Catalytic Mechanism of Enterococcal Kanamycin Kinase (APH(3′)-IIIa): Viscosity, Thio, and Solvent Isotope Effects Support a Theorell−Chance Mechanism†

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Received February 19, 1996; Revised Manuscript Received April 18, 1996⊗

ABSTRACT: Bacterial resistance to the aminoglycoside antibiotics is manifested primarily through the production of enzymes which covalently modify these drugs. The *Enterococci* and *Staphylococci* produce an ATP-dependent kinase, APH(3′)-IIIa, which phosphorylates such antibiotics as kanamycin, amikacin, and neomycin, and this enzyme shows a Theorell−Chance kinetic mechanism by traditional product and analogue inhibitor analysis and by the alternative substrate diagnostic [McKay, G. A., & Wright, G. D. (1995) *J. Biol. Chem. 270*, 24686−24692]. We report that the APH(3′)-IIIa exhibits small solvent ($V^{H}/V^{D} \approx 1.50$) and thio effects ($V^{ATP}/V^{ATP\gamma S} = 2$) indicating hydroxyl group deprotonation and nucleophilic attack on ATP do not significantly contribute to the overall steady-state rate. The enzymatic rates were determined with the viscogens PEG 8000, glycerol, and sucrose, and these experiments demonstrate that ATP binding and ADP release are diffusion controlled and that ADP release is solely rate limiting for APH(3′)-IIIa. In addition, the slope of *V*/*K* for ATP vs relative viscosity is greater than the theoretical limit of 1, suggesting a possible enzyme conformational change upon binding of ATP. This new experimental evidence supports a Theorell−Chance mechanism for APH(3′)-IIIa.

A thorough understanding of the mechanism of enzymes which inactivate antibiotics is lacking in all but a very few cases. Proteins which contribute to the detoxification of antibiotics are responsible for a dramatic increase in the crisis of antibiotic resistance which is currently a major health concern. A good example of the forced obsolescence of a group of antibiotics as a result of the emergence of resistance enzymes can be found in the history of aminoglycoside antibiotics. Waksman discovered the aminocyclitol/aminoglycoside antibiotics streptomycin (Schatz et al., 1944) and neomycin (Waksman & Lechevalier, 1949) in the 1940s, and these were rapidly incorporated into the clinical antimicrobial therapies of the day despite some serious side effects such as oto- and nephrotoxicity. The discovery of kanamycin by Umezawa and co-workers in 1957, a potent antimicrobial compound with fewer side effects, was to be a harbinger for the isolation of several similar aminoglycoside drugs within the next decade. However, the rapid emergence and dispersion of kanamycin resistance phenotypes within the microbial community diluted the effectiveness of this class of compounds. In fact, the widespread dissemination of genes encoding enzymes which transferred a phosphate group to the 3′-hydroxyl position of kanamycin (APH(3′))[1] effectively eliminated most 3′-OH bearing aminoglycoside antibiotics from clinical use in the 1970s, and now these are rarely used with the exception of the semisynthetic drug amikacin. The emergence of at least seven different classes of APH(3′) isozymes over the last 25 years has essentially obviated the clinical use of several broad-spectrum aminoglycoside antibiotics (Shaw et al., 1993).

One approach to reclaim the usefulness of 3′-OH-containing aminoglycosides is the synthesis of specific inhibitors of these enzymes. 3′-Deoxyaminoglycosides such as tobramycin are competitive inhibitors of APH(3′) (McKay & Wright, 1995) and have found extensive clinical use. Recently, mechanism-based inactivators of APH(3′)-Ia and APH(3′)-IIa have been described in which a nitro group is incorporated at C2′ (Roestamadji et al., 1995). Enzyme-catalyzed phosphorylation of the 3′-OH which is adjacent to the 2′-nitro group permits elimination of inorganic phosphate and generation of an electrophilic nitro alkene which can undergo Michael addition with active site nucleophiles. This is a good example of the inactivation potential of such compounds. The development of β-lactamase inhibitors has greatly benefited from the rich knowledge of the enzymology and structure of β-lactamases. Similarly, an understanding of the mechanism of aminoglycoside-metabolizing enzymes will contribute to the development of novel enzyme inhibitors.

The enterococcal enzyme, APH(3′)-IIIa, has the broadest specificity of all known APH(3′) enzymes (Shaw et al., 1993) which makes this isozyme ideal as a prototypical APH. We have overexpressed APH(3′)-IIIa in *Escherichia coli* and determined its substrate specificity (McKay et al., 1994a). The enzyme regiospecifically phosphorylates 4,6-disubstituted aminocyclitol aminoglycosides at the 3′-hydroxyl of the 6-aminoglucose ring (McKay et al., 1994a) and will phosphorylate the 5″-hydroxyl of the ribose ring in 4,5-disubstituted aminocyclitol aminoglycosides such as lividomycin A (Thompson et al., 1996). Classical analysis of steady-state kinetic parameters and product and dead-end inhibition have indicated that the enzyme operates by a Theorell−Chance kinetic mechanism (Scheme 1) (McKay

† This research was funded by a grant from the Medical Research Council of Canada and by a Natural Sciences and Engineering Council of Canada Postgraduate Scholarship to G.A.M.

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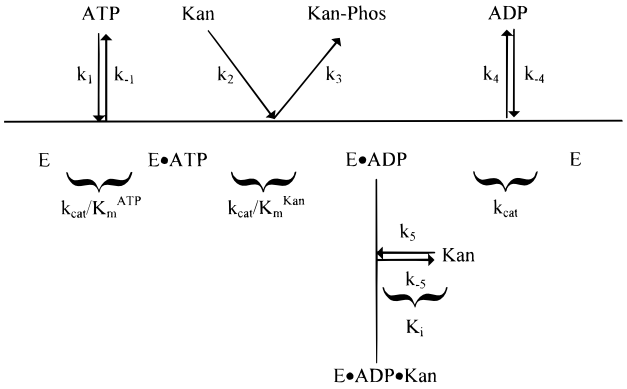
⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: APH, aminoglycoside phosphotransferase; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

S0006-2960(96)00388-1 CCC: $12.00

Scheme 1



& Wright, 1995). ATP is in rapid equilibrium and binds first, this is followed by binding of aminoglycoside, phosphate transfer and immediate release of phosphorylated drug. Finally, ADP is released. In order to further probe the contribution of each step to the overall rate of the reaction, we have used viscosity, solvent isotope, and nucleotide thio effects. This has permitted an analysis of the thermodynamics of the reaction and has enabled the independent identification of ADP release as the rate-limiting step in catalysis, as predicted for a Theorell−Chance mechanism.

MATERIALS AND METHODS

ATPγS, ATP, and aminoglycosides were from Sigma. D_2O (99.9%) was from Isotec. 3′-Phosphokanamycin A was prepared by minor modification (Thompson et al., 1996) of the reported method (McKay et al., 1994a).

The concentration of 3′-phosphokanamycin A was determined in triplicate using ninhydrin assay from a standard curve generated with kanamycin A. The concentration of standard kanamycin A was first determined by titration with APH(3′)-IIIa. APH(3′)-IIIa was purified as previously described (McKay et al., 1994a).

Enzyme Assay. APH(3′)-IIIa activity was monitored by coupling ADP release following ATP-dependent aminoglycoside phosphorylation to the pyruvate kinase/lactate dehydrogenase coupled enzyme system (McKay et al., 1994a). The reverse reaction, formation of ATP from 3′-phosphokanamycin and ADP, was monitored at 340 nm by coupling ATP synthesis to hexokinase and glucose-6-phosphate dehydrogenase. In a typical experiment 980 μL of assay buffer (100 mM HEPES-HCl, pH 7.5, 7.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM $NADP^+$, 2 mM glucose, 9.4 units of hexokinase, and 1.9 units glucose-6-phosphate dehydrogenase, 1 mM ADP) was mixed with 10 μL of phosphorylated aminoglycoside solution. The solution was preincubated for 10 min at 37 °C, and the assay was initiated by addition of 5 μL of enzyme solution (0.12 nmol).

ATPase activity was monitored by performing the assay in the absence of kanamycin A or in the presence of the 3′-deoxyaminoglycoside tobramycin. Phosphate release was monitored by the method of Lanzetta et al. (1979).

Viscosity Experiments. Assays were performed with glycerol (0−30%), sucrose (0−30%), and PEG 8000 (6.7%). The viscosity of solutions was determined in quadruplicate relative to a 50 mM HEPES-HCl, pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, at 25 °C using an Ostwald viscometer. The rate of the pyruvate kinase/lactate dehydrogenase coupled enzyme system was unaffected by the presence of these viscogens under the conditions used.

Solvent Isotope Effects. Reaction mixtures were prepared in D_2O including all substrates. Stock enzyme solutions were added in H_2O to a maximum final concentration of 5% (v/v). pD values were determined by measuring pH and adding 0.4 units (pD = pH + 0.4).

Data Analysis. Data were fit to eq 1 for normal Michaelis−Menten kinetics or eq 2 for substrate-inhibited reaction by nonlinear least-squares methods using the computer program Grafit (Leatherbarrow, 1992).

$$v = V_{max}S/(K_m + S) \quad (1)$$

$$v = V_{max}S/(K_m + S + S^2/K_i) \quad (2)$$

All data points were used to obtain the kinetic parameters without further weighting. Errors indicated for these parameters are standard errors obtained from the best fit of the data by eq 1 or 2.

RESULTS

Solvent Isotope and Thio Effects. Since aminoglycoside phosphorylation implies at least one deprotonation event (3′-OH), we sought to evaluate the effect of substituting this OH for OD by performing the reaction in D_2O. Solvent effects were minor at two pDs (6.5 and 7.5) for both kanamycin A and ATP with k_{cat}^H/k_{cat}^D of 1.5−1.6 (Table 1).

Similarly, substitution of ATPγS for ATP resulted in only a small V_{max} change, ($k_{cat}^{ATP}/k_{cat}^{ATP\gamma S} \approx 2$) (Table 1).

Viscosity Effects. In an effort to elucidate which catalytic step was rate limiting, we determined steady-state kinetic parameters for APH(3′)-IIIa in solvents of varying viscosity. We sought to distinguish between effects due to global viscosity changes versus effects on diffusion-controlled equilibria by monitoring effects due to the macroviscogen PEG 8000 and the microviscogens glycerol and sucrose (Blacklow et al., 1988). The polymer PEG 8000 had no effect on the relative k_{cat} or k_{cat}/K_m when either kanamycin A or ATP was varied (Table 2). This is an important result which demonstrates that the macroviscosity of the solutions has no effect on the enzymatic rates as predicted for polymeric species which do not affect the rate of diffusion of small molecules (Blacklow et al., 1988). Glycerol and sucrose, on the other hand, had a dramatic effect on k_{cat} when either substrate was varied (Figure 1, Table 2). Additionally, k_{cat}/K_m was affected for ATP but not for the aminoglycoside substrate kanamycin A. Similar results were obtained with another aminoglycoside, amikacin, which has a 20-fold higher K_m (245 μM) than kanamycin but an unchanged k_{cat} (McKay et al., 1994a).

Kanamycin A and other aminoglycosides, except amikacin, show substrate inhibition. We see no significant effect due to glycerol or sucrose-induced viscosity changes in K_i (Table 3).

ATPase Activity and Steady-State Kinetics of the Reverse Reaction. No ATPase activity was observed in the absence or presence of tobramycin by either the phosphate release or the ADP release assays. The availability of purified 3′-phosphokanamycin A permitted determination of kinetic parameters in the reverse (ATP forming) reaction by coupling ATP to hexokinase/glucose-6-phosphate dehydrogenase. Data obeyed typical Michaelis−Menten kinetics and were fit to

Table 1: Solvent Isotope and Thio Effects for APH(3′)-IIIa

varied substrate	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)	k_{cat}^H/k_{cat}^D	$k_{cat}^{ATP}/k_{cat}^{ATP\gamma S}$
ATP					
pH 7.5[a]	27.7 ± 3.7	1.76 ± 0.08			
pD 7.5	11.6 ± 1.7	1.08 ± 0.04		1.63	
kan A					
pH 6.5	4.44 ± 0.86	1.73 ± 0.05	2.36 ± 0.48		
pD 6.5	16.21 ± 0.72	1.18 ± 0.05	1.28 ± 0.34	1.47	
pH 7.5[a]	12.6 ± 2.6	1.79 ± 0.09	6.4 ± 1.7		
pD 7.5	3.8 ± 0.8	1.18 ± 0.04	2.0 ± 0.4	1.50	
ATPγS, pH 7.5	49.5 ± 6.9	0.842 ± 0.24			2.09 ± 0.095

[a] McKay et al. (1994a).

Table 2: Viscosity Effects on APH(3′)-IIIa

viscogen	varied substrate	fixed substrate	$(k_{cat}°/k_{cat})^{\eta}$ [a]	$((k_{cat}/K_m)°/k_{cat}/K_m)^{\eta}$ [a]
glycerol	ATP	kan A (0.125 mM)	1.03 ± 0.05	1.58 ± 0.22
glycerol	ATP	kan A (1 mM)	1.37 ± 0.14	2.10 ± 0.19
glycerol	kan A	ATP (1 mM)	1.29 ± 0.03	0.15 ± 0.06
glycerol	ATP	amikacin (2.5 mM)	0.93 ± 0.12	2.23 ± 0.13
glycerol	ATP	amikacin (0.1 mM)	0.56 ± 0.03	0.56 ± 0.07
glycerol	amikacin	ATP (1 mM)	1.00 ± 0.08	0.06 ± 0.26
sucrose	ATP	amikacin (2.5 mM)	0.76 ± 0.10	0.83 ± 0.11
sucrose	kan A	ATP (1 mM)	1.05 ± 0.05	−0.12 ± 0.13
sucrose	amikacin	ATP (1 mM)	0.76 ± 0.08	−0.02 ± 0.06
PEG 8000	kan A	ATP (1 mM)	0.02 ± 0.02	0.00 ± 0.04
PEG 8000	ATP	kan A (1 mM)	0.02 ± 0.00	−0.05 ± 0.02

[a] $k_{cat}°$ and $(k_{cat}/K_m)°$ are the rates with no added viscogen. Values are the slopes of plots for either $k_{cat}°/k_{cat}$ or $(k_{cat}/K_m)°/k_{cat}/K_m$ vs relative viscosity of the solution (e.g., Figure 1).

eq 1. The K_m for ADP was 21.6 ± 3.3 μM, and the K_m for 3′-phosphokanamycin A was 1320 ± 170 μM. The k_{cat} for the reverse direction was 0.11 ± 0.01 s^{-1}.

DISCUSSION

The aminoglycoside antibiotics are especially useful for the treatment of hospital-acquired (nosocomial) infections. The frequency of nosocomial infections caused by *Enterococci* and *Staphylococci* is second only to those caused by *E. coli* (Spera & Farber, 1992). These Gram positive cocci often harbor the *aph(3′)-IIIa* gene, which renders them insensitive to a number of aminoglycoside antibiotics, including kanamycin and lividomycin (Trieu-Cuot & Courvalin, 1983). Additionally, many of these strains also produce the bifunctional APH(2″)-AAC(6′) enzyme which confers resistance to the clinically important 3′-deoxyaminoglycosides tobramycin and gentamicin C (Ferretti et al., 1986). There is therefore considerable interest in understanding the molecular mechanism of aminoglycoside inactivation in these opportunistic pathogens.

We have overexpressed and characterized the aminoglycoside kinase APH(3′)-IIIa (McKay et al., 1994a) and performed initial identification of potential active site residues by affinity labeling (McKay et al., 1994b). The kinetic mechanism of APH(3′)-IIIa was determined by classical product and dead-end substrate analogue inhibition to be Theorell−Chance (McKay & Wright, 1995). This mechanism is a special case of an ordered BiBi mechanism in which there is no contribution of the E·A·B ⇄ E·P·Q (here E·ATP·Kan ⇄ E·Kan-phosphate·ADP) equilibrium to the steady-state rate. For APH(3′)-IIIa, we find that ATP binds first with rapid equilibrium followed by binding of the aminoglycoside, release of the phosphoaminoglycoside, and finally dissociation of ADP (Scheme 1). The latter release is predicted to be rate limiting for a Theorell−Chance enzyme. In an effort to independently investigate the contributions of ADP release and other steps to the overall rate, we have examined solvent isotope, thio, and viscosity effects for APH(3′)-IIIa.

The simplest chemical mechanism by which phosphorylation of aminoglycosides can occur is by direct attack of nucleophilic hydroxyl on the γ-phosphate of ATP. An alternative double-displacement mechanism has been suggested solely on the basis of primary sequence alignment where the γ-phosphate is first transferred to an enzyme residue (possibly a histidine) followed by attack of the aminoglycoside hydroxyl (Kocabiyik & Perlin, 1992; Martin et al., 1988). Both mechanisms imply a deprotonation of the secondary 3′-OH (or primary 5″-OH for 4,5-disubstituted aminocyclitols) of aminoglycosides to increase the nucleophilicity of the attacking hydroxyl (Scheme 2). Alternatively, deprotonation may occur prior to binding of the drug to the enzyme, but this is highly unlikely given the expected pK_a of approximately 16 for a primary or secondary alcohol (March, 1985).

In a solution of D_2O, in which all aminoglycoside hydroxyls are deuterated, one would expect a significant solvent isotope effect if enzyme-mediated hydroxyl group deprotonation contributes to the overall rate. The difference in zero-point energy of H−O and D−O is 6 kJ mol^{-1} (Kyte, 1995); therefore, if only vibrational energy is considered, then the maximal isotope effect is expected to be approximately 10 when proton transfer is solely rate limiting. We observe only a small solvent effect (∼1.5) on k_{cat} for both kanamycin and ATP. This value is consistent with a minor contribution of proton transfer to the rate of aminoglycoside phosphorylation by APH(3′)-IIIa. Since the pK_a's of potentially important catalytic residues are perturbed by

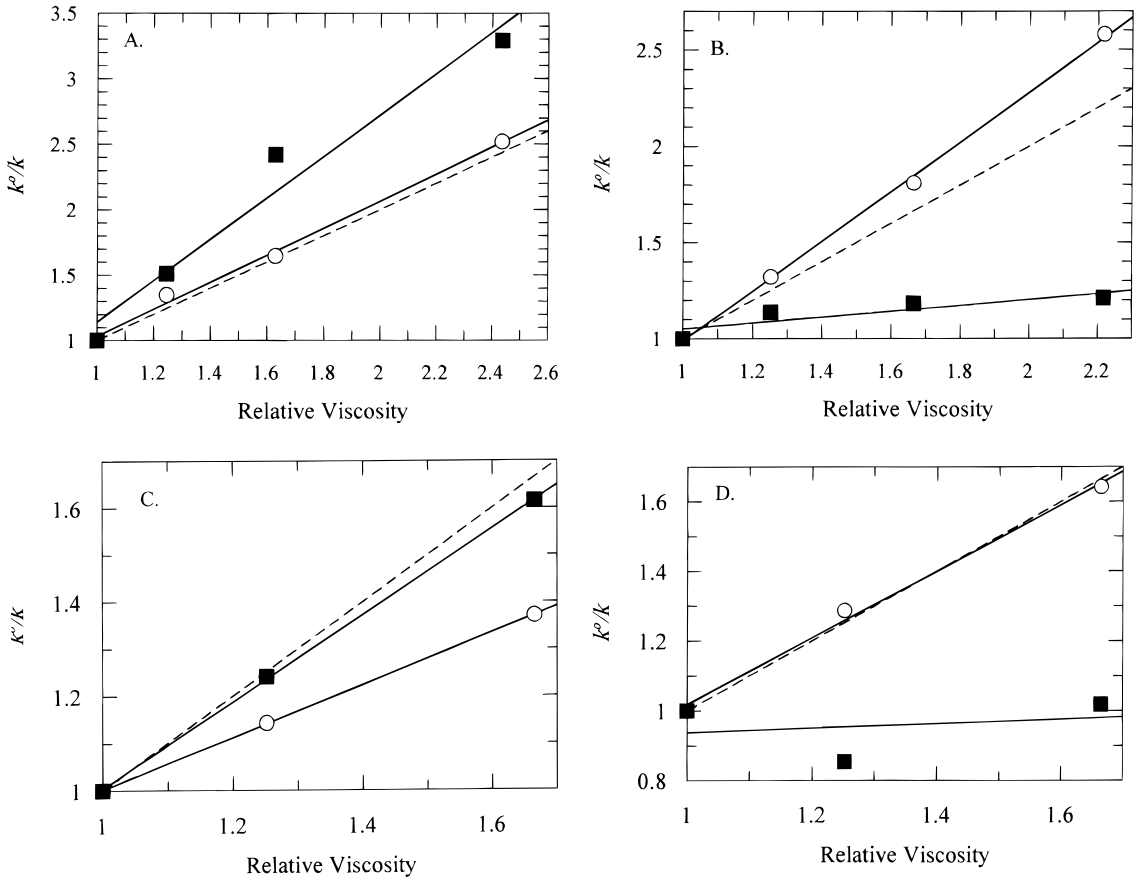
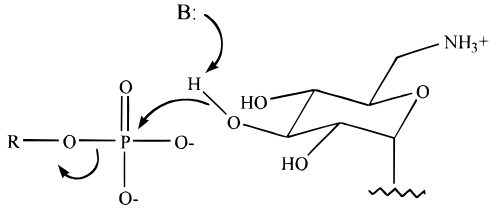


FIGURE 1: Viscosity effects on the rates of APH(3′)-IIIa-catalyzed reactions. All experiments were performed in a glycerol-containing buffer consisting of 50 μM HEPES, pH 7.5, 10 mM $MgCl_2$, 40 mM KCl, 0.7 mM NADH, 2.5 mM phosphoenol pyruvate, and 10 μL of pyruvate kinase/lactate dehydrogenase mix. The dotted line has a slope of 1. (A) Variable ATP at constant kanamycin A (125 μM). (B) Variable kanamycin A at constant ATP (1 mM). (C) Variable ATP at a constant amikacin (2.5 mM). (D) Variable amikacin at constant ATP (1 mM). (○) $k_{cat}{}^o/k_{cat}$, (■) $(k_{cat}/K_m)^o/(k_{cat}/K_m)$.

Table 3: Effect of Viscosity on Substrate Inhibition by Kanamycin A

relative viscosity	$(K_i^\circ/K_i)^\eta$
1	1.00
1.252 (glycerol)	0.95
1.810 (glycerol	1.04
2.580 (glycerol)	0.97
1.200 (sucrose)	1.36
1.666 (sucrose)	0.82
2.301 (sucrose)	0.73

Scheme 2



D_2O (Schowen, 1977), it is important to perform solvent isotope experiments at more than one pH(D). The solvent isotope effect for APH(3′)-IIIa-dependent phosphorylation of kanamycin A was unchanged at pD 6.5 and 7.5. Such a modest solvent isotope effect indicates hydroxyl proton abstraction does not contribute significantly to the rate of phosphorylation as expected for a Theorell−Chance mechanism. This is consistent as well with other kinases such as the C-terminal Src kinase ($k_{cat}{}^H/k_{cat}{}^D = 1.2$) (Cole et al., 1994) and protein kinase A ($k_{cat}{}^H/k_{cat}{}^D = 1.6$) where product release and not proton transfer is partially rate determining (Yoon & Cook, 1987).

Substitution of sulfur for oxygen in nucleotides (phosphothioates) has proven to be a highly useful tool for studying enzyme mechanisms (Eckstein, 1983). The sulfur-substituted phosphorus (P−S) is significantly less electrophilic than the P−O group, and this results in a slower rate of catalysis where nucleophilic attack on the phosphoryl center contributes to the rate-limiting step. The ratio of the rate for the reaction with the oxygen-bearing molecule to the thio analogue has been termed the thio effect. For example, the C-terminal Src kinase shows a thio effect of 10−20 (Cole et al., 1994), demonstrating the significant contribution of nucleophilic attack on the γ-phosphate to the overall rate. The K_m for ATPγS was only 1.8-fold higher than ATP for APH(3′)-IIIa, therefore nucleotide binding is minimally affected by the S to O substitution. Similarly, the thio effect (ratio of k_{cat}s for ATP and ATPγS) was 2. This small effect is consistent with a negligible contribution of phosphate transfer chemistry to the overall rate.

Both the solvent isotope and thio effects imply that phosphate transfer chemistry, either deprotonation of the nucleophilic hydroxyl or nucleophilic attack on the phosphorus center, does not contribute significantly to the rate-limiting catalytic event(s) as predicted by a Theorell−Chance mechanism. In order to further examine the rates of the antibiotic inactivating reaction, we turned to experiments in which the viscosity of the reaction mixture was modified. This is a highly useful method for the determination of rates

which are controlled by diffusion (Adams & Taylor, 1992; Bazelyansky et al., 1986; Blacklow et al., 1988; Brouwer & Kirsch, 1982; Caldwell et al., 1991; Cole et al., 1994; Hardy & Kirsch, 1984; Simopoulos & Jencks, 1994). In a simple biomolecular reaction, the rate of collision (or dissociation) is inversely proportional to the viscosity of the medium (Kramers, 1940). By altering the viscosity of the enzyme assay solution, one can discern the effects on the apparent first- (k_{cat}) and second-order (k_{cat}/K_m) rate constants.

An important caveat in such experiments is that the viscogen should not directly interact with the enzyme. This can be examined by performing viscosity studies with poor substrates which are known to be nondiffusion limited (Brouwer & Kirsch, 1982). Alternatively, when such substrates are unavailable, a "poor enzyme" such as a catalytically impaired site mutant can be substituted (Blacklow et al., 1988). In either case, one expects no viscosity effect. Unfortunately, we have no access to such substrates and our current survey of site mutants has not provided an adequate "poor enzyme". Nonetheless, we can turn to the phenomenon of substrate inhibition as an appropriate control. Neither glycerol nor sucrose has a marked effect on aminoglycoside K_i, indicating that these molecules are not blocking the active site of APH(3′)-IIIa. Furthermore, we can state that the E·ADP complex does not accumulate along the reaction pathway due to the change in viscosity (Gates & Northrop, 1988).

At saturating levels of ATP, there is no viscosity effect on k_{cat}/K_m for the aminoglycoside substrates kanamycin A and amikacin with APH(3′)-IIIa. This was gratifyingly consistent with our proposed kinetic mechanism since, as the second substrate entering in a Theorell−Chance mechanism, V/K for aminoglycosides is not expected to be sensitive to solvent viscosity and has no contribution to the rate-limiting step(s) of catalysis (Scheme 1). Under the same conditions, k_{cat} for APH(3′)-IIIa is dramatically affected by solvent viscosity. The average slope at saturating substrate (either ATP or aminoglycoside) in both glycerol and sucrose is 1.02, demonstrating that V_{max} is clearly diffusion limited. V_{max} is solely determined by ADP release in this case; therefore, this is the rate-limiting step in aminoglycoside detoxification.

Unexpectedly, the slope effect on V/K for ATP at saturating amounts of aminoglycoside is greater than 1 for kanamycin and amikacin in glycerol and approaches 1 with amikacin in sucrose. The maximal theoretical value for the ratios of maximal rates is predicted to be 1 (Brouwer & Kirsch, 1982). Upward deviation from this value could mean there is one or more other diffusion-controlled equilibrium which contributes to V/K^{ATP} under these conditions (Simopoulous & Jencks, 1994). A possible candidate for such a phenomenon is the contribution of a diffusion-controlled enzyme conformational change upon ATP binding, as V/K for ATP should only reflect initial binding to the enzyme (Scheme 1). Such a viscosity dependent structural change has been noted for example with triosephosphate isomerase (Sampson & Knowles, 1992). We reasoned that a conformational change would be likely with APH(3′)-IIIa which transfers the γ-phosphate of ATP to a hydroxyl group of aminoglycosides. One would therefore expect some nonspecific phosphate transfer to water in the absence of aminoglycoside substrates; however, we find no detectable ATP hydrolysis activity nor do we observe phosphate transfer to compounds such as 6-aminomethylglucoside, an analogue of the phosphate-accepting sugar of kanamycin A (McKay et al., 1994a). These observations are reminiscent of the mechanism of hexokinase. This enzyme performs similar chemistry and uses a dramatic shift in enzyme conformation to bring substrate ATP and glucose together and therefore avoid significant ATP hydrolysis in the absence of available glucose (Bennett & Steitz, 1980). We therefore sought independent evidence for such a change with APH(3′)-IIIa upon substrate and substrate analogue (ATP, ADP, tobramycin, and combinations of these) binding using several techniques such as perturbation of tryptophan fluorescence, changes in UV spectra, circular dichroism, and hydrolysis of ATP in the presence of aminoglycosides that do not possess a 3′-hydroxyl such as tobramycin. No evidence for structural changes was observed using these techniques, although this does not rule out subtle alterations in enzyme conformation which could account for the substrate specificity and viscosity effects discussed above. Consistent with this hypothesis is the observation that native and substrate (ATP and analogues + aminoglycosides)-bound APH(3′)-IIIa crystallize in distinct space groups, suggesting that the enzyme adopts a different shape upon substrate binding (W.-C. Hon and A. M. Berghuis, personal communication).

Table 4: Apparent Microscopic Rate Cosntants for APH(3′)-IIIa with ATP and Kanamycin A as Substrates

constant[a]	values	ΔG (kcal/mol)
k_1 [b]	6.35×10^4 s^{-1} M^{-1}	11.3
k_{-1} [c]	0.11 s^{-1}	19.5
k_2 [d]	1.40×10^5 s^{-1} M^{-1}	10.8
k_3 [e]	86.4 s^{-1} M^{-1}	15.4
k_4 [f]	1.76 s^{-1}	17.8
k_{-4} [g]	4.68×10^3 s^{-1} M^{-1}	12.9

[a] Constants are described in Scheme 1. [b] $k_1 = k_4/K_m^{ATP}$. [c] $k_{-1} = k_{cat(reverse)}$. [d] $k_2 = k_4/K_m^{kan}$. [e] $k_3 = k_{cat(reverse)}/K_m^{kan\text{-}phosphate}$. [f] $k_4 = k_{cat(forward)}$. [g] $k_{-4} = k_{cat(reverse)}/K_m^{ADP}$.

These viscosity studies therefore support a Theorell−Chance kinetic mechanism and identify ATP binding and ADP release as diffusion controlled processes. Under physiological conditions, ATP is not limiting and aminoglycoside is, therefore only ADP release contributes to the enzyme rate and is completely rate limiting. While no direct physical evidence could be obtained for an ATP-dependent protein conformational change, the observed slopes of >1 for V/K^{ATP} are suggestive of such a change and indeed a Theorell−Chance mechanism is expected to incorporate such changes (Gates & Northrop, 1988). This would also be consistent with a lack of ATPase activity in the enzyme. Further elucidation of such changes awaits solution of the three-dimensional structure in the presence and absence of ATP and analogues, which is currently in progress.

Thermodynamics of the Proposed Mechanism for APH(3′)-IIIa. Our data are consistent with a Theorell−Chance kinetic mechanism with ADP dissociation as the rate-limiting step:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot A \xrightarrow{k_2} (E\cdot A\cdot B \rightleftarrows E\cdot P\cdot Q) \xrightarrow{k_3} E\cdot Q \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q$$

By evaluating the steady-state parameters of APH(3′)-IIIa in the forward and reverse (ATP-forming) directions, we can set some lower limits on a series of microscopic rate

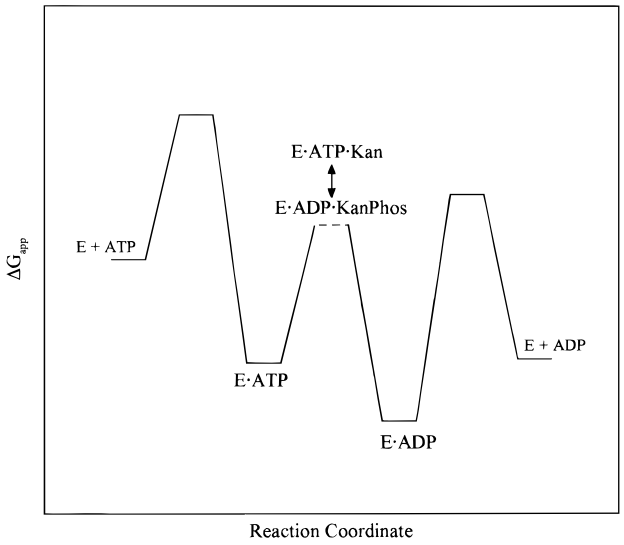


FIGURE 2: Energetic profile for APH(3′)-IIIa. The profile was constructed using 1 M reactants. The height of the ternary complex cannot be accurately determined using steady-state methods and is therefore shown as a broken line.

constants (Table 4), recognizing that some of these incorporate other equilibria (e.g., conformational change) and these are presented diagramatically in Figure 2. For a Theorell−Chance enzyme $k_{-1} = k_{cat(reverse)}$ and should also equal $K_{ia}k_1$. However, there is over a 10-fold difference in the value observed for $k_{cat(reverse)}$ (0.11 s^{-1}) and that calculated from the product of k_1 and K_{ia} (1.65 s^{-1}). This discrepancy is consistent with our suggestion of a possible conformational change upon ATP binding: E + ATP $\rightleftarrows$ E·ATP $\rightleftarrows$ E*·ATP. As predicted by the mechanism, the E·ADP complex is a low-energy complex.

Summary. The antibiotic resistance enzyme APH(3′)-IIIa is a clinically significant enzyme that operates by a Theorell−Chance kinetic mechanism. Consistent with this mechanism are our observations that aminoglycoside deprotonation and nucleophilic attack at the γ-phosphate of ATP are not rate limiting. ATP binding and ADP release are diffusion-controlled equilibria, and ADP release is rate limiting in the forward direction, observations which also support Theorell−Chance kinetics. The tight binding of ATP and ADP (K_m = 24 and 22 μM, respectively) and the suggestion that the substrate-bound enzyme undergoes a conformational change both advocate that bisubstrate analogues which incorporate aminoglycoside and nucleotide characteristics should be good inhibitors of this enzyme and may be useful as chemotherapeutic agents. Such compounds could possibly "freeze" APH(3′)-IIIa in a closed conformation and should be more effective than simple competitive inhibitors of aminoglycoside binding.

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BI9603884