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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).

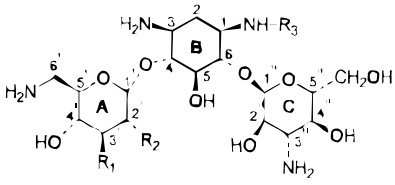
Initial velocity studies. The kinetic parameters, V_{max} and V/K_m, were determined in both the forward and reverse directions. The maximal velocity for the reverse direction is reduced about 73-fold when compared to that of the forward reaction.

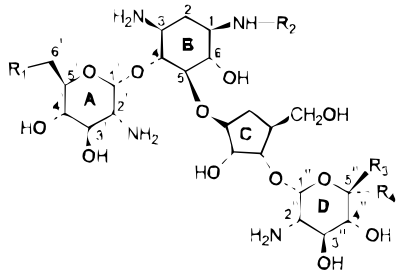
TABLE 2

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.

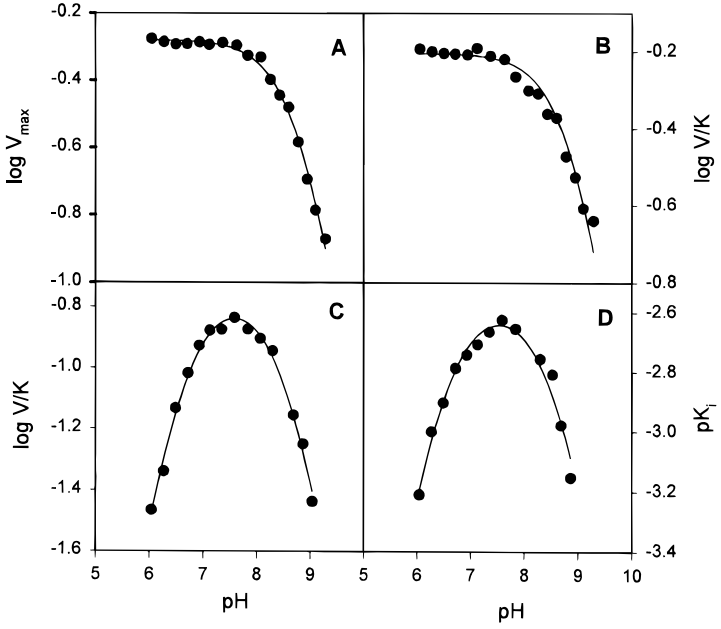


FIG. 2. The pH rate profiles for KNTase. (A) pH rate profile of V_{max} for the wild-type enzyme. The concentration of MgATP was varied from 0.124 to 5.0 mM. The concentration of kanamycin A was fixed at 0.05 mM. The concentration of free Mg^{2+} was held at 5.0 mM. The solid line was obtained by fitting 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].