Selden, L. A., Gershman, L. C., Kinosian, H. J., & Estes, J. E. (1987) *FEBS Lett. 217*, 89–93.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Storrer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 1–5.
Straub, F., & Feuer, G. (1950) *Biochim. Biophys. Acta 4*, 455–470.
Thames, K. E., Cheung, H. C., & Harvey, S. C. (1974) *Biochem. Biophys. Res. Commun. 60*, 1252–1261.
Tsien, R. Y. (1980) *Biochemistry 19*, 2396–2404.
Waechter, F. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1821–1822.
Waechter, F., & Engel, J. (1975) *Eur. J. Biochem. 57*, 453–459.
Waechter, F., & Engel, J. (1977) *Eur. J. Biochem. 74*, 227–232.
Wang, Y.-L., & Taylor, D. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5503–5507.
Wanger, M., & Wegner, A. (1983) *FEBS Lett. 162*, 112–116.
Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Zimmerle, C. T., & Frieden, C. (1986) *Biochemistry 25*, 4899–4906.
Zimmerle, C. T., Patane, K., & Frieden, C. (1987) *Biochemistry 26*, 6545–6552.

Substrate Specificities and Structure–Activity Relationships for the Nucleotidylation of Antibiotics Catalyzed by Aminoglycoside Nucleotidyltransferase 2″-I†

Cynthia A. Gates‡ and Dexter B. Northrop*
Division of Pharmaceutical Biochemistry, School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706
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ABSTRACT: Aminoglycoside nucleotidyltransferase 2″-I (formerly gentamicin adenylyltransferase) conveys antibiotic resistance to Gram-negative bacteria by transfer of AMP to the 2″-hydroxyl group of 4,6-substituted deoxystreptamine-containing aminoglycosides. The kinetic constants of thirteen aminoglycoside antibiotics and the magnesium chelates of eight nucleotide triphosphates were determined with purified enzyme. Eleven of the antibiotics exhibit substrate inhibition attributed to secondary binding of the aminoglycoside to an enzyme–AMP–aminoglycoside complex. Maximal velocities vary by only 4-fold, versus variation of values of V_{max}/K_m for the aminoglycosides of nearly 4000-fold, consistent with a Theorell–Chance kinetic mechanism as proposed for this enzyme [Gates, C. A., & Northrop, D. B. (1988) *Biochemistry* (second of three papers in this issue)] with the added specification that the binding of aminoglycosides is in rapid equilibrium. Under these conditions, V_{max}/K_m becomes k_{cat}/K_d, where k_{cat} is the net rate constant for catalysis (but not turnover) and K_d is the dissociation constant of aminoglycosides from a complex with enzyme and nucleotide. Values of k_{cat} fall closely together into three distinct sets, with the 3′,4′-dideoxygentamicins > gentamicins > kanamycins. These sets reflect unusual structure–activity correlations which are specific for catalysis but have nothing to do with the maximal velocity of this enzyme. The contribution of individual functional groups to binding was evaluated according to K_d values generated from substrate inhibition; specifically, binding is reduced by esterification at the 6″-carbon, hydroxylation of the 2′-carbon, unsaturation of the 4′,5′ carbon–carbon bond, methylation of the 6′-carbon or the 6′-amino groups, and ethylation of the 1-amino group. Comparisons between gentamicins and kanamycins are inconsistent with a common site of adenylylation at the 2″-hydroxyl but suggest either the 3′- or 4′-hydroxyl of the former. Unfortunately for the search for better antibiotics, most structure–activity relationships of enzymatic activity parallel antibiotic activity, with two exceptions being alkylation of the 1-amino group and stereochemical repositioning of the 5-hydroxyl group.

Aminoglycoside nucleotidyltransferase 2″-I [ANT(2″)-I][1] (EC 2.7.7.46) catalyzes the transfer of nucleotides to the 2″-hydroxyl group of 4,6-substituted deoxystreptamine-containing aminoglycoside antibiotics, accompanied by the release of pyrophosphate, according to the reaction:

$$\text{Mg–ATP} + \text{aminoglycoside} \rightarrow \text{Mg–PP}_i + \text{2″-AMP–aminoglycoside}$$

ANT(2″)-I was first identified as the biochemical basis for R factor mediated resistance to gentamicin by Benveniste and Davies (1971), who demonstrated a requirement for ATP in

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* Author to whom correspondence should be addressed.
‡ Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

[1] The enzyme was originally designated AAD(2″) by the plasmid nomenclature group, but the change to ANT(2″) was suggested since the nucleotide transferred to the antibiotic is not limited to adenine (Van Pelt & Northrop, 1984). The enzyme was further defined as ANT(2″)-I upon the discovery of a second aminoglycoside nucleotidyltransferase, designated ANT(2″)-II, with a different substrate range than that of ANT(2″)-I (Coombe & George, 1981).

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the inactivation of gentamicin and kanamycin by cell-free extracts of *Escherichia coli* K12 containing pJR66. This plasmid originated from a gentamicin-resistant strain of *Klebsiella pneumoniae*, isolated by Martin et al. (1971). Naganawa et al. (1971) identified the product of kanamycin inactivation by *E. coli* extracts containing pJR66 as adenylylation of the 2″-hydroxyl group. Further transfers of pJR66 to other recipient bacteria revealed that resistance was conferred by two plasmids, one containing the genes encoding a phosphotransferase [APH(3′)] and an adenylyltransferase [AAD(3″)] and the other containing genes for ANT(2″)-I plus APH(3′) and AAD(3″). The latter plasmid was designated pJR66b (Yagisawa & Davies, 1979). Yagisawa later constructed a chimeric plasmid containing an *Eco*RI restriction fragment cloned into a pVH52 vector and named the plasmid pMY10 (personal communication).

Taking advantage of the fact that *E. coli* W677 containing pMY10 produced larger amounts of ANT(2″)-I, Van Pelt and Northrop (1984) developed a spectrophotometric assay, purified the enzyme, and made a preliminary kinetic characterization, noting that the substrate specificity is not stringent for either nucleotides or aminoglycosides. Previous substrate profiles based on the relative efficiency of aminoglycosides to accept a [^{14}C]adenosine monophosphate moiety from ATP had indicated that ANT(2″)-I can use a number of aminoglycosides as substrates (Smith & Smith, 1974; Lombardini & Cheng-Chu, 1980; Angelatou et al., 1982; Bongaerts & Molendijk, 1984). However, there were quantitative difficulties with the radioactivity assay (Goldman & Northrop, 1975).

The predominant resistance mechanism to aminoglycoside antibiotics among nosocomial pathogens in the United States is due to ANT(2″)-I (Shimizu et al., 1985). Its diversity of aminoglycoside substrates renders multiple resistance to the bacteria carrying this single enzyme. Because catalytic features of the enzyme must determine the characteristics of the bacterial resistance to antibiotics, precise determination of the kinetic parameters of the substrates is needed. By use of purified, stable enzyme and an accurate spectrophotometric assay, the activity of ANT(2″)-I has been measured with a variety of antibiotics and nucleotides. This led to the identification of a Theorell–Chance kinetic mechanism described in the second and third of three papers in this issue (Gates & Northrop, 1988a,b). The present report examines the kinetic features of substrates of ANT(2″)-I and correlates these to structural variations.

Experimental Procedures

Materials. Gentamicins C_1, C_{1a}, C_2, A, B, and B_1, sisomicin, 5-epiisomicin, and *N*-ethylsisomicin (netilmicin) were the gifts of Dr. George Miller, Schering Corp. Dr. F. Leitner and Dr. R. P. Elander, both of Bristol Laboratories, donated kanamycins A and B. Tobramycin and nebramycin factor 4 were the gifts of Dr. Marvin Gorman and William Fields, respectively, of Lilly Research Laboratories. All aminoglycosides were provided as sulfate salts. ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and TTP were purchased as sodium salts from Sigma. NADP, UDP-glucose, glucose 1,6-bisphosphate, dithiothreitol, sodium ethylenediaminetetraacetate (NaEDTA), UDP-glucose pyrophosphorylase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and 2-(cyclohexylamino)-ethanesulfonic acid (CHES) buffer were also purchased from Sigma. *E. coli* W677 containing the chimeric plasmid MY10 was the gift of Dr. Julian Davies, of the Pasteur Institute, Paris. Two electrophoretic variants of aminoglycoside nucleotidyltransferase 2″-I were purified 43- to 85-fold from *E. coli* W677/pMY10 by a modification of the method of Van Pelt and Northrop (1984). Enzyme preparations that had specific activities ranging from 1.1 to 1.7 international units (IU)/mg in the standard assay at pH 9.1 were used in kinetic studies.

Enzyme Assays. Aminoglycosides nucleotidyltransferase activity was measured by a modification of the coupled enzyme assay of Van Pelt and Northrop (1984) which links the production of pyrophosphate to UDP-glucose pyrophosphorylase, phosphoglumutase, and glucose-6-phosphate dehydrogenase reactions. The rate of increase of absorbance of NADPH at 340 nm was monitored continuously, assuming an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}, by using either a Gilford 240 spectrophotometer (0.32-mm slit width) or a Cary 118 spectrophotometer (3.00-mm slit width). Full-scale settings of 0.02–2.0 absorbance units were used. Both spectrophotometers were equipped with specially designed cuvette holders that increased the temperature equilibration 5-fold (i.e., equilibration half-life was 0.71 min) by maximizing contact with cuvettes and could accommodate semimicrocuvettes with path lengths up to 10 cm. A Haake circulating water bath maintained the temperature in the cuvette compartment at 25 °C. One unit of ANT(2″)-I activity is defined as the production of 1 μmol of NADPH/min.

Assay mixtures contained 0.1 M CHES buffer, titrated to pH 9.1 at 25 °C with sodium hydroxide, 0.5 mM UDP-glucose, 1.2 mM glucose 1,6-bisphosphate, 0.2 mM NADP, 0.3 mM dithiothreitol, 5 units/mL UDP-glucose pyrophosphorylase, 13 units/mL phosphoglucomutase, 4 units/mL glucose-6-phosphate dehydrogenase, 0.01 mM NaEDTA, and variable concentrations of aminoglycoside, nucleotide, magnesium acetate, and enzyme. Total volumes of 0.25 mL in a 10-mm path length, self-masking quartz cuvette or 1.0 mL in a 50-mm path length optical glass cuvette were used. A solution containing the coupling enzymes and their substrates, dithiothreitol, and NADP was kept on ice. Reactions were initiated by addition of either nucleotide or aminoglycoside substrates, by use of a paddle, to the remaining components which were preincubated in the cuvette compartment for at least 2.5 min. The free magnesium ion concentration was maintained at 10.0 mM, on the basis of calculations of the apparent stability constant of the nucleotide triphosphate according to the equation of Adolfsen and Moudrianakis (1978), and the volume of a stock solution of magnesium acetate required for varied concentrations of nucleotide was determined according to the equations described by Morrison (1979). Concentrations of nucleotide triphosphates were determined by measurements of absorbance at 260 nm at pH 7.0, or by measurements of absorption change in an assay of activity with hexokinase (Orme-Johnson et al., 1977). The concentration of aminoglycoside was determined by enzymatic assays with ANT(2″)-I or aminoglycoside acetyltransferase (6′)-IV as described previously (Van Pelt & Northrop, 1984; Radika & Northrop, 1984a). Stock dilutions of ANT(2″)-I were prepared in 0.1 M 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid (TAPS) buffer, pH 8.1 at 5 °C. Protein concentration was determined by measuring absorbance at 280 and 260 nm, on the basis of the equation of Warburg and Christian (1948).

Data Analysis. Data were fitted to eq 1 and 2, representing the absence and presence of substrate inhibition, respectively.

$$v = VA/(K + A) \quad (1)$$

$$v = VA/(K + A + A^2/K_I) \quad (2)$$

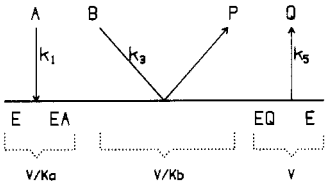
In these equations, v is the measured velocity, V is maximal velocity, K is the Michaelis–Menten constant of either nu-

Table I: Kinetic Constants of Nucleotide Substrates[a]

substrate	V (units/mg)	K (mM)	V/K
Mg–dGTP	0.93 ± 0.02	0.067 ± 0.03	13.82 ± 0.57
Mg–dATP	2.58 ± 0.05	0.27 ± 0.02	9.56 ± 0.49
Mg–GTP	2.97 ± 0.10	0.46 ± 0.03	6.41 ± 0.28
Mg–TTP	2.05 ± 0.10	0.56 ± 0.08	3.63 ± 0.37
Mg–ATP	4.70 ± 0.17	1.32 ± 0.14	3.56 ± 0.26
Mg–dCTP	1.85 ± 0.07	1.19 ± 0.10	1.55 ± 0.08
Mg–UTP	3.85 ± 0.20	2.74 ± 0.31	1.41 ± 0.09
Mg–CTP	2.95 ± 0.11	3.19 ± 0.21	0.92 ± 0.03

[a] Assayed at pH 9.1 with 49.5 μM gentamicin B.

Scheme I

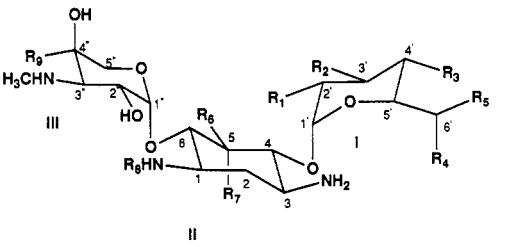


cleotide or aminoglycoside, and K_I is a substrate inhibition constant. Data were fit to these equations by nonlinear regression using the BASIC program of Duggleby (1984) on a NorthStar Horizon computer.

Results and Discussion

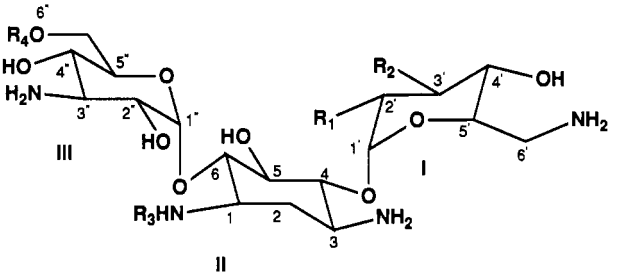
Nucleotide Specificity. Structure–activity relationships of nucleotides were examined by determining the activity of ANT(2″)-I as a function of varied concentrations of the magnesium chelate of eight nucleotides, including three purine, four pyrimidine, and three deoxy nucleotides. Apparent kinetic constants were determined for each, in the presence of gentamicin B at 6.6 times its Michaelis–Menten constant, and the results are shown in Table I. All eight nucleotides were substrates. In contrast to the substrate inhibition observed by Van Pelt and Northrop (1984) with reference to free nucleotides, the magnesium chelates do not cause substrate inhibition, consistent with the assignment of the chelate as the true substrate and absence of dead-end inhibition by free magnesium. The maximal velocities varied 5-fold, with the highest value given by Mg–ATP, presumably the natural substrate. Values of V/K_a varied 15-fold, with Mg–ATP and Mg–GTP ranking midway between the higher purine deoxyribonucleotides and lower pyrimidine compounds.

The second paper of three in this issue presents evidence that ANT(2″)-I obeys a Theorell–Chance kinetic mechanism (Gates & Northrop, 1987a). In its traditional form, this mechanism can be represented by the shorthand notation of Cleland (1963) as illustrated in Scheme I, where A represents magnesium nucleotide, B is aminoglycoside, P is magnesium pyrophosphate, Q is nucleotidylated aminoglycoside, and the enzyme is represented by the horizontal line with reaction progress proceeding from left to right. Substrate inhibition arises from the binding of B to EQ in a dead-end fashion. Portions of the reaction contributing to kinetic parameters are indicated below the mechanism. Values of V are determined solely by k_5; values of V/K_a and V/K_b are equal to k_1 and k_3, respectively, the diffusion-controlled bimolecular rate constants. These definitions provide several important implications to interpretations of the data in Table I. First, values of V/K_a for alternative substrates ought to be nearly identical. The values in Table I differ by more than expected from bimolecular rate constants for compounds of such structural similarity, indicating that the mechanism of ANT(2″)-I is not as simple as depicted in Scheme I but requires a conformational change interspersed between the binding of nucleotides and



Compound	R1	R2	R3	R4	R5	R6	R7	R8	R9
Gentamicin C_{1a}	NH_2	H	H	H	NH_2	OH	H	H	CH_3
Gentamicin C_2	NH_2	H	H	CH_3	NH_2	OH	H	H	CH_3
Gentamicin C_1	NH_2	H	H	CH_3	NH_2CH_3	OH	H	H	CH_3
Sisomicin	NH_2	H	4′	H	NH_2	OH	H	H	CH_3
5-epiSisomicin	NH_2	H	4′	H	NH_2	H	OH	H	CH_3
Netilmicin	NH_2	H	4′	H	NH_2	OH	H	CH_2CH_3	CH_3
Gentamicin A	NH_2	OH	OH	H	OH	OH	H	H	H
Gentamicin B	OH	OH	OH	H	NH_2	OH	H	H	CH_3
Gentamicin B_1	OH	OH	OH	CH_3	NH_2	OH	H	H	CH_3

FIGURE 1: Structures of gentamicins and related aminoglycosides.



Compound	R1	R2	R3	R4
Kanamycin A	OH	OH	H	H
Amikacin	OH	OH	L-AHBA	H
Kanamycin B	NH_2	OH	H	H
Tobramycin	NH_2	H	H	H
Nebramycin 4	NH_2	OH	H	$CONH_2$

FIGURE 2: Structure of kanamycins and related aminoglycosides. AHBA = 4-amino-1-hydroxybutyryl.

aminoglycosides. A second implication of Scheme I is that the Michaelis–Menten constants have no kinetic significance because they are ratios of different parameters from dissimilar steps.

Aminoglycoside Specificity. ANT(2″)-I has been reported to adenylylate a number of gentamicins and kanamycins whose structures are illustrated in Figures 1 and 2, respectively. These aminoglycosides all consist of three rings, and the site of modification by ANT(2″)-I is in ring III. All of the substrates for ANT(2″)-I are 4,6-substituted aminoglycosides that contain the deoxystreptamine ring but differ in the number and positions of amino and hydroxyl groups. The 2″-hydroxyl and the 3-amino groups are common to all substrates as are the 5- and 4″-hydroxyl groups, but the stereochemical orientation differs among the latter.

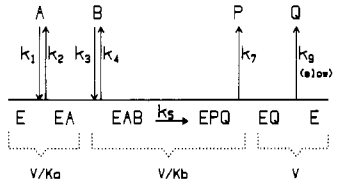
The structure–activity relationships of aminoglycosides were examined by determining activity of ANT(2″)-I as a function of varied concentrations of nine antibiotics of the gentamicin group and four from the kanamycin group. Table II presents the apparent kinetic constants obtained in the presence of

Table II: Kinetic Constants of Aminoglycoside Substrates[a]

substrate	V (IU/mg)	K (μM)	V/K (L mg^{-1} min^{-1})	K_I (μM)
gentamicin C_{1a}	3.5 ± 0.2	0.6 ± 0.1	5.97 ± 0.72	20.1 ± 2.5
gentamicin C_2	3.9 ± 0.2	1.0 ± 0.1	3.74 ± 0.72	56.6 ± 5.6
gentamicin B	4.8 ± 0.3	7.5 ± 1.2	0.64 ± 0.06	186.0 ± 26
sisomicin	10.1 ± 0.6	18.8 ± 2.5	0.54 ± 0.04	193.5 ± 22.9
gentamicin C_1	8.9 ± 0.3	24.9 ± 1.8	0.36 ± 0.01	1294.0 ± 194
tobramycin	10.2 ± 0.9	42.7 ± 0.2	0.24 ± 0.01	81.2 ± 10.9
gentamicin B_1	12.9 ± 0.6	54.9 ± 4.2	0.23 ± 0.01	343.7 ± 38.3
kanamycin B	7.4 ± 0.7	31.7 ± 4.4	0.23 ± 0.01	85.7 ± 12.2
nebramycin 4	6.9 ± 1.0	50.1 ± 15.4	0.14 ± 0.02	179.9 ± 43.4
gentamicin A	5.2 ± 0.2	114.0 ± 1.5	0.046 ± 0.002	
kanamycin A	5.3 ± 0.4	126.0 ± 18.3	0.042 ± 0.003	484.2 ± 68.9
5-episisomicin	3.0 ± 0.2	103.7 ± 20.8	0.029 ± 0.004	
netilmicin	5.7 ± 1.2	3478.0 ± 1030	0.0016 ± 0.0002	5124.0 ± 153

[a] Assayed at pH 9.1 with 10.2–11.4 mM Mg-ATP.

Scheme II



Mg–ATP at 7.7–8.6 times its Michaelis–Menten constant. The values of V of the aminoglycosides vary from each other by about 4-fold; in sharp contrast, the values of V/K_b vary by nearly 4000-fold. This extreme disparity between kinetic parameters is very much in keeping with the definition of V illustrated in Scheme I, in which catalysis is kinetically insignificant, but the changes in V/K_b are incompatible with its being defined solely by k_3. Three orders of magnitude variation are inconsistent with a single, diffusion-controlled rate constant and therefore require that a catalytic step be included that is significant to V/K_b but not to V, as indicated in Scheme II. To retain the quality of a Theorell–Chance mechanism, it is necessary only that k_5 and k_7 greatly exceed k_9, such that catalysis remains insignificant to V. The definition of V/K_b of Scheme II is

$$\frac{V}{K_b} = \frac{k_3 k_5}{k_4 + k_5} \tag{3}$$

but under conditions of rapid equilibrium, $k_4 \gg k_5$, and V/K becomes

$$\frac{V}{K_b} = \frac{k_3 k_5}{k_4} = \frac{k_{cat}}{K_d} \tag{4}$$

where k_{cat} is the net rate constant for catalysis and K_d is the dissociation constant for the aminoglycoside.

An important feature of the aminoglycoside specificity is substrate inhibition, as illustrated in Figure 3, and it is observed with nearly all aminoglycosides.[2] The third paper of three in this issue (Gates & Northrop, 1988b) presents evidence that the substrate inhibition constants are true dissociation constants and that inhibitory binding of aminoglycosides is very similar to substrate binding. Substrate inhibition can therefore be

[2] Because the Michaelis–Menten constant is sometimes only half the value of the substrate inhibition constant, velocities deviate significantly from the uninhibited rate, even at moderate concentrations of aminoglycoside. This requires that accurate measurements of activity be made at extremely low concentrations of aminoglycoside (e.g., [S] < $K/4$), that many data points be collected in order to define the curvature of reciprocal plots, and that values for V be derived from a very long effective extrapolation which can only be accurately obtained by nonlinear regression with rate equations incorporating substrate inhibition.

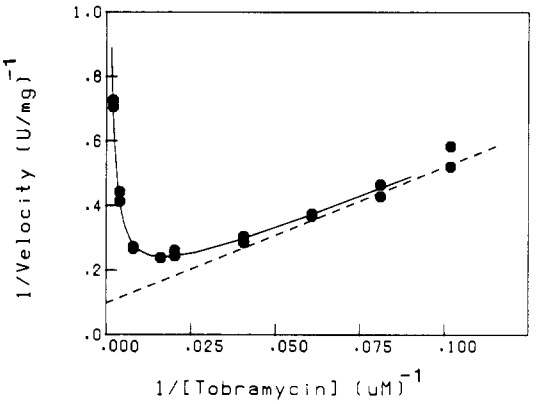


FIGURE 3: Double-reciprocal plot of ANT(2″)-I reaction velocity as a function of tobramycin concentration. The solid line represents a fit to eq 2, and the dashed line designates the position of $1/V$ and K/V. The concentrations of Mg–ATP and free Mg^{2+} were fixed at 10.4 and 10.0 mM, respectively. The concentration range of tobramycin was 9.8–491.2 μM.

Table III: Values of k_{cat} Derived from the Product of $(V/K) \times K_I$

substrate	set	k_{cat}	substrate	set	k_{cat}
tobramycin	I	19 ± 7	gentamicin B	II	119 ± 36
kanamycin B	I	20 ± 7	netilmicin	II	120 ± 3
kanamycin A	I	20 ± 9	gentamicin C_{1a}	II	120 ± 59
nebramycin 4	I	25 ± 19	gentamicin C_2	III	212 ± 84
gentamicin B_1	II	75 ± 24	gentamicin C_1	III	466 ± 166
sisomicin	II	105 ± 40			

exploited to extract k_{cat} from eq 4 by multiplying V/K by K_I to cancel K_d. Table III lists the products of these two kinetic constants for those aminoglycosides that displayed substrate inhibition. The values obtained fall closely together within three sets. The first set of four aminoglycosides, which give the lowest values of product, do not seem to be significantly different from each other despite a nearly 6-fold difference in values of V/K (refer to Table II), suggesting that these aminoglycosides undergo catalysis at a very similar rate and that they differ from each other primarily in binding. More striking are data for the second set of five which also do not seem to be significantly different from each other despite inclusion of the best and worst substrates with a nearly 4000-fold difference in values of V/K. These aminoglycosides also undergo catalysis at a very similar rate and differ primarily in binding.

Nevertheless, the data of the second set, all gentamicins, are significantly different from that of the first, all kanamycins. The structural difference between the kanamycin and gentamicin families of aminoglycosides rests in ring III, called kanosamine and garosamine, respectively. These structures contain the site of nucleotidylation, the 2″-hydroxyl group;

Table IV: Kinetic Effects of Structural Differences between Aminoglycosides

position	antibiotic	group	set	ΔK_I (x-fold)
6″-C	kanamycin B	OH	I	
	nebramycin 4	$CONH_2$	I	+2.1
2′-C	kanamycin B	NH_2	I	
	kanamycin A	OH	I	+5.6
3′-C	tobramycin	H	I	
	kanamycin B	OH	I	1.0
4′–5′	gentamicin C_{1a}	C—C	II	
	sisomicin	C=C	II	+9.6
1-N	sisomicin	H	II	
	netilmicin	CH_2CH_3	II	+26.5
6′-C	gentamicin B	H	II	
	gentamicin B_1	CH_3	II	+1.8
6′-N	gentamicin C_2	H	III	
	gentamicin C_1	$NHCH_3$	III	+22.7

hence, their major structural differences are consistent with changes in rates of catalysis. Similarly, the third set of two aminoglycosides, which give the highest values on Table III, are also gentamicins but differ from set II by the absence of hydroxyl groups in positions 3′ and 4′ of ring I. The data show that the presence of these hydroxyl groups on ring I impedes catalysis.

Because many pairs of the aminoglycosides differ from each other by a single structural component, a comparison of values of K_I within sets should identify specific contributions to binding. Ratios of values of K_I were calculated from the data of Table II, grouped together according to sets of Table III, and are presented in Table IV. The first comparison shows that a carboxylamine in position 6″ of nebramycin 4 raises the K_I about 2-fold, a substantial reduction in binding, probably due to steric interference. The next shows that the replacement of the amine at position 2′ with a hydroxyl group raises the K_I over 5-fold, strongly implicating the 2′-amine group in binding, consistent with results from pH kinetics (Gates & Northrop, 1988b). The last comparison from set I shows that the 3′-hydroxyl neither contributes nor deters binding. The comparisons from set II show that the distortion of ring III caused by the double bond of sisomicin reduces binding nearly 10-fold, that the additional ethyl group of netilmicin reduces binding more than 25-fold, and that the methyl group at position 6′ of gentamicin B_1 reduces binding nearly 2-fold; all are probably due to steric interactions. Comparing data from the last set, the aminomethyl at the 6′-position of gentamicin C_2 reduces binding over 20-fold, and this is also probably a steric effect.

Not included in Tables III and IV are comparisons with 5-episisomicin and gentamicin A, because substrate inhibition was not detected within the concentrations examined (e.g., 5-episisomicin was assayed at concentrations up to 50-fold greater than the K_I of sisomicin). The former differs from sisomicin by having an axial instead of equatorial hydroxy group in the 5-position of ring I, and its value for V/K_b is reduced considerably, suggesting that most of that reduction would be canceled as above, consistent with reduced binding due to significant steric hindrance by the axial hydroxyl. The latter differs from gentamicin B by a shift in an amino group from the 6′- to the 2′-position on ring I; hence, the undetectable substrate inhibition shows that the group in the 2′-position does not contribute to binding—in direct contradiction to the results discussed above in the comparison of kanamycins A and B. The differences between the gentamicins and kanamycins may be severe enough to preclude binding to the enzyme in the same orientation; rather, the relative positions of rings I and III may exchange places to allow binding of gentamicin in an inverse mode, such that the site occupied by the 6′-amino group of kanamycins is occupied by 3″-amino group of the gentamicins, leaving the 2′ group exposed to solvent. A similar form of inverse binding was proposed by Williams and Northrop (1978) to explain the extreme sensitivity of AAC(3)-I to dead-end inhibition by neomycin. Neomycin being an inhibitor, it was not possible to test the inverse binding hypotheses, but in the present case, inverse binding should implicate a different position of nucleotidylation. To date, the site of modification by this enzyme has been established for 3′,4′-dideoxykanamycin only (Naganawa et al., 1971), and the site of modification of other aminoglycosides is based on inference. Gentamicins A, B, and B_1 share common hydroxyls at the 3′- and 4′-positions, and their possible modification via inverse binding needs to be investigated.

ANT(2″)-I is capable of modifying aminoglycosides of considerable structural variation but does not accept all aminoglycosides as substrates. Those that it does accept have the 5-hydroxyl group in common, and this group must be in an equatorial position for optimum binding. Other than this, the enzyme does not seem to require a hydroxyl group at any particular position, other than the one that is nucleotidylated. Among amino groups, the three at positions 1, 3, and (with the lone exception of gentamicin A) 6′ are common to all the substrates in Table II, and modifications at or near the 6′ reduce binding.

Significance of Structure–Activity Relationships. One strategy for combating enzyme-mediated bacterial resistance involves the production of semisynthetic antibiotics derived from chemical modification of existing drugs (Umezawa, 1982). This strategy seeks structural changes that will make an antibiotic invulnerable to enzymatic modification but will not affect antimicrobial activity. Unfortunately, many structure–activity relationships of ANT(2″)-I parallel the antibiotic activities. Like the enzyme, antibiotic activity has little dependence on hydroxyl groups and a graduated dependence on the number and position of amino groups. These play an important role in antibiotic activity according to the series 2′,6′-diamino > 6′-amino > 2′-amino > no amino (Benveniste & Davies, 1973), but gentamicins containing the 2′- and 6′-amino groups are also better substrates of ANT(2″)-I (see Table II). Antibiotics with maximal antimicrobial activity contain amino groups at the 1-, 3-, 2′-, 6′, and 3″-positions, and for example, acylation of the 3-amino group and dimethylation of the 3″-amino substituent abolishes antimicrobial activity. Nevertheless, antimicrobial activity is retained in the C series of gentamicins in which the 6′-position is N- and C-methylated. Similarly, looking at ring substitutions, the gentamicins are generally better antibiotics than the kanamycins (Moellering, 1983), and again ANT(2″)-I displays higher values of k_{cat} in Table III for the gentamicins than for the kanamycins.

Structure–activity correlations for AAC(3)-I (Williams & Northrop, 1976) and AAC(6′) (Radika & Northrop, 1984a) also identified similarities between enzymatic and antimicrobial requirements, but they did find some exceptions. Resistant bacteria containing an acetyltransferase decrease antibiotic activity by acylation of the amino groups required for efficient binding of the antibiotic to the ribosome; hence, alkylation of the 6′-amino group appears to be a promising type of modification to reduce acetyltransferase activity and still retain some antibiotic potency. In the case of AAC(3)-I, hydroxylation of the 4′-carbon reduces enzymatic activity but not antibiotic activity. A particularly important site of modification that leads to the retention of antibiotic activity is the 1-amino group in ring II, such as that found in the semisyn-

thetic and clinically important amikacin and netilmicin (see Figure 1). Amikacin is not a substrate for ANT(2″)-I, but netilmicin is, with the lowest value for V/K_b in Table II.

Because neither the 1-amino nor the 5-hydroxyl group are critical to antibiotic activity, modification of these positions has been a strategy of medicinal chemists in recent years to develop semisynthetic antibiotics that will be effective against resistant strains of bacteria (Moellering, 1983). Such modifications have been pursued in the context of enzymatic activity as measured in the presence of excess antibiotic and thus were not targeted toward a specific kinetic parameter. At best, changes in enzymatic activity in the presence of excess antibiotic might be due to changes in V, which varies little among known substrates of ANT(2″)-I, whereas at worst, they might be due to changes in K_I, which would be seen in a misleading fashion: modifications that reduce binding will actually enhance the inhibited enzymatic activity and perhaps then be abandoned, despite a commensurate but unmeasured reduction in V/K, which in turn would lead to a useful reduction in resistance because microbial resistance correlates with V/K and not with V (Radika & Northrop, 1984b). Consequently, the values of V/K in Table II could assist the medicinal chemist in targeting modifications with higher probabilities of successful clinical application in combating resistance.

Registry No. AAD(2″), 61642-42-0; Mg-dGTP, 89705-26-0; Mg-dATP, 74386-14-4; Mg-GTP, 22139-68-0; Mg-TTP, 72781-90-9; Mg-ATP, 1476-84-2; Mg-dCTP, 79295-52-6; Mg-UTP, 22188-71-2; Mg-CTP, 7358-19-2; gentamicin C_{1a}, 26098-04-4; gentamicin C_2, 25876-11-3; gentamicin B, 36889-15-3; sisomicin, 32385-11-8; gentamicin C_1, 25876-10-2; tobramycin, 32986-56-4; gentamicin B_1, 36889-16-4; kanamycin B, 4696-76-8; nebramycin 4, 51736-76-6; gentamicin A, 13291-74-2; kanamycin A, 59-01-8; 5-episisomicin, 55870-64-9; netilmicin, 56391-56-1; kanamycin, 8063-07-8.

References

Adolfsen, R., & Moundriankis, E. N. (1978) *J. Biol. Chem. 253*, 4378–4379.

Angelatou, F., Litsas, S. B., & Kontomichalou, P. (1982) *J. Antibiot. 35*, 235–244.

Benveniste, R., & Davies, J. (1971) *FEBS Lett. 14*, 293–296.

Benveniste, R., & Davies, J. (1973) *Antimicrob. Agents Chemother. 4*, 402–409.

Bongaerts, G. P. A., & Molendijk, L. (1984) *Antimicrob. Agents Chemother. 25*, 234–237.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.

Coombe, R. G., & George, A. M. (1981) *Antimicrob. Agents Chemother. 20*, 75–80.

DeHertough, D. A., & Lerner, S. A. (1985) *Antimicrob. Agents Chemother. 27*, 670–671.

Duggleby, R. G. (1984) *Comput. Biol. Med. 14*, 447–455.

Gates, C. A., & Northrop, D. B. (1988a) *Biochemistry* (second of three papers in this issue).

Gates, C. A., & Northrop, D. B. (1988b) *Biochemistry* (third of three papers in this issue).

Goldman, P. R., & Northrop, D. B. (1975) *Biochem. Biophys. Res. Commun. 66*, 1408–1413.

Koch, K. F., Rhoades, J. A., Hagaman, E. W., & Wenkert, E. (1974) *J. Am. Chem. Soc. 96*, 3300–3305.

Lombardini, J. B., & Cheng-Chu, M. (1980) *Int. J. Biochem. 12*, 427–431.

Martin, C. M., Ikari, N. S., Zimmerman, J., & Waitz, J. A. (1971) *J. Infect. Dis. 124*, s24–s29.

Mitsuhashi, S. (1975) in *Drug Action and Drug Resistance* (Mitsuhashi, S., Ed.) pp 269–275, University of Tokyo Press, Tokyo, Japan.

Moellering, R. C., Jr. (1983) *Rev. Infect. Dis. 5* (*Suppl. 2*), S212–S232.

Morrison, J. F. (1979) *Methods Enzymol. 63*, 257–293.

Naganawa, H., Yagisawa, M., Kondo, S., Takeuchi, T., & Umegawa, H. (1971) *J. Antibiot. 24*, 913–915.

Orme-Johnson, W. H., Hageman, R., Henzl, M., Ludden, P., Raushel, F., Reinhart, G., Redina, A., Toce, J., & Williams, J. (1977) *Enzymology and Metabolic Techniques*, University of Wisconsin Department of Biochemistry, Madison, WI.

Radika, K., & Northrop, D. B. (1984a) *Biochemistry 23*, 5118–5122.

Radika, K., & Northrop, D. B. (1984b) *Antimicrob. Agents Chemother. 25*, 479–482.

Shimizu, K., Kumada, T., Hsieh, W.-C., Chung, H.-Y., Chong, Y., Hare, R. S., Miller, G. H., Sabatelli, F. J., & Howard, J. (1985) *Antimicrob. Agents Chemother. 28*, 282–288.

Umezawa, H. (1982) *Trends Antibiot. Res.: Genet., Biosynth., Actions New Subst., Proc. Int. Conf., 1982*, 1–15.

Van Pelt, J. E., & Northrop, D. B. (1984) *Arch. Biochem. Biophys. 230*, 250–263.

Warburg, O., & Christian, W. (1942) *Biochem. Z. 310*, 384–421.

Williams, J. W., & Northrop, D. B. (1978) *J. Biol. Chem. 253*, 5908–5914.

Yagisawa, M., & Davies, J. E. (1979) *J. Antibiot. 32*, 250–253.