, a few bifunctional enzymes, thought to result from the fusion of two genes, are known to exist (4).

The bifunctional AAC(6′)-Ie/APH(2″)-Ia[2] enzyme is the most clinically important aminoglycoside-modifying enzyme in Gram-positive bacteria, responsible for high-level resistance in both *Enterococcus* and *Staphylococcus* (3, 4). The gene for this unique enzyme encodes an N-terminal AAC(6′) domain and a C-terminal APH(2″) domain. Both domains can function independently (5) and have been reported to inactivate, through phosphorylation and/or acetylation, most aminoglycoside antibiotics (6, 7) (Fig. 2).

Earlier enzymological studies of AAC(6′)-Ie/APH(2″)-Ia have led to the notion that APH(2″)-Ia is unique among aminoglycoside phosphotransferases due to its extremely broad substrate specificity (6). It was reported that both 4,6-disubstituted and 4,5-disubstituted aminoglycosides and neamine, an atypical aminoglycoside, are substrates for APH(2″)-Ia when ATP is used as the cosubstrate (6). This has led to the assumption that aminoglycoside antibiotics cannot be used for the treat-

[2] The abbreviations used are: AAC(6′)-Ie/APH(2″)-Ia, aminoglycoside(6′) acetyltransferase Ie/aminoglycoside(2″) phosphotransferase Ia; AAC, aminoglycoside *N*-acetyltransferase; APH, aminoglycoside *O*-phosphotransferase; DLU, density light unit; MIC, minimal inhibitory concentration.

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