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Kinetic Mechanism of Kanamycin Nucleotidyltransferase from *Staphylococcus aureus*[1]

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Kanamycin nucleotidyltransferase (KNTase) catalyzes the transfer of the adenyl group from MgATP to either the 4′ or 4″-hydroxyl group of aminoglycoside antibiotics. The steady state kinetic parameters of the enzymatic reaction have been measured by initial velocity, product, and dead-end inhibition techniques. The kinetic mechanism is ordered where the antibiotic binds prior to MgATP and the modified antibiotic is the last product to be released. The effects of altering the relative solvent viscosity are consistent with the release of the products as the rate-limiting step. The pH profiles for V_{max} and V/K_{ATP} show that a single ionizable group with a pK of ~8.9 must be protonated for catalysis. The V/K profile for kanamycin as a function of pH is bell-shaped and indicates that one group must be protonated with a pK value of 8.5, while another group must be unprotonated with a pK value of 6.6. An analysis of the kinetic constants for 10 different aminoglycoside antibiotics and 5 nucleotide triphosphates indicates very little difference in the rate of catalysis or substrate binding among these substrates.

Key Words: kanamycin nucleotidyltransferase; antibiotic modification.

INTRODUCTION

Aminoglycoside antibiotics are a diverse group of natural and semisynthetic antimicrobial agents. These compounds consist of amino sugars that are linked to an aminocyclitol moiety through glycosidic bonds (*1*). Binding of these antibiotics to the bacterial 30S ribosomal subunit interferes with normal protein translation and is bactericidal. (*2*). Unfortunately, bacterial resistance to these compounds has emerged as a significant public health concern, due, in part, to the unregulated usage of the aminoglycosides in certain environments (*3*). The specific mechanisms for bacterial

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antibiotic resistance can include alterations in cellular permeability, modification of the target site, and chemical derivatization of the antibiotic itself (*4*). Enzymatic modification of the aminoglycoside antibiotics is the most common mechanism for the acquisition of bacterial resistance to these particular pharmaceuticals (*5*).

The principal mechanism of bacterial resistance to this class of antibiotics can be arranged into three groups, based on the specific site of modification of the drug. One or more of the sugar hydroxyl groups can either be phosphorylated or adenylylated by ATP. Alternatively, the amino groups may be acetylated by acetylCoA (*5*–*7*). Kanamycin nucleotidyltransferase (KNTase[3]), as originally isolated from the gram-positive bacterium *Staphylococcus aureus*, catalyzes the transfer of the nucleoside monophosphate group from ATP to those aminoglycoside antibiotics that possess an equatorially oriented hydroxyl group at either the 4′- or 4′-position (*8*–*11*). The reaction with the aminoglycoside kanamycin is illustrated below in Eq. [1].

$$\text{Kanamycin} + \text{MgATP} \leftrightarrows 4'\text{-AMP-Kanamycin} + \text{MgPP}_i \qquad [1]$$

The three-dimensional structure of KNTase has been determined by X-ray crystallographic techniques in the Holden laboratory (*12*,*13*). A structure at 3.0 Å resolution was solved without bound ligands (*12*) and a 2.5 Å structure, complexed with kanamycin and a nonhydrolyzable ATP analog, was solved shortly thereafter (*13*). The X-ray crystal structure shows that KNTase is a homodimer. The individual subunits consist of two structural domains of approximately equal size and the two active sites are located at the dimer interface.

The emergence of bacterial resistance has virtually eliminated the practical utility of these antibiotics as effective pharmaceuticals. More effective aminoglycoside antibiotics and potential inhibitors of the modification enzymes need to be developed and synthesized. This task would be aided by a more detailed knowledge of the chemical and biological interactions between the aminoglycoside modifying enzymes and their associated substrates. Since KNTase is one of only three proteins in this class of enzymes where detailed structural information is available, it is an ideal candidate for a more thorough investigation of the chemistry and mechanism of those enzymes that specifically inactivate aminoglycoside antibiotics. We report herein, through the use of initial velocity, dead-end inhibitors, product inhibition, and the effects of pH and solvent viscosity, the kinetic and chemical mechanism of KNTase.

MATERIALS AND METHODS

Materials. A thermostable variant (D80Y) of kanamycin nucleotidyltransferase was expressed in *Escherichia coli* and then isolated using a modification of previously described protocols (*11*,*12*,*14*). Paromomycin sulfate was purchased from ICN. The remaining antibiotics, nucleotides, buffers, coupling enzymes, and other chemicals were purchased from Sigma.

Assay of kanamycin nucleotidyltransferase. The catalytic activity of kanamycin

[3] Abbreviations used: KNTase, kanamycin nucleotidyl transferase; Pipes, piperazine-*N*,*N*′-bis-[2-ethanesulfonic acid]; Ches, 2-[*N*-cyclohexylamino]ethanesulfonic acid; Taps, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; MES, 2[*N*-morpholino]ethanesulfonic acid.

nucleotidyltransferase was assayed in both the forward and reverse directions by monitoring the appearance of NADH at 340 nm with a Gilford 260 spectrophotometer. In the forward reaction, the activity of KNTase was measured using the assay developed by Van Pelt and Northrop (*15*). The KNTase activity was monitored spectrophotometrically by coupling the production of pyrophosphate to UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase. Assay mixtures for the adenylylation of the antibiotic contained 0.1 M Pipes, pH 7.0, 0.5 mM UDP-glucose, 0.5 mM glucose 1,6-bisphosphate, 0.2 mM NAD, 0.3 mM dithiothreitol, 0.01 mM EDTA, 2 units/ml UDP-glucose pyrophosphorylase, 10 units/ml phosphoglucomutase, 16 units/ml glucose-6-phosphate dehydrogenase, and variable concentrations of nucleotide, aminoglycoside, $MgCl_2$, and enzyme. The free magnesium ion concentration was maintained at 5.0 mM.

The activity of KNTase in the reverse reaction was obtained spectrophotometrically by linking the production of ATP to hexokinase and glucose-6-phosphate dehydrogenase. Assay mixtures contained 0.1 M Pipes, pH 7.0, 16 units/ml hexokinase, 10 units/ml glucose-6-phosphate dehydrogenase, 0.2 mM NAD, 1.0 mM glucose, and various concentrations of PP_i, $MgCl_2$, AMP-kanamycin, and enzyme. The temperature was maintained at 25°C with a circulating water bath. Protein concentrations were determined by the method of Bradford, using bovine serum albumin as the standard (*16*).

Synthesis of AMP-kanamycin A. AMP-kanamycin was synthesized following a modification of the method used to isolate AMP-tobramycin (*10*). The reaction mixture contained 0.52 mmol of ATP, 0.1 mmol of kanamycin A, 23.2 mmol sodium acetate, 11.5 mmol of $MgCl_2$, 0.8 mmol of dithiothreitol, 0.4 mg of KNTase, and 500 units of pyrophosphorylase in a total volume of 900 ml at pH 5.8. The incubation was carried out in a shaker bath at 35°C for 26 h. The reaction mixture was then diluted to 2 liters with distilled water, heated for 10 min at 80°C, filtered, and applied to an Amberlite CG-50 column (NH_4^+ form, approximately 100 ml). The column was washed with 2 liters of distilled water and then AMP-kanamycin was eluted using a 0.08 to 0.8% linear gradient of ammonium hydroxide. The absorbance at 260 nm was used to identify those fractions containing the modified kanamycin. The ^{31}P NMR spectrum indicated that the product was a mixture of the 4′- and 4″-adenylylated kanamycin.

Solvent viscosity effects. The effect of solvent viscosity was determined by adding the appropriate amount of viscosogen to the standard assay mixtures. Glycerol and polyethylene glycol, with an average molecular weight of 8000 (PEG8000), were used as the two viscosogens. The relative viscosity for the glycerol samples were obtained by linear interpolation of previously published data (*17*). The values for the glycerol solutions were as follows (w/w%, η_{rel}): 11, 1.3; 22, 1.7; 35, 2.9; and 43, 4.2. The relative viscosities for the PEG8000 solution were obtained as previously described (*18*) and are as follows (w/w%, η_{rel}): 1.7, 1.6; 3.3, 2.0; and 6.7, 3.6.

pH profiles. The pH effects on KNTase activity were measured at values of pH from 6.0 to 10.0. The buffers, Mes, Pipes, Taps, and Ches, were used within one pH unit of their pK values and titrated to the desired pH by NaOH. The time courses were linear throughout the incubation and assay period and no precipitation was

observed, indicating that the enzymes were stable and insensitive to the changes of pH and solvent conditions. The pH was remeasured after each assay.

Inhibition studies. Product and dead-end inhibition experiments were conducted by varying the concentrations of one substrate at various fixed levels of inhibitor. The remaining substrate was kept at a fixed concentration.

Data analysis. The variation of the initial velocities with the concentration of nucleotide and aminoglycoside were fit to either Eqs. [2] or [3], using the computer programs HYPER and SUBIN, representing the absence and presence of substrate inhibition, respectively. Data corresponding to an intersecting initial velocity pattern with two substrates were fitted to Eq. [4] using the program SEQUEN. Inhibition constants were obtained by fitting the data to Eqs. [5], [6], or [7], using the computer programs UNCOMP, COMP, and NONCOMP, representing uncompetitive inhibition, competitive inhibition, and noncompetitive inhibition, respectively (*19*), where v is the initial velocity, V is the maximum velocity, K_m is the Michaelis constant, A and B are the concentrations of substrate, I is the concentration of inhibitor, K_i is the substrate inhibition constant, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant. The pH profiles were fit to Eqs. [8] and [9], where y is V/K or V, K_a, and K_b is dissociation constants of the groups that ionize, c is the constant, and H is the concentration of hydrogen ion.

$$v = VA/(K_a + A) \quad [2]$$

$$v = VA/(K_a + A + A^2/K_i) \quad [3]$$

$$v = VAB/(K_aB + K_bA + AB + K_{ia}K_b) \quad [4]$$

$$v = VA/(K + A\,(1 + I/K_{ii})) \quad [5]$$

$$v = VA/(A + K\,(1 + I/K_{is}) \quad [6]$$

$$v = VA/(K\,(1 + I/K_{is}) + A(1 + I/K_{ii})) \quad [7]$$

$$\log y = \log\,(c/(1 + K_b/H)) \quad [8]$$

$$\log y = \log\,(c/(1 + H/K_a + K_b/H)) \quad [9]$$

RESULTS

Nucleotide triphosphate specificity. The structure–activity relationships between the nucleotide substrate and KNTase were evaluated by determining the apparent kinetic parameters for various magnesium chelated nucleotides, including two purines (ATP and GTP) and three pyrimidines (CTP, TTP, and UTP). All five nucleotide triphosphates were substrates for the enzyme and the maximal velocities varied from 0.19 to 0.73 s^{-1}. The V/K values for the nucleotides varied by less than two-fold (0.7 to 1.3 s^{-1} mM^{-1}). The kinetic constants are presented in Table 1.

Specificity of aminoglycoside substrates. The structure–activity relationship between the aminoglycoside substrate and the enzyme was examined by measuring the activity of KNTase as a function of the concentration of the different aminoglycoside antibiotics, including five compounds from the kanamycin family and five from the neomycin family (Fig. 1). The concentration of MgATP was fixed at 5 mM. The

TABLE 1

Apparent Kinetic Parameters of Aminoglycoside and Nucleotide Substrates[a]

Substrate[b]	V_{max} (sec^{-1})	K_m (μM)	K_i (mM)
Kanamycin	0.49 ± 0.01	5 ± 1	1.1 ± 0.1
Bekanamycin	1.2 ± 0.1	11 ± 1	0.17 ± 0.01
Tobramycin	1.3 ± 0.1	12 ± 1	0.10 ± 0.01
Butirosin	0.90 ± 0.02	15 ± 1	1.4 ± 0.1
Dibekacin	0.44 ± 0.01	15 ± 1	0.54 ± 0.05
Amikacin	0.62 ± 0.01	24 ± 1	3.6 ± 0.3
Neomycin	0.89 ± 0.01	10 ± 1	5.3 ± 0.5
Ribostamycin	0.85 ± 0.02	12 ± 1	0.55 ± 0.05
Paromomycin	0.88 ± 0.03	17 ± 1	0.37 ± 0.03
Lividomycin	0.56 ± 0.01	35 ± 1	2.3 ± 0.2
MgATP	0.51 ± 0.01	420 ± 10	
MGCTP	0.24 ± 0.01	190 ± 8	
MgUTP	0.19 ± 0.01	150 ± 5	
MgTTP	0.21 ± 0.01	301 ± 20	
MgGTP	0.73 ± 0.01	940 ± 20	

[a] Enzymatic activity was assayed at pH 7.0, 25°C with 100 mM Pipes buffer. The kinetic data were fitted to Eq. [2] or [3].

[b] The concentration of MgATP was fixed at 5.0 mM when the antibiotic was varied and the concentration of kanamycin A was held at 0.05 mM when the nucleotide was varied.

maximal velocity varied about 3-fold and the *V*/*K* values for the various antibiotics varied by nearly 7-fold. All of the compounds tested exhibited substrate inhibition at elevated levels. However, the inhibition constant, K_i, for the aminoglycoside antibiotics varied from one other by nearly 53-fold, with the lowest value given by tobramycin (Table 1).

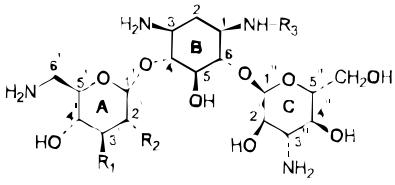
Initial velocity studies. The kinetic parameters, V_{max} and V/K_m, were determined in both the forward and reverse directions. The maximal velocity for the reverse direction is reduced about 73-fold when compared to that of the forward reaction.

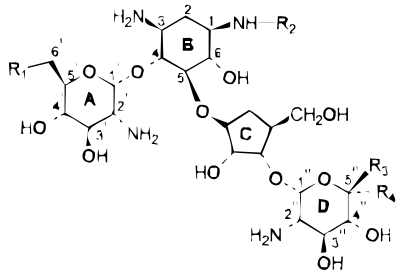
TABLE 2

Initial Velocity Data and Kinetic Parameters for Reactions Catalyzed by KNTase[a]

		K_m (μM)		K_i (μM)		
Varied substrate	Pattern type	MgATP or $MgPP_i$	Kanamycin or AMP-Kanamycin	MgATP or $MgPP_i$	Kanamycin or AMP-Kanamycin	V_{max} (sec^{-1})
MgATP *vs* Kanamycin	Intersecting	170 ± 30	20 ± 2	230 ± 60	27 ± 9	0.51 ± 0.01
$MgPP_i$ *vs* AMP-kanamycin	Intersecting	250 ± 40	26 ± 1	490 ± 50	52 ± 10	0.007 ± 0.001

[a] The enzyme was assayed at pH 7.0, 25°C in 100 mM Pipes buffer.





	R_1	R_2	R_3		R_1	R_2	R_3	R_4
Amikacin	OH	OH	r	Butirosin	NH_2	r	no D-ring	
Bekanamycin	OH	NH_2	H	[a]Lividomycin	OH	H	H	w
Dibekacin	H	NH_2	H	Neomycin	NH_2	H	H	w
Kanamycin A	OH	OH	H	Paromomycin	OH	H	w	H
Tobramycin	H	NH_2	H	Ribostamycin	NH_2	H	no D-ring	

r = $COCHOHCH_2CH_2NH_2$. w = CH_2NH_2. [a]lividomycin contains no 3'-OH.

FIG. 1. (A) Structures of kanamycin and related aminoglycoside antibiotics. (B) Structures of neomycin and related aminoglycoside antibiotics.

The initial velocity patterns in either direction were intersecting. The results are summarized in Table 2.

Inhibition studies. Product and dead-end inhibition studies were conducted to determine the basic kinetic mechanism for the enzymatic modification of aminoglycoside antibiotics by KNTase. When AMP-kanamycin was used as a product inhibitor, the inhibition pattern was competitive *versus* kanamycin and noncompetitive *versus* MgATP. The dead-end inhibition by spectinomycin, was competitive *versus* kanamycin and noncompetitive *versus* MgATP. Both AMPCPP and AMP were found to be competitive inhibitors *versus* MgATP. An uncompetitive inhibition pattern was obtained with either MgAMPCPP or MgAMP when kanamycin A was used as the variable substrate. The results are summarized in Table 3.

Effect of pH. The pH profiles for the enzymatic reaction were conducted as a function of the concentrations of both nucleotide and antibiotic over the pH range from 6.0 to 10. In the V_{max} *versus* pH profile, a slope of -1 is observed above pH 8.5 and a plateau is seen below pH 7.5 (Fig. 2A), indicating that one ionizable group must be protonated for catalytic activity. The pK value, as determined from a fit of

TABLE 3

Inhibition Patterns for Kanamycin Nucleotidyltransferase

Varied substrate	Inhibitor	Fixed substrate[a]	Pattern type	K_{ii} (mM)	K_{is} (mM)
MgATP	AMP	Kanamycin	C		1.6 ± 0.1
MgATP	AMPCPP	Kanamycin	C		3.5 ± 0.3
Kanamycin	AMP	MgATP	UC	13 ± 1	
Kanamycin	AMPCPP	MgATP	UC	16 ± 1	
Kanamycin	Spectinomycin	MgATP	C		24 ± 3
MgATP	Spectinomycin	Kanamycin	NC	110 ± 33	17 ± 2
MgATP	AMP-kanamycin	Kanamycin	NC	0.34 ± 0.02	0.55 ± 0.08
Kanamycin	AMP-kanamycin	MgATP	C		0.077 ± 0.006

[a] The concentration of kanamycin was fixed at 0.05 mM. The concentration of MgATP was held at 1.0 mM. The concentration of AMP-kanamycin was fixed at 0.032 mM.

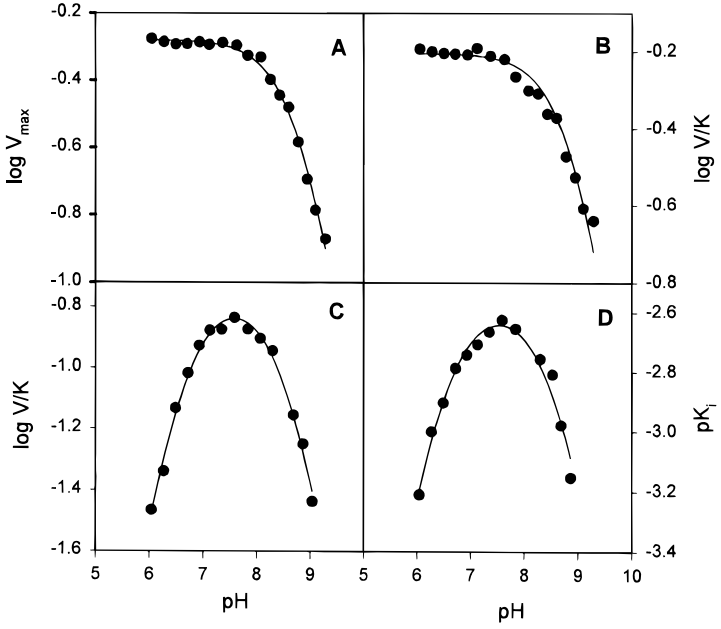


FIG. 2. The pH rate profiles for KNTase. (A) pH rate profile of V_{max} for the wild-type enzyme. The concentration of MgATP was varied from 0.124 to 5.0 mM. The concentration of kanamycin A was fixed at 0.05 mM. The concentration of free Mg^{2+} was held at 5.0 mM. The solid line was obtained by fitting the data to equation 8. (B) pH rate profile of V/K for MgATP. The assay condition is the same as A. The solid line was obtained by fitting the data to Eq. [8]. (C) pH rate profile of V/K for kanamycin A. The concentration of kanamycin A was varied from 5.0 to 400 μM. The concentration of MgATP was fixed at 5 mM. The concentration of free Mg^{2+} was held at 5.0 mM. The solid line was obtained by fitting the data to Eq. [9]. (D) Profile of pK_i for kanamycin A as a function of pH. The assay condition is the same as C. The solid line was obtained by fitting the data to Eq. [9].

the data to Eq. [8], is 8.8 ± 0.1. The pH dependence of *V/K* for MgATP is illustrated in Fig. 2B. The profile is similar with a pK value of 9.0 ± 0.1, representing one ionizable group that must be protonated for substrate activity. The pH profile of *V/K* for kanamycin A as the variable substrate is bell-shaped with pK values of 6.6 ± 0.1 and 8.5 ± 0.1 (Fig. 2C). The *V/K* profile for the antibiotic indicates two ionizable groups, where one must be protonated and the other must be unprotonated for maximum activity. The pH profile for the substrate inhibition constant for kanamycin is shown in Fig. 2D. It is bell shaped with pK values of 6.7 ± 0.1 and 8.5 ± 0.1.

Solvent viscosity studies. The effect of solvent viscosity on the kinetic parameters, *V/K* and *V,* for the nucleotide and antibiotic was measured using glycerol and PEG8000 as viscosogens. The macroviscosogen PEG8000 does not alter the kinetic parameters for either of the substrates (data not shown). However, the effect of glycerol is characterized by a decrease in the relative values of *V* and *V/K* with increasing solvent viscosity. The slopes of these plots for the effect on the *V/K* values for kanamycin and nucleotide are 1.1 and 0.45, respectively, while the slope for the effect on V_{max} is 1.05. These results are illustrated in Figs. 3A and 3B. No significant effect was observed on the substrate inhibition constant, K_i.

Magnesium ion effect. The effect of excess magnesium ion on the initial velocity of the enzymatic reaction was measured as a function of the ratio of Mg^{2+} to ATP. The assays were carried out at 25°C, pH 7.0. The concentrations of ATP and kanamycin were fixed at 0.5 mM and 50 μM, respectively. Under these conditions, the optimal velocity occurs at 2.0 mM Mg^{2+}. Inhibition by magnesium ion was observed at a concentration above 7 mM. These results are illustrated in Fig. 4.

DISCUSSION

Substrate specificity. The three-dimensional structure of KNTase, complexed with kanamycin and the nonhydrolyzable ATP analog, AMPCPP, has been solved (*13*).

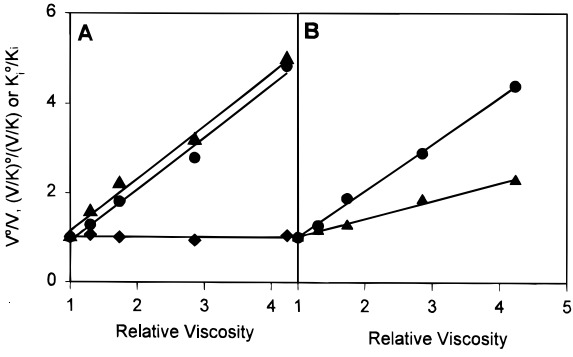


FIG. 3. Viscosity effects on KNTase. (A) The effect of glycerol as a viscosogen on the relative values of V_{max} (●), *V/K* (▲), and K_i (♦), with kanamycin A as the varied substrate. The concentration of MgATP was fixed at 4 mM. The concentration of free Mg^{2+} was held at 5.0 mM. (B) The effect of glycerol as viscosogen on the relative values of V_{max} (●) and *V/K* (▲), with MgATP as the varied substrate. The concentration of kanamycin A was fixed at 0.05 mM. The concentration of free Mg^{2+} was held at 5.0 mM. Enzymatic activity was assayed at pH 7.0, 20°C, with 100 mM Pipes buffer.

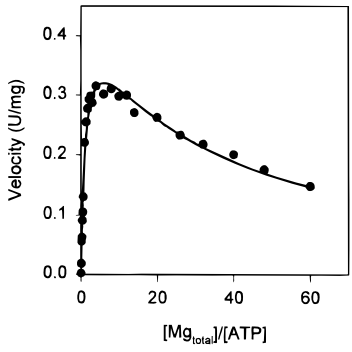


FIG. 4. The effect of magnesium ion on the activity of kanamycin nucleotidyltransferase. The concentration range of $MgCl_2$ was varied from 0.0 to 60 mM. The concentration of ATP and kanamycin were kept at 0.5 mM and 0.05 mM, respectively. The solid line was obtained by fitting the data to Eq. [3].

This structure is crucial for a determination of potential interactions between active site residues and functional groups of the substrates. Based on the X-ray crystal structure, the major portion of the antibiotic binding site consists of a ring of seven negatively charged amino acid residues that include D50, E52, E67, E76, E141, E142, and E145. Four of these residues, E67, E76, E141, and E145, plus K74, interact with the amino sugar ring **A** through hydrogen bonds (Fig. 5). The two amino groups of the deoxystreptamine ring **B** interact with the side chains of E141 and S94, but relatively few interactions are found between the aminoglycoside ring **C** and the protein (*13*). KNTase has a rather broad substrate specificity. Ten different aminoglycoside antibiotics, five of them from the kanamycin family and five from the neomycin group, were used to investigate the structure–activity relationship between the substrate and the enzyme. All of these aminoglycoside antibiotics have similar structures for rings **A** and **B**, which interact extensively with the side chains of amino acid residues in

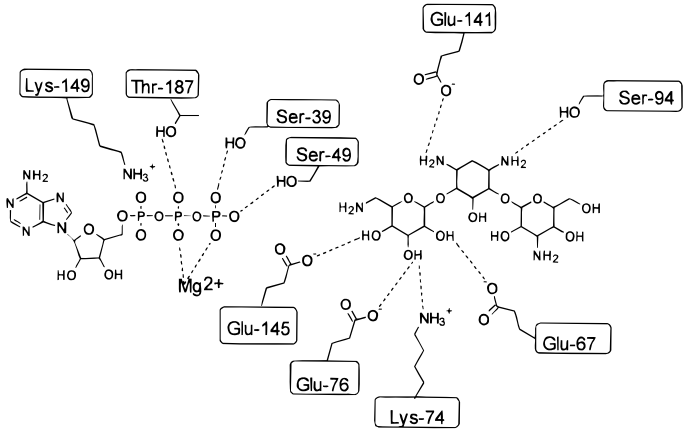


FIG. 5. Representation of the binding interaction at the active site of KNTase (*13*).

the active site. The major structural difference between the kanamycin and neomycin family of aminoglycosides lies in the third ring (Fig. 1). The kanamycin group has a six-membered ring **C** while the neomycin family has a five-membered ring **C** plus a six-membered ring **D**. Relatively small effects on the rate of catalysis and substrate binding by the different antibiotics were observed. Due to the internal symmetry within the kanamycin group, it is possible that rotation about ring **B** would orient ring **C** to be in a position such that the 4″-hydroxyl group could be adenylylated by ATP. This symmetry is not possible with the neomycin family.

KNTase is also able to accept a number of different nucleotides as substrates. Relatively small differences in *V* and *V*/*K* were observed with five different nucleotide triphosphates (ATP, GTP, UTP, TTP, and CTP) (Table 1). Since there are very few specific interactions between the nucleotide base and the amino acid residues in the active site (Fig. 5), it was anticipated that there would be minor differences in the catalytic constants for either catalysis or binding by the different nucleotide triphosphates.

Kinetic mechanism of KNTase. The catalytic activity of kanamycin nucleotidyltransferase was examined in both directions. The rate of the forward reaction was 73 times faster than the reverse reaction. The kinetic mechanism that is most consistent with the data obtained through initial velocity and inhibition studies is an ordered Bi–Bi mechanism, where the antibiotic binds before the nucleotide and pyrophosphate leaves the active site prior to the adenylylated antibiotic. Substrate inhibition was also observed. The initial velocity patterns determined with KNTase for both directions are intersecting, indicating that the antibiotic and nucleotide must bind before any products leave the active site (*20*).

The order of substrate addition was investigated using substrate analogues and dead-end inhibitors of both substrates. Spectinomycin, a kanamycin A analog, was found to be a competitive inhibitor of kanamycin A and a noncompetitive inhibitor of MgATP. The noncompetitive inhibition pattern of spectinomycin *versus* MgATP in a sequential mechanism is consistent with the binding of the antibiotic to the enzyme prior to nucleotide binding (*21*). AMP and AMPCPP, nonhydrolyzable ATP analogues, were found to be competitive inhibitors of ATP. Uncompetitive inhibition patterns by both AMP and AMPCPP with respect to kanamycin A were obtained, and these results thus support kanamycin A as the first substrate to bind to the enzyme.

Product inhibition experiments were conducted to determine the order of product release and to gain more information about the order of substrate addition. Adenylylated kanamycin was analyzed as a product inhibitor for the forward reaction. The competitive production inhibition pattern of AMP-kanamycin *versus* kanamycin and the noncompetitive production inhibition pattern of AMP-kanamycin *versus* ATP set the last product to be released as the adenylylated antibiotic and the first substrate to bind as the antibiotic. The latter result is consistent with the substrate analogue dead-end inhibition studies.

In an ordered kinetic mechanism, substrate inhibition has typically been caused by the second substrate binding to an enzyme–product complex in a dead-end fashion (*20*). Substrate inhibition, in this instance, however, was observed by the aminoglycoside, the first substrate to bind to the enzyme. Hence, kanamycin may be able to bind to the aminoglycoside binding pocket of the E•AMP–kanamycin complex prior to

final product release. This scenario would dictate that the antibiotic portion of AMP–kanamycin leave its binding site, while the nucleotide portion remains in the binding site. The specific binding site exposed by partial release of the aminoglycoside portion may then allow a second molecule of kanamycin to bind to the enzyme and form a dead-end complex. Such binding may prevent the enzyme from undergoing a conformational change that may be required for release of the nucleotide portion of the final product. A very similar mechanism was originally proposed by Gates and Northrop to explain the substrate inhibition that was observed in the reaction catalyzed by gentamicin adenylyltransferase (*22–24*)

An alternative explanation for the observed substrate inhibition by the aminoglycoside antibiotic may result from the closeness of the two antibiotic binding sites to one another. The distance between the ends of the two kanamycin molecules is only 3.5 Å (*13*). Steric interactions between these two antibiotic molecules may be possible within the dimer. The 4′-hydroxyl group of the bound antibiotic is about 5.0 Å away from the α-phosphorus of the nucleotide triphosphate analog, which is too far for a direct in-line displacement. It is likely that the enzyme undergoes a further conformational change prior to catalysis in order to reduce this distance. Interactions between the two antibiotic molecules in the dimer may inhibit the enzyme from undergoing a conformational change. Further investigations will be required to provide a more detailed picture of the origin of the substrate inhibition.

Effect of pH. The effect of pH on the kinetic parameters of both substrates was conducted to determine the required state of protonation of the ionizable groups. It was anticipated that an enzyme-base is required to abstract a proton from the hydroxyl group of kanamycin A prior to nucleophilic attack on the α-phosphorus of MgATP. The pH profile for V_{max} indicates that a single group with a pK value of about 8.8 must be protonated for maximal activity. The same ionizing group is observed in the pH profile of V/K for MgATP, an indication that this group may be involved in both binding and catalysis. Since the pK values for the phosphoryl groups of MgATP are below 6.1, it may be concluded that this ionizable group originates from one of the side chains at the active site of the protein. The most likely candidate for this ionizable group is the ε-amino group of a lysine residue since there are no histidine residues within the active site. The positively charged ε-amino group of K149 interacts with the negatively charged α-phosphoryl group of MgATP (*13*). Protonation of this residue may be required to facilitate an ion-pair complex and activation of the α-phosphoryl group for nucleophilic attack.

The pH profile of V/K for kanamycin exhibits two ionizable groups, a group with a pK_a value of 6.6 must be unprotonated and a group with a pK_a value of 8.5 must be protonated for substrate activity. There are two likely candidates for the ionizable group with a pK_a of 8.5. It may originate from the ε-amino group of K74 since it interacts with the 3′-hydroxyl group of kanamycin *via* a hydrogen bond. Alternatively, the amino groups of the antibiotic itself may be required to be protonated. Since the amino groups at carbons 1 and 3 interact with S34 and E141 in the active site (Fig. 5), one, or both, of them may be essential for catalytic activity. The pK_a values for the amino groups at carbons 1 and 3 have been estimated to be 6.2 and 7.4, respectively, in tobramycin using NMR methods (*25*). The second ionizable group with a pK value of 6.6 could be a carboxyl group of glutamate. Four different glutamate groups, E67,

E76, El41, and E145, have been found to interact with the amino and/or hydroxyl groups of the antibiotic substrate (Fig. 5). The most likely candidate is the carboxylate from E145, which is poised to abstract the proton from the 4′-OH of kanamycin. However, no ionization is observed in the pH profiles for V_{max} with a pK near 6. Either the pK value is shifted outside of the experimental range in the enzyme/substrate/product complex or the ionization-state of this residue has no effect on the release of products.

Solvent viscosity effects. Solvent viscosity studies have been a highly effective tool in the elucidation of the dependence of an enzymatic reaction on a diffusion-controlled step (*18, 26–28*). The effect of solvent viscosity on the rate of the KNTase reaction was measured by determining the sensitivity of the kinetic parameters, V_{max} and V/K, to changes in solvent viscosity. In an enzymatic reaction, the diffusion-controlled rate constants of association and dissociation between enzyme and ligands are inversely proportional to the relative viscosity (*27*). Shown below is a simplified kinetic mechanism for the reaction catalyzed by KNTase:

$$E \underset{k2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k4}{\overset{k_3 B}{\rightleftharpoons}} EAB \xrightarrow{k_5} EPQ \xrightarrow{k_7} E + \text{products}$$

In this model, A is kanamycin, B is MgATP, k_1, k_2, k_3, and k_4 are the rate constants for the binding of kanamycin and MgATP, and k_5 represents the catalytic step. This latter step is assumed to be essentially irreversible. The release of the products is represented by k_7. The rate expressions for the kinetic parameters, V, V/K_a and V/K_b, can be derived using the net rate constant method of Cleland (*29*).

$$V = (E_t)\, k_5 k_7 / k_5 + k_7) \quad [10]$$

$$V/K_a = (E_t)\, k_1 \quad [11]$$

$$V/K_b = (E_t)\, k_3 k_5 / (k_5 + k_4) \quad [12]$$

The rate constants k_1, k_2, k_3, k_4, and k_7 are presumed to represent diffusional events between the enzyme and substrates, and will be dependent on the changes in solution viscosity.

The effect of solvent viscosity on the V/K values for ATP, $(V/K_b)_o$ /(V/K_b), can be expressed as a function of the relative viscosity of the solution (η_{rel}), where

$$(V/K_b)_o/(V/K_b) = (k_5 \eta_{rel} + k_4) / (k_5 + k_4) \quad [13]$$

$(V/K_b)_o$ and (V/K_b) are the V/K values for nucleotide in the absence and presence of the viscosogen, respectively. The slope of the linear equation provides a measure of the sensitivity of the kinetic parameters to the change in solvent viscosity. The slope from Eq. [13] is given by the following equation:

$$(V/K_b)_\eta = k_5/(k_5 + k_4) \quad [14]$$

Therefore, the value of $(V/K_b)_\eta$ can vary from 0 to 1 depending on the relative magnitude of k_5 and k_4. When the substrate is very sticky, $(V/K_b)_\eta$ approaches the limiting value of 1. When the substrate is nonsticky, $(V/K_b)_\eta$ approaches zero. A slope of 0.45 was observed for MgATP, which indicates that k_4 and k_5 are approximately the same.

Since the rate constant for product dissociation, represented by k_7, is also a diffusion limited step, the relationship between the maximal velocity (V) and relative viscosity (η_{rel}) can be expressed by the following equation:

$$V_o/V = (k_5\eta_{rel} + k_7)/(k_5 + k_7) \quad [15]$$

A plot of V_o/V relative to η_{rel} should be linear and the slope of the line will be given by

$$V_\eta = k_5/(k_5 + k_7) \quad [16]$$

This equation predicts that the value of V_η can vary from 0 to 1 depending on the relative magnitude of k_5 and k_7. When the chemical step is much greater than product release, V_η approaches the limit of 1 and product release is rate limiting. When the rate of product release (k_7) becomes large, V_η approaches the limit of 0 and the overall rate of the enzymatic reaction is primarily dependent on the rate of chemical catalysis. A value of 1.0 was obtained on V_{max} and thus, the rate of product release is slower than the rate of the chemical step. With kanamycin, the slope effect on V/K at saturating levels of ATP is expected to be 1 according to the following equation.

$$(V/K_a)_o/(V/K_a) = \eta_{rel} \quad [17]$$

A slope of 1.1 is observed (Fig. 4) and thus supports the simple model for the kinetic mechanism.

Summary. The modification of antibiotic by KNTase follows an ordered Bi–Bi kinetic mechanism, with the aminoglycoside antibiotic being the first substrate to bind and the adenylylated antibiotic being the last product to be released. The overall rate of the reaction appears to be limited by the release of the products.

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