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Correlation of Aminoglycoside Resistance with the K_ms and V_{max}/K_m Ratios of Enzymatic Modification of Aminoglycosides by 2″-*O*-Nucleotidyltransferase

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A clinical isolate, *Serratia marcescens* 75, was found to be susceptible to netilmicin and yet had a high level of aminoglycoside 2″-*O*-nucleotidyltransferase activity for netilmicin. Kinetic studies of the partially purified enzyme revealed substrate inhibition for gentamicin and tobramycin at concentrations greater than 10^{-2} mM, but this was not observed for netilmicin. The MICs of the aminoglycosides tested exhibited a good inverse correlation with the K_m values for the enzyme and a direct correlation with the V_{max}/K_m ratios of the enzyme.

Enzymatic modification of aminoglycosides does not necessarily confer resistance to the drugs (1, 3, 4, 10). We describe a clinical isolate, *Serratia marcescens* 75, which was highly susceptible to netilmicin and yet had a high level of aminoglycoside 2″-*O*-nucleotidyltransferase [ANT(2″)] activity for netilmicin when assayed with excess aminoglycoside substrate. We examined the relationship between the MICs of netilmicin, tobramycin, and gentamicin and the kinetic parameters of the ANT(2″) enzyme which modified each of these aminoglycosides.

S. marcescens 75 was tested for susceptibility to aminoglycosides by an agar dilution method (7). Inocula of 10^4 CFU of cells grown overnight in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) were applied with a pronged replicator (7) onto Mueller-Hinton agar (BBL) containing the test antibiotics in twofold dilutions. The strain was highly resistant to gentamicin and tobramycin, but susceptible to netilmicin (Table 1).

Sonic extracts of *S. marcescens* 75 were prepared for the assay of aminoglycoside-modifying activity as previously described (9). Aminoglycoside-modifying activity was assayed by adsorption of the radioactive product onto phosphocellulose filter disks (Whatman P-81) by the method of Haas and Dowding (5). For the adenylylating assay, the final concentrations of reagents were as follows: 0.033 M Tris-hydrochloride, pH 7.8; 7.5 mM $MgCl_2$; 2.5 mM dithiothreitol; 0.33 mM [^{14}C]ATP (4 Ci/mol) (Amersham Corp., Arlington Heights, Ill.); and 0.167 mM aminoglycoside. The reaction mixture plus enzyme was incubated for 20 min at 37°C.

Sonic extracts of *S. marcescens* 75 had substantial adenylylating activity for gentamicin, tobramycin, netilmicin, and kanamycin, but none for amikacin. This was compatible with ANT(2″), except that netilmicin is said to be a poor substrate. Under the conditions of our assay, the activity for netilmicin was ca. 40% greater than that for gentamicin and roughly comparable to that for tobramycin. The sonic extracts had negligible acetylating or phosphorylating activity when assayed similarily with [^{14}C]acetyl coenzyme A or ^{32}P, respectively.

To demonstrate that the activity of the ANT(2″) was solely responsible for the high-level resistance to gentamicin and tobramycin and yet did not confer resistance to netilmicin, even in another genetic background, we transferred resistance to tobramycin into *Escherichia coli* JSRO-N, a plasmid-free, nalidixic acid-resistant recipient. Transconjugants were selected with a frequency of 5×10^{-6} per donor cell on Mueller-Hinton agar containing tobramycin at 12.5 μg/ml and nalidixic acid as the counterselecting agent at 25 μg/ml. The MICs of gentamicin, tobramycin, and netilmicin were 50, 50, and 1.56 μg/ml, respectively. Sonic extracts from two transconjugants chosen for study had adenylylating activity for gentamicin, tobramycin, netilmicin, and kanamycin at ca. 50% the level of the activity from the donor strain. Again, there was negligible acetylating or phosphorylating activity.

An 18-liter culture of *S. marcescens* 75 grown in brain heart infusion broth containing 15 μg of gentamicin per ml was incubated with agitation at 37°C until the optical density at 600 nm was 1.5. The cells were harvested, washed twice with 0.15 M NaCl, and centrifuged again. The final pellet was suspended in 100 ml of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.9, containing 10% glycerol and 0.01% β-mercaptoethanol) and sonicated on ice. The sonic extract was centrifuged at 4°C at 18,000 × *g* for 30 min. The extract supernatant was subjected to streptomycin sulfate and ammonium sulfate precipitations. Dialysis against HEPES buffer was then performed to remove the salt. The suspension was concentrated in the Amicon micro-concentrator system, and the sample was then loaded onto a Sephadex G75 Superfine chromatography column. Elution from the G75 column gave a 25-fold purification of the ANT(2″) enzyme (6). Further attempts to purify the enzyme with DEAE chromatography or affinity chromatography with tobramycin as the ligand were unsuccessful.

The Sephadex G75-purified fractions were used to conduct the enzyme kinetic studies. Scrupulous attention to enzyme storage conditions was necessary due to enzyme instability in vitro. We observed ca. a 50% loss of enzyme activity when it was stored at 0°C for 24 h. No appreciable loss of activity was noted when it was stored at −70°C. Therefore, the partially purified enzyme was stored in multiple vials at −70°C until needed for kinetic assays.

For determining enzyme kinetics for the partially purified ANT(2″), the standard adenylylating assay was modified as follows. The total volume of the reaction mixture was increased to 0.250 ml; the final concentration of [^{14}C]ATP (8

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TABLE 1. Aminoglycoside susceptibility of *S. marcescens* 75 and the kinetic parameters of its ANT(2″)

Aminoglycoside	MIC (μg/ml)	V_{max}[a]	K_m (mM)	V_{max}/K_m
Netilmicin	1.56	7.7	6.8×10^{-2}	1.1×10^{2}
Gentamicin	50	3.5	5.9×10^{-4}	5.9×10^{3}
Tobramycin	100	7.0	5.0×10^{-4}	1.4×10^{4}

[a] Expressed as nanomoles per minute per milligram of protein.

Ci/mol) was 0.66 mM. The presence of substrate inhibition required careful measurements of velocities at very low aminoglycoside concentrations. The substrate concentrations varied between 0.167 mM and 4.2 μM. The reaction mixture was preincubated at 37°C before starting the reaction with 0.05 ml of enzyme. For assays with substrate concentrations above 0.01 mM, samples of 0.045 ml were spotted onto phosphocellulose disks at 1-min intervals for a total of 5 min. With lower substrate concentrations, samples were obtained every 30 s for 2.5 min. Controls contained no enzyme. All runs were done at least in duplicate. Kinetics were performed for gentamicin, netilmicin, and tobramycin. Plots of [S]/V versus [S] were used for determination of the K_m/V_{max} values for each aminoglycoside.

In the presence of substrate excess (0.167 mM aminoglycoside), the adenylylating assay was linear for ca. 12 min for gentamicin and tobramycin and ca. 20 min for netilmicin. When aminoglycoside concentrations were varied, substrate inhibition was noted for gentamicin and tobramycin at concentrations greater than 10^{-2} mM. No substrate inhibition was noted for netilmicin. K_m and V_{max} values obtained from plots of [S]/V versus [S] are shown in Table 1. The K_m for netilmicin was ca. 100 times greater than that for gentamicin and tobramycin. The V_{max} values for netilmicin and tobramycin were similar and were twice that for gentamicin. Thus, the V_{max}/K_m ratio for tobramycin, with an MIC of 100 μg/ml, was 100 times greater than the V_{max}/K_m for netilmicin (MIC of 1.56 μg/ml) and approximately twice that for gentamicin (MIC of 50 μg/ml).

Several investigators have recognized that aminoglycoside-modifying activity, when assayed with excess aminoglycoside substrate, does not necessarily correlate with levels of resistance to the various aminoglycosides (1, 4, 10). Our findings of a low MIC of netilmicin despite high ANT(2″) activity in *S. marcescens* 75 confirm this. Vastola et al. (10) concluded that the discrepancy between modifying activity and MICs could be explained on the basis of how good a substrate the aminoglycoside was for the enzyme, i.e., the K_m. For ANT(2″), 3-*N*-acetyltransferase [AAC(3)-I], and 2′-*N*-acetyltransferase [AAC(2′)], they showed that aminoglycosides which had low MICs had high K_ms. However, Radika and Northrop (8) and Bongaerts and Molendijk (2) reported a poor correlation between MICs and the K_m values for 6′-*N*-acetyltransferase [AAC(6′)] and ANT(2″) enzymes, respectively. These investigators maintained that only the V_{max}/K_m ratio was predictive of the MICs. In contrast, we found that both the K_m and V_{max}/K_m of ANT(2″) from *S. marcescens* 75 were indicators of resistance: a low K_m yielded a high MIC; a high V_{max}/K_m yielded a high MIC.

It is possible that our kinetic data for ANT(2″) and those of Bongaert and Molendijk differ because the enzyme was expressed in different bacteria, i.e., *S. marcescens* 75 in our studies and *E. coli* L58058.1 in theirs. However, our conjugation experiments showed that the ANT(2″) enzyme, when expressed in the *E. coli* transconjugants, had approximately the same aminoglycoside-modifying activity and MICs as did the *Serratia* donor strain.

Finally, we confirmed the previous report (2) that substrate inhibition of ANT(2″) occurred at aminoglycoside concentrations greater than 10^{-2} mM for gentamicin and tobramycin, but not for netilmicin. It is unclear whether substrate inhibition has any mechanistic significance. However, it is interesting to note that *S. marcescens* 75 was highly resistant to gentamicin and tobramycin, both of which exhibited substrate inhibition, whereas it was highly susceptible to netilmicin, which did not show substrate inhibition. It may be that substrate inhibition, in addition to K_m and V_{max}/K_m, is an important indicator of the ability to confer aminoglycoside resistance. On the other hand, the lack of substrate inhibition may simply reflect the lower saturation of the enzyme with netilmicin than with gentamicin or tobramycin at the concentrations tested.

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