Kinetic Mechanism of Aminoglycoside Phosphotransferase Type IIIa

EVIDENCE FOR A THEORELL-CHANCE MECHANISM

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Bacterial resistance to aminoglycoside-aminocyclitol antibiotics is mediated primarily by covalent modification of the drugs by a variety of enzymes. One such modifying enzyme, the 3'-aminoglycoside phosphotransferase, which is produced by Gram-positive cocci such as Enterococcus and Streptococcus inactivates a broad range of aminoglycosides by ATP-dependent phosphorylation of specific hydroxyl residues on the antibiotics. Through the use of dead-end and product inhibitor studies, we present the first detailed examination of the kinetic mechanism for the 3'-aminoglycoside phosphotransferase-Ill a. Initial velocity patterns deduced from steady-state kinetics indicate a sequential mechanism with ordered binding of ATP first followed by aminoglycoside. Dead-end inhibition by AMP and adenyllyl-imidodiphosphate is competitive versus ATP and noncompetitive versus kanamycin A. Dead-end inhibition by tobramycin, a kanamycin analogue lacking a 3'-OH, is competitive versus both kanamycin A and uncompetitive versus ATP, indicative of ordered substrate binding where ATP must add prior to aminoglycoside addition. Product inhibition by kanamycin phosphate is noncompetitive versus ATP when kanamycin A is held at sub-saturating concentrations (Km(kana)A), whereas no inhibition is observed when the concentration of kanamycin A is held at 10 Km(kana). This is consistent with kanamycin phosphate being the first product released followed by ADP release. The patterns of inhibition observed support a mechanism in which ATP binding precedes aminoglycoside binding followed by a rapid catalytic step. Product release proceeds in an ordered fashion where kanamycin phosphate is released quickly followed by a slow release of ADP. Aminoglycoside substrates, such as kanamycin A, show substrate inhibition that is noncompetitive versus ATP. This indicates binding of the aminoglycosides to the slowly dissociating (EADP) complex at high drug concentrations. These experiments are consistent with a Theorell-Chance kinetic mechanism for 3'-aminoglycoside phosphotransferase-Ill a.

The development of antibiotic chemotherapy during the early part of the century has been paralleled by an increase in bacterial resistance to these drugs. Microorganisms have evolved that elude the cytotoxic effect of antibiotics by a variety of means including alteration of targets and chemical modification. The aminoglycoside-aminocyclitol antibiotics are one class of drugs subject to the latter mechanism of resistance.

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This family of antimicrobial agents includes streptomycin, gentamicin, and kanamycin A as well as many others (Fig. 1) (1). The aminoglycosides can be grouped into three classes: 1) 4,6-disubstituted deoxystreptamine compounds such as kanamycin, 2) 4,5-disubstituted deoxystreptamine aminoglycosides such as paromomycin, and 3) those compounds without a deoxystreptamine ring, which include such drugs as streptomycin. They are used world wide but are subject to a broad spectrum of enzymatic inactivations. Aminoglycosides can be rendered ineffective to the target bacteria by chemical modification catalyzed by a variety of enzymes including the acetyltransferases, the O-phosphotransferases, and the N-acetyltansferases (2). The O-phosphotransferases are widely distributed in nature and are comprised of at least 20 different phosphotransferases depending upon the regiospecificity of hydroxyl group modification (for review, see Ref. 3). One particular phosphotransferase, 3'-aminoglycoside phosphotransferase-Ill a, produced by the opportunistic pathogens Enterococci (4) and Staphylococci (5), regiospecifically modifies the 3' position of the 6-aminoglucone ring of kanamycin A and phosphorylates a variety of 4,5-disubstituted deoxystreptamine aminoglycosides as well (6).

To date, few aminoglycoside modifying enzymes have been subject to extensive kinetic or mechanistic studies. The 3'-aminoglycoside acetyltransferase-I was demonstrated to follow a random kinetic mechanism (7) as was the bifunctional 6'-acetyltransferase 2'-phosphotransferase enzyme (8). A third acetyltransferase (AAC(6'-4)) has been established to follow a rapid equilibrium random sequential kinetic mechanism (9). The 2'-aminoglycoside nucleotidyltransferase was shown to inactivate the antibiotics through a Theorell-Chance kinetic mechanism (10) where the release of the nucleotidylated aminoglycoside is the rate-determining step in the reaction. The aminoglycoside phosphotransferases have not been subject to rigorous kinetic analysis with the exception of the 3'-aminoglycoside phosphotransferase-Ill a, where the groundwork has been laid by initial structure-function analysis (6). We report herein, through the use of initial velocity studies, dead-end inhibitors, product inhibition patterns, and substrate inhibition, that 3'-aminoglycoside phosphotransferase-Ill a follows an ordered substrate addition and an ordered product release, which limits the rate of reaction and thus supports the occurrence of a Theorell-Chance mechanism.

MATERIALS AND METHODS

Chemicals—Kanamycin A, tobramycin, amikacin, paromomycin, AMP, ATP, HEPES, and pyruvate kinase/lactate dehydrogenase were obtained from Sigma. AMP-PNP1 was from Boehringer Mannheim. 3'-Aminoglycoside phosphotransferase-Ill a was purified from E. coli BL21(DE3)pET TSACG1 as described previously (6). Phosphotranslated kanamycin was purified by modification2 of our previous method (6).

1 The abbreviation used is: AMP-PNP, adenylyl-imidodiphosphate.

2 P. R. Thompson and G. D. Wright, unpublished results.
Enzyme Assays—Aminoglycoside phosphotransferase-IIIa activity was monitored by coupling the release of ADP to a pyruvate kinase/lactate dehydrogenase reaction as described previously (6). Unless otherwise indicated, kanamycin A was held at 100 µM as the fixed second substrate, and ATP was held at 1 mM as the first substrate in an ordered Bi Bi system.

Data Analysis—Kinetic data for varied substrate concentrations were fit to Equations 1 and 2 by nonlinear least-squares using the computer program Grafit (11). Kinetic data for varied substrate concentrations were fit to Equation 3 by nonlinear least-squares using the computer program Grafit (11). Initial velocities were fit to Equation 4 by nonlinear least-squares fitting to the equations by nonlinear regression (11).

Initial velocity patterns were fit to Equation 5 by nonlinear least-squares fitting to the equations by nonlinear regression (11).

Initial Velocity Patterns—As a first step in the determination of the kinetic mechanism of 3'-aminoglycoside phosphotransferase-IIIa, the velocity of reaction with kanamycin A, a 4,6-disubstituted deoxystreptamine aminoglycoside, was determined at several fixed concentrations of ATP. Double-reciprocal plots of 1/v versus 1/[kan A] are displayed in Fig. 2. The observed pattern of intersecting lines in these plots is indicative of a sequential rather than a ping-pong mechanism. Paromomycin, a 4,5-disubstituted deoxystreptamine aminoglycoside also exhibits an intersecting pattern of initial velocity plots (data not shown) indicating that 3'-aminoglycoside phos-
photontransferase-IIIa follows a sequential kinetic mechanism regardless of the class of aminoglycoside substrate analyzed. A $K_{ia}$ value for ATP of $26.0 \pm 8.4 \mu M$ is indistinguishable from the $K_{m(\text{ATP})}$ of $27.7 \pm 3.7 \mu M$ previously reported (6), consistent with rapid equilibrium binding of ATP (12).

Dead End Inhibitors—Substrate analogue inhibitors of aminoglycoside (tobramycin) and nucleotide (AMP) were assayed as inhibitors of both substrates in order to elucidate substrate binding order. These results are summarized in Table I. Tobramycin (no 3'-OH) was found to be a potent competitive inhibitor of kanamycin A with a $K_{i}$ of $0.58 \pm 0.40 \mu M$ as well as a competitive inhibitor of amikacin with a $K_{i}$ of $0.35 \pm 0.02 \mu M$. It was also found to be an uncompetitive inhibitor of ATP with a $K_{i}$ of $0.64 \pm 0.02 \mu M$ (Fig. 3).

AMP was found to be a weak competitive inhibitor of ATP (Table I; Fig. 4a) with a $K_{i}$ of $4.9 \pm 0.59 \mu M$. It was also found to be a noncompetitive inhibitor of kanamycin A with a $K_{i}$ of $10 \pm 2.4 \mu M$ and a $K_{i}$ of $7.8 \pm 0.51 \mu M$ (Table I, Fig. 4b). This is consistent with an ordered Bi Bi substrate addition where ATP is the obligate first substrate followed by aminoglycoside addition.

A second nucleotide analogue, AMP-PNP (a nonhydrolyzable ATP isostere) was found to be a competitive inhibitor of ATP (Table I) with a $K_{i}$ of $350 \pm 50 \mu M$. It was also found to be a noncompetitive inhibitor of amikacin with a $K_{i}$ of $990 \pm 80 \mu M$ and a $K_{i}$ of $1.8 \pm 0.74 \mu M$. These results are consistent with inhibition results obtained using AMP and indicate an order of obligate ATP addition first followed by aminoglycoside addition.

Substrate Inhibition—At high concentrations of aminoglycoside, a decrease in the rate of reaction is observed. This is indicative of nonproductive binding of the drug to a catalytically incompetent form of the enzyme. We initially examined substrate inhibition by paromomycin as it exhibited strong inhibition (6). Over a wide range of paromomycin concentrations ($2 K_{i(par)}$ to $75 K_{i(par)}$), the family of reciprocal plots shows a decrease in the slopes ($K_{m(\text{ATP}),\text{par}}/V_{\max}$) approaching the normal limit of $K_{m(\text{ATP})}/V_{\max}$. As the concentration of aminoglycoside continues to increase, the family of plots generated forms a series of parallel lines indicative of uncompetitive inhibition. The slope replot therefore decreases over low concentrations of aminoglycoside reaching a minimum above $10 K_{i(par)}$ (Fig. 5b; Table I). Over the same range of paromomycin concentrations, the intercept replot exhibits a typical nonlinear pattern as it approaches a minimum and then increases again (Fig. 5a; Table I). To more closely examine the nature of the substrate inhibition, a second aminoglycoside substrate, kanamycin A, was chosen as the fixed substrate at several saturating levels. The aminoglycoside in this case was held at saturating levels in order to avoid confusing the replot data with initial velocity effects and to limit the results to substrate inhibition effects. The inhibition is again uncompetitive with a linear intercept replot (Fig. 5c; Table I), and a standard slope replot (not shown) was associated with linear uncompetitive inhibition. This indicates nonproductive binding of kanamycin to the (E-ADP) complex suggesting at least partial rate-limiting ADP product release. This linear intercept uncompetitive inhibition is observed for both classes of aminoglycoside substrates.

Product Inhibition—Due to the limitations of the coupled assay, which consumes product ADP, only kanamycin phosphate was available for use as a product inhibitor. Kanamycin phosphate was found to be a noncompetitive inhibitor of ATP ($[\text{kanamycin A}] = K_{m(\text{kan})}$) with a $K_{i}$ of $10 \pm 7.1 \mu M$ and a $K_{i}$ of $3.7 \pm 0.47 \mu M$ (Fig. 6a; Table I). Inhibition of ATP by kanamycin phosphate was eliminated by increasing the concentration of kanamycin A to $126 \mu M$ ($10 K_{m(\text{kan})}$) (Fig. 6b; Table I).
I). The results are consistent with a rapid release of the first product, kanamycin phosphate followed by a relatively slow release of ADP as the second product. This is indicative of a Theorell-Chance mechanism, a specialized case of the ordered Bi Bi mechanism.

**Alternative Substrates—**

3'-Aminoglycoside phosphotransferase-IIIa has a broad aminoglycoside substrate range, which makes it amenable to analysis by the alternative substrate method of Radika and Northrop (13). For a series of both 4,5- and 4,6-disubstituted deoxystreptamine antibiotics, we see $k_{cat}$ vary only 1.1-fold and $k_{cat}/K_m(\text{ATP})$ vary 1.4-fold (Table II). This results in a series of essentially coincident lines in reciprocal plots (not shown) indicative of an ordered sequential mechanism, specifically a Theorell-Chance mechanism where ATP binds first followed by the aminoglycoside.

**DISCUSSION**

Determination of the kinetic mechanism of the aminoglycoside detoxifying enzyme, 3'-aminoglycoside phosphotransferase-IIIa required the combination of several techniques. Double-reciprocal plots of initial velocity data have an intersecting pattern of lines indicative of a sequential mechanism and excluding the possibility of a ping-pong mechanism. This intersecting pattern of lines is observed for kanamycin A as well as for paromomycin (4,5-disubstituted aminoglycoside) and amikacin (which shows no substrate inhibition). This demonstrates that the 3'-aminoglycoside phosphotransferase-IIIa follows a sequential mechanism for all classes of aminoglycosides as well as for the “poor” substrate amikacin. A $K_{ia}$ of 26.0 ± 8.4 μM with kanamycin A as the fixed substrate was calculated fitting data to Equation 3. This value is in agreement with a $K_m(\text{ATP})$ of 27.7 ± 3.7 μM reported previously (6). This indicates that $K_m(\text{ATP}) = K_{ia}$, where $K_{ia} = k_{-3}k_3$ and $K_m(\text{ATP}) = k_3k_d/k_3(k_3 + k_d)$ and suggests that binding of ATP is in rapid equilibrium (12).

Substrate binding order was probed using substrate analogue dead-end inhibitors of both the nucleotide and aminoglycoside substrates. Tobramycin was found to be a potent competitive inhibitor of aminoglycosides and an uncompetitive inhibitor of ATP. The observation of uncompetitive inhibition in a sequential mechanism is diagnostic of an ordered mechanism where ATP binds prior to aminoglycoside binding (inhibitor binding) (14). Both the nonhydrolyzable ATP analogue
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Inhibition was observed with a $K_{m}$ involving holding kanamycin A at saturating concentrations for ATP analyzed as a product inhibitor of ATP. Initial studies of enzymatic activity, this obviates the use of ADP as a product couplet to pyruvate kinase/lactate dehydrogenase for an assay order of product release. Given that ADP product release is required prior to aminoglycoside binding.

AMP-PNP and AMP were found to be competitive inhibitors of ATP and noncompetitive inhibitors of kanamycin A. These data support an ordered substrate addition where ATP binding is required prior to aminoglycoside binding.

Product inhibition studies were initiated to elucidate the order of product release. Given that ADP product release is coupled to pyruvate kinase/lactate dehydrogenase for an assay of enzymatic activity, this obviates the use of ADP as a product inhibitor. Kanamycin phosphate, which we generated using purified 3′-aminoglycoside phosphotransferase-IIIa, was therefore analyzed as a product inhibitor of ATP. Initial studies involved holding kanamycin A at subsaturating concentrations ($K_{m}$) while varying ATP. A pattern of noncompetitive inhibition was observed with $K_{i}$ of 10 ± 7.1 mM and $K_{i}$ of 3.7 ± 0.47 mM. When kanamycin A was held at saturating concentrations ($K_{m}$), no inhibition was observed. This pattern of inhibition is consistent with release of kanamycin phosphate first followed by release of ADP. The observed elimination of product inhibition by saturation with kanamycin A excludes a simple ordered Bi Bi kinetic mechanism. It is consistent, however, with a Theorell-Chance mechanism, which is a special case of the ordered Bi Bi mechanism where the central complex (E-ATP-kan $\rightleftharpoons$ E-kan-phos-ADP) becomes kinetically insignificant and does not contribute to the rate of reaction as presented in Scheme I (15). This mechanism involves a rapid release of kanamycin phosphate followed by a slower rate-limiting release of ADP as the second product. The existence of the ternary complex has been established by initial velocity studies, although its existence is transient and kinetically insignificant, therefore the product inhibition patterns are consistent with a ping-pong mechanism.

Also compatible with the existence of a Theorell-Chance mechanism is a 1.15-fold difference in $k_{cat}$ values and a 1.38-fold difference in $k_{cat}/K_{m}$ values determined for ATP with several aminoglycoside fixed second substrates (Table II). This marginal increase in both $k_{cat}$ values and $k_{cat}/K_{m}$ values is consistent with a Theorell-Chance mechanism, where the fixed substrate is the second substrate in the binding order (13). In a double-reciprocal family of plots with ATP as the variable substrate, all plots collapse into a single overlapping line. As presented in Scheme I, changing the second substrate (i.e. aminoglycoside) would have very little effect upon the overall rate of reaction, given that it is released prior to the proposed rate-limiting segment of the mechanism. As the rate-limiting segment of the reaction is dependent upon the nucleotide substrate, one would expect a more significant $k_{cat}$ and $k_{cat}/K_{m}$ effect by using alternative fixed nucleotide substrates other than ATP (13). This, however, is not possible when using the coupled PK/LDH system.

Uncompetitive substrate inhibition (intercept linear) by aminoglycosides versus ATP is indicative of nonproductive binding of the aminoglycoside, at high concentrations, to the (E-ADP) enzyme form following kanamycin phosphate release (Scheme I). The formation of this (E-ADP-I) complex (where I is the aminoglycoside) must exist given that the nonproductive binding of the aminoglycoside must be isolated from ATP addition by irreversible steps (in order to observe uncompetitive inhibition) (16). The release of ADP from the (E-ADP-I) complex occurs more slowly than the catalytic step and subsequent kanamycin phosphate release. The slow release of ADP allows this form of the enzyme to accumulate, and thus at high kanamycin substrate concentrations the aminoglycoside is able to bind the (E-ADP-I) complex and partially block ADP release from the (E-ADP-I) complex.

Biological Significance of the Mechanism—At typical intracellular concentration of ATP (3 mM in Salmonella typhi-murium (17)), the enzyme will be saturated with this first substrate. Upon encountering an aminoglycoside molecule, catalysis occurs, followed by rapid ejection of the inactive phosphorylated drug. The (E-ADP-I) complex then undergoes slow dissociation to give free enzyme. This suggests then that the enzyme would loose its capacity to protect the cell effectively at high aminoglycoside concentrations. One proposal for explaining the cytotoxic effects of aminoglycosides advanced by Davis (18) suggests that the first step in inhibition of cell growth is the entry of small amounts of aminoglycoside into the cell. Only after intracellular membrane damage occurs will large quantities of drug penetrate the cell. Our observation that the minimal inhibitory concentration of aminoglycosides is positively correlated with $k_{cat}/K_{m}$ (6), the determining rate at low am-

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3 Others have noted similar effects with other aminoglycoside modifying enzymes, in fact with much more significant correlation coefficients, e.g. Ref. 19.
noglycoside concentrations indicates that drug inactivation is important at sub-
K_m concentrations of drug, consistent with our observed mechanism. No correlation with minimal inhibi-
tory concentration is observed with k_cat, the rate at infinite
concentration of aminoglycoside; this is not surprising as the
enzyme shows substrate inhibition at these levels of drug,
therefore the capacity to detoxify the agents is in fact decreasing
at high levels of drug, and the organism is losing the battle
against the antibiotic. Therefore, evolution has provided a cat-
alyst that is suited to the inactivation of low levels of ami-
noglycoside, consistent with Davis' hypothesis for the mode of
action of these antibiotics.

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