Molecular Mechanism of Aminoglycoside Antibiotic Kinase APH(3')-IIIa

ROLES OF CONSERVED ACTIVE SITE RESIDUES*

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The aminoglycoside antibiotic kinases (APHs) constitute a clinically important group of antibiotic resistance enzymes. APHs share structural and functional homology with Ser/Thr and Tyr kinases, yet only five amino acids are invariant between the two groups of enzymes and these residues are all located within the nucleotide binding regions of the proteins. We have performed site-directed mutagenesis on all five conserved residues in the aminoglycoside kinase APH(3')-IIIa: Lys44, Glu60, Asp190, Asn195, and Glu208, which coordinate two Mg2+ ions, Mg1 and Mg2. Previous structural and mutagenesis evidence have demonstrated that Lys44 interacts directly with the phosphate groups of ATP; mutagenesis of invariant Glu60, which forms a salt bridge with the e-amino group of Lys44, demonstrated that this residue does not play a critical role in ATP recognition or catalysis. Results of mutagenesis of Asp190 were consistent with a role in proper positioning of the aminoglycoside hydroxyl during phosphoryl transfer but not as a general base. The Mg1 and Mg2 ligand Asp208 was found to be absolutely required for enzyme activity and the Mg2 ligand Asn195 is important for MgATP recognition. The mutagenesis results together with solvent isotope, solvent viscosity, and divalent cation requirements are consistent with a dissociative mechanism of phosphoryl transfer where initial substrate deprotonation is not essential for phosphoryl transfer and where Mg2 and Asp208 likely play a critical role in stabilization of a metaphosphate-like transition state. These results lay the foundation for the synthesis of transition state mimics that could reverse aminoglycoside antibiotic resistance in vivo.

Aminoglycoside antibiotics (see Fig. 1 below) are widely used in the treatment of bacterial infections caused by both Gram-positive and Gram-negative pathogens yet the specific details of the molecular mechanisms of resistance to this class of antibiotics remain largely unknown. Aminoglycosides act first by disrupting protein expression and interfering with translation at the level of appropriate aminocyt-tRNA recognition at the ribosomal A site; this is followed by a series of secondary effects, including membrane damage, which combine to arrest growth and kill the cell (1). The ribosomal receptor for most aminoglycoside antibiotics, including gentamicin C, kanamycin, and neomycin, is the 16 S rRNA of the 30 S subunit. The location of aminoglycoside binding site has been inferred by chemical footprinting analyses (2, 3) and through the elucidation of the structure of aminoglycosides in complex with oligomers that model the 30 S rRNA binding site by NMR methods (4, 5) and more recently by direct crystallographic determination of the complexes of the small ribosomal subunit with aminoglycosides (6). These studies suggest a tight complex between rings I and II of the aminoglycosides with the rRNA and highlight important interactions between conserved aminoglycoside amino and hydroxyl functional groups and the nucleic acids. Given the close fit between drug and target, it is not surprising that bacteria have evolved resistance mechanisms that decorate the aminoglycosides by covalent modification resulting in disruption of the complexes with a consequent decrease in ribosome affinity (7, 8).

Aminoglycoside-modifying enzymes are widely distributed among bacterial pathogens and include O-phosphoryltransferases (kinases), N-acetyltransferases, and O-adenyltransferases. The aminoglycoside kinases (abbreviated APH)1 include a number of enzymes with differing aminoglycoside substrate specificities and regiospecificities of phosphoryl transfer. Despite this broad tolerance for aminoglycoside substrates, all APHs share a common active site motif that resembles the active site residues found in the Ser/Thr/Tyr protein kinase superfamily (9). This similarity mirrors the three-dimensional structural homology between aminoglycoside and protein kinases (10). In particular, five amino acid residues are 100% conserved between aminoglycoside and protein kinases: Lys44, Glu60, Asp190, Asn195, and Asp208 (APH(3')-IIIa numbering; see Fig. 2 below).

Site-directed mutagenesis and affinity labeling studies on the broad spectrum aminoglycoside kinase APH(3')-IIIa found in Gram-positive pathogens such as staphylococci, streptococci, and enterococci, have supported the importance of several of the conserved residues. For example, Lys44 was implicated in ATP binding in APH(3')-IIIa and is homologous to an invariant Lys in protein kinases with a similar role (10, 11). Asp190 aligns with an Asp residue widely described in the protein kinase literature as an active site base necessary for deprotonating the incoming nucleophilic hydroxyl, and indeed mutation of Asp190 to Ala in APH(3')-IIIa has a major impact on catalysis (10). These studies along with the demonstration that APHs can

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1 The abbreviations used are: APH, aminoglycoside kinase; MALDI-TOF, matrix-assisted laser desorption time-of-flight mass spectrometry; PCR, polymerase chain reaction; WT, wild type; PEG, polyethylene glycol; MIC, minimal inhibitory concentration; SIE, solvent isotope effect; PKA, protein kinase A; SVE, solvent viscosity effect.

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phosphorylate some protein and peptide substrates (12) support the functional link between aminoglycoside and protein kinases. APH(3')-IIIa has also been determined to be regiospecifically phosphorylated by 4,6-disubstituted deoxystreptamine aminoglycosides such as kanamycin and amikacin at the 3'-hydroxyl exclusively and at the 3'- and 5'-hydroxyls of 4,5-disubstituted deoxystreptamine aminoglycosides such as neomycin and ribostamycin (Fig. 1) (13) with ADP release governing the maximal rate in the steady state (14).

The determination of the structures of APH(3')-IIIa in complex with various substrates and inhibitors together with the revelation of the structural similarity with Ser/Thr/Tyr protein kinases has immensely aided our understanding of the mechanism of this enzyme. Guided by studies on the better-characterized protein kinases, we have examined the roles of conserved residues lining the active site of this enzyme and characterized their contribution to catalysis.

**MATERIALS AND METHODS**

**Chemicals**—Ribostamycin, lividomycin A, neomycin B, butirosin, amikacin, β-NADH, phosphoenolpyruvate, and pyruvate kinase/lactate dehydrogenase enzymes were from Sigma Chemical Co. (St. Louis, MO). Kanamycin A was from Bioshop (Burlington, Ontario, Canada). All oligonucleotide primers were synthesized at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

**Site-Directed Mutagenesis**—The Gln60→Ala, Asp190→Asn, and Asp208→Ser site mutants were prepared by the “Megaprimer” methodology (15) using primers described in Table I. Briefly, the primers pGlu60Ala, pAsp190Ala, and pAsp190Glu were appropriately combined with primers p3'–PCR or p5'–PCR complimentary to ends of aminoglycoside antibiotics (Fig. 1) to generate the megaprimers by PCR using the plasmid pETSACG1 as template, and this primer was used to amplify the complete gene on ice for up to 60 min. The reaction was quenched by the addition of 1 mM phenylmethylsulfonyl fluoride, and the products were separated by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) on the Harvard Microchemistry Center (Boston, MA).

**Kinetic Assay**—The kinetic assay used to monitor APH(3')-IIIa activity has previously been described (16). The assay measures the production of ADP generated upon aminoglycoside phosphorylation and couples that production to the oxidation of β-NADH using the enzymes pyruvate kinase and lactate dehydrogenase. The rate of ADP production was determined by monitoring the decrease in absorbance at 340 nm. Initial rates were fit by nonlinear least squares methods to Eq. 1, or Eq. 2,

$$v = k_{cat} [E] [S] / K_m + [S]$$

$$v = k_{cat} [E] [S] / (K_m + [S])(1 + [S]/K_i)$$

if substrate inhibition was detected, using Grafit version 3.0 (19). ATP was fixed at 1 mM, and kanamycin A was fixed at 100 μM, when measuring the steady-state kinetic parameters for aminoglycosides substrates and ATP, respectively.

**Table 1**

| Oligonucleotide | Sequence |
|-----------------|----------|
| 5'-PCR primer   | 5'-CGAAGCTTGGACTAAACAAATCCATCCAG-3' |
| 3'-PCR primer   | 5'-GCTCTAGAATATGCTTAAGAAG-3' |
| pGlu60Ala       | 5'-GTGGAGCGGCGCAAGGATAG-3' |
| pAsp190Ala      | 5'-GCCTGTCGGAGGCCGCGCGCGC-3' |
| pAsp190Glu      | 5'-GCTGTCGGAGGCCGCGCGCGC-3' |
| pAsp208Glu      | 5'-GCTGTCGGAGGCCGCGCGCGC-3' |

**FIG. 1. Representative structures of aminoglycoside antibiotics.** 2-Deoxystreptamine aminoglycosides are divided into two groups based on substitution of the 2-deoxystreptamine ring (ring II). 4,6-Disubstituted aminoglycosides include kanamycin A, and the semi-synthetic aminoglycosides amikacin and isepamicin which are alkylated at position N1 on ring II 4-amino-2-hydroxybutyryl and 3-amino-2-hydroxypropionyl groups, respectively. 4,5-Disubstituted aminoglycosides include ribostamycin, neomycin, butirosin (ribostamycin alkylated at position N1 on ring II by a 4-amino-2-hydroxybutyryl group) and lidovidomycin, which lacks the hydroxyl group at position 3'. Sites of phosphorylation catalyzed by APH(3')-IIIa are indicated by arrows.

**FIG. 2. The active site of APH(3')-IIIa.** All the residues that are absolutely conserved among APHs and ePKs are shown: Lys44, Glu60, Asp190, Asn195, and Asp208.
Metal Ion Dependence—The metal ion (M$^2$+) dependence of both initial rates and the steady-state kinetic parameters of APH(3')-IIIa were examined. To measure the effect on initial rates, the concentrations of ATP (1 mM) and kanamycin (100 μM) were fixed, and the concentration of MgCl$_2$ varied between 0.1 and 10 mM. To ensure that the coupled assay system was active at such low concentrations of Mg$^{2+}$, ADP was added and the rate of reaction was determined. Similarly, for studies using Mn$^{2+}$ as the divalent metal, conditions were established where the rate of ADP turnover by pyruvate kinase was not rate-limiting. At all concentrations of Mn$^{2+}$, the rate of ADP turnover was faster than the rate of aminoglycoside phosphorylation. The initial rates were fit to either Eq. 1 or Eq. 2, depending on whether or not Mg$^{2+}$ inhibition was noted. The best fits of the data were obtained when the initial rates were plotted against the concentration of free Mg$^{2+}$ and not when plotted against the concentration of total Mg$^{2+}$. The concentration of free Mg$^{2+}$ was calculated using the parameters described previously (20).

Measurement of Solvent Isotope and Solvent Viscosity Effects for APH(3')-IIIa—The solvent isotope and solvent viscosity effects on the Asn$^{195}$ → Ala and the Glu$^{60}$ → Ala were determined by the methods previously described for WT APH(3')-IIIa (14). For solvent isotope studies the H$_2$O content was ≥3.5% (v/v), and for solvent viscosity studies glycerol was used as the microviscogen unless otherwise noted. Briefly, glycerol was added to the coupled enzyme assay buffer to concentrations from 0 to 30% (w/v), and the steady-state kinetic parameters for ATP were determined. The concentration of kanamycin A was fixed at 125 μM. Assays were carried out in duplicate at 37 °C. The relative viscosity of the glycerol-containing assay buffer was determined in quadruplicate using an Ostwald viscometer, as described in Ref. (14). Macroviscous controls were performed in all cases using PEG 8000.

Determination of the Minimal Inhibitory Concentration (MIC) of Kanamycin A—The minimal inhibitory concentration (MIC) is defined as the lowest concentration of kanamycin A required to completely inhibit growth of E. coli BL21(DE3)/pETSACG1. To determine MICs, cultures were streaked out over LB agar with ampicillin (50 μg/ml) as the selection marker and grown overnight at 37 °C. From this, single colonies were selected and restreaked on LB agar with ampicillin and grown at 37 °C for 18–24 h. A few colonies were picked and suspended in sterile 0.85% (w/v) NaCl to achieve an OD$_{625}$ of 0.08–0.1. These suspensions were added to 95 μl of Mueller-Hinton broth in sterile microtiter plate wells containing serial dilutions of kanamycin A (0–512 μg/ml). The final dilution corresponds to ~5 × 10$^5$ colony forming units/ml. A sterile lid was placed over the microtiter plate, and it was allowed to incubate at 37 °C for 16–20 h before being visually inspected for bacterial growth.

RESULTS

Protease Susceptibility of APH(3')-IIIa

Because protease sensitivity can be a sensitive marker of changes in protein structure, we examined the susceptibility of APH(3')-IIIa to various proteases in the presence and absence of substrates in an effort to ensure that changes in the activity of the site mutants were not the result of significant changes in protein conformation. Exposure of the WT enzyme to subtilisin results in the cleavage of the enzyme at positions His$^{78}$ and Glu$^{157}$, which are in exposed loop regions (Fig. 3B), as determined by MALDI-TOF mass spectrometry, and addition of tobramycin completely protected against proteolysis whereas addition of ATP alone gave only modest protection (Fig. 3A). All of the site mutants described in this work showed protease sensitivity that was indistinguishable from the effects on the WT enzyme.

Analysis of APH(3')-IIIa Mutants

Glu$^{60}$→Glu$^{60}$ is completely conserved in both protein and aminoglycoside kinases, forming a salt bridge with Lys$^{14}$ (Fig. 2). The Glu$^{60}$ → Ala mutant nonetheless did not show significant changes in either $k_{cat}$ or $K_m$ with respect to the WT enzyme (Table II). The only significant effect was a 5-fold increase in neomycin B $k_{cat}/K_m$ for the 4,6-disubstituted deoxyystreptamine-containing neomycin B. In vivo kanamycin resistance data parallel the little change in in vitro effects as assessed by MIC studies in liquid culture (Table III).

Asn$^{195}$—Asn$^{195}$ is also absolutely conserved among the APHs, and the protein kinases and is involved in coordinating Mg$^2+$ (see Fig. 2 for Mg nomenclature). Mutation of this residue to Ala resulted in no changes in $k_{cat}$ but had a greater than 5-fold increase in $k_{cat}/K_m$ for ATP (Table II). Furthermore, although $k_{cat}/K_m$ for aminoglycosides was largely unaffected, greater than 5-fold increases were recorded for amikacin, isepamicin, and butirosin, aminoglycosides substituted at N1. The kanamycin MIC was also reduced to 64 μg/ml, correlating the important role in enzyme activity with the biologically relevant phenotype of drug resistance (Table III).

Asp$^{190}$—Asp$^{190}$ is equivalent to the invariant protein kinase Asp in Hanks’ consensus sequence VI that has been ascribed the role of active site base. The Asp$^{190}$ → Ala mutant of APH(3')-IIIa has previously been shown to be over 500-fold less active than the WT enzyme (10). To further explore the role of Asp$^{190}$ in the catalytic mechanism of APH(3')-IIIa, Asp$^{190}$ → Glu and Asp$^{190}$ → Asn mutants were generated. Both mutants were severely impaired in phosphoryl transfer capacity and in most cases only estimates of $k_{cat}$ were possible (Table II). The importance of Ala$^{190}$ was also supported by a greater than 32-fold decrease in MIC (Table III).
Mechanism of Aminoglycoside Antibiotic Kinase

Steady-state kinetic parameters for the WT and mutant APH(3')-IIIa proteins

| Substrate          | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) |
|--------------------|------------|----------------------|-----------------------------------|
| ATP                | 27.7 ± 3.7 | 1.76 ± 0.08          | $6.35 \times 10^4$                |
| Kanamycin A        | 12.6 ± 2.6 | 1.79 ± 0.09          | $1.42 \times 10^5$                |
| Amikacin           | 245 ± 27   | 2.46 ± 0.11          | $1.00 \times 10^4$                |
| Isepmicin          | 198 ± 28.5 | 1.41 ± 0.35          | $0.71 \times 10^4$                |
| Neomycin B         | 7.72 ± 0.9 | 2.08 ± 0.07          | $2.69 \times 10^3$                |
| Butirosin          | 34.3 ± 3.1 | 2.92 ± 0.07          | $5.88 \times 10^3$                |
| Ribostamycin       | 9.3 ± 1.8  | 1.89 ± 0.10          | $1.76 \times 10^5$                |
| Lilividomycin A    | 31.6 ± 5.1 | 3.97 ± 0.25          | $1.26 \times 10^5$                |

| Substrate          | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) |
|--------------------|------------|----------------------|-----------------------------------|
| APH(3')-IIIa-Glu$^{60} \to$ Ala | ATP | 12.5 ± 2.3 | 0.62 ± 0.02 | $4.96 \times 10^4$ |
| AKanamycin A       | 7.8 ± 2.7  | 0.51 ± 0.02 | $6.54 \times 10^4$ |
| Amikacin           | 57.6 ± 6.0 | 1.02 ± 0.03 | $1.77 \times 10^4$ |
| Neomycin B         | 51.8 ± 11.5 | 2.60 ± 0.15 | $5.01 \times 10^4$ |
| APH(3')-IIIa-Asn$^{195} \to$ Ala | ATP | 113 ± 10   | 1.70 ± 0.06 | $1.50 \times 10^4$ |
| AKanamycin A       | 15.6 ± 2.7 | 2.03 ± 0.07 | $1.30 \times 10^5$ |
| Amikacin           | 1020 ± 260 | 0.83 ± 0.09 | $0.83 \times 10^5$ |
| Neomycin B         | 4.3 ± 1.0   | 1.34 ± 0.05 | $2.27 \times 10^5$ |
| Isepmicin          | 624 ± 149   | 0.56 ± 0.09 | $1.38 \times 10^3$ |
| Butirosin          | 59.0 ± 17.0 | 0.53 ± 0.04 | $8.98 \times 10^2$ |
| Ribostamycin       | 5.8 ± 1.0   | 1.86 ± 0.05 | $3.21 \times 10^5$ |
| Lilividomycin A    | 13.4 ± 3.2  | 3.05 ± 0.14 | $2.61 \times 10^5$ |
| APH(3')-IIIa-Asp$^{190} \to$ Asn | Kanamycin A | 0.013 ± 0.001$^{b}$ | 140 |
| APH(3')-IIIa-Asp$^{190} \to$ Glu | ATP | 108 ± 11   | 0.047 ± 0.001 | $4.35 \times 10^2$ |
| AKanamycin A       | 334 ± 37   | 0.043 ± 0.001 | $1.28 \times 10^2$ |
| Amikacin           | ≤0.011 ± 0.0004$^{b}$ | 220 |
| Neomycin B         | ≤0.011 ± 0.0003$^{b}$ | 190 |

$^a$ Steady-state kinetic parameters for the aminoglycoside substrates are taken from Ref. 16, whereas the steady-state kinetic parameters for ATP are taken from Ref. 10.

$^b$ The rates for the phosphorylation of amikacin and neomycin B were too slow to accurately measure the steady state kinetic parameters. Approximations for the $k_{cat}$ values are given.

### Table III

**MIC of kanamycin A for APH(3')-IIIa site mutants**

Comparison of liquid culture MICs obtained using *E. coli* BL21(DE3)/pETSACG1 in Mueller-Hinton broth as described under "Materials and Methods."

| APH(3')-IIIa | MIC of kanamycin A (μg/ml) |
|--------------|---------------------------|
| WT           | >512                       |
| None$^a$     | 8                          |
| Glu$^{60} \to$ Ala | 512                      |
| Asp$^{190} \to$ Ala | 16                        |
| Asp$^{208} \to$ Asn | 16                        |
| Asp$^{208} \to$ Glu | 16                        |
| Asn$^{195} \to$ Ala | 64                        |
| Asp$^{208} \to$ Ala | 8                          |
| Asp$^{208} \to$ Asn | 8                          |
| Asp$^{208} \to$ Glu | 8                          |

$^a$ Negative control *E. coli* BL21(DE3)/pET22b.

### Metal Ion Dependence of WT and Mutant APH(3')-IIIa Proteins

Mg$^{2+}$ is a required element for APH(3')-IIIa rate enhancement, and other metal ions, for example Mn$^{2+}$, do not support catalysis at a concentration of 10 mM (16). The fact that a number of invariant active site residues are also metal ligands suggested that the active site mutants generated in this study might have altered metal ion dependencies. As a first test of this possibility, we examined the ability of the Asn$^{195} \to$ Ala mutant to use Mn$^{2+}$ as the divalent cation (Table IV). Unlike the WT enzyme, the Asn$^{195} \to$ Ala mutant was quite active in 15 mM MnCl$_2$ (Fig. 4).

The choice of divalent cation also had a significant impact on the rate of reaction for the Asn$^{195} \to$ Ala mutant. The $k_{cat}$ measured for the Mn$^{2+}$-catalyzed phosphorylation of kanamycin A was decreased 4.6-fold (Table IV). There is a corresponding decrease in the $k_{cat}$ determined when ATP is the variable substrate. A comparison of the rates of reaction determined at 15 mM MnCl$_2$ to those determined at 10 mM MgCl$_2$ is reasonable, because the rate of the Mn$^{2+}$ catalyzed reaction varies little between 5 and 15 mM MnCl$_2$.

Asp$^{208}$—The crystal structure of APH(3')-IIIa:ADP demonstrates that Asp$^{208}$ is a ligand for both Mg$1$, providing two of the six possible Mg$1$ coordination positions, and Mg$2$, providing one coordination ligand (10), and we have previously shown that Asp$^{208}$ is critical to the protein kinase activity of APH(3')-IIIa (12); an Asp$^{208}$ → Ala mutant is at least 1800-fold less active than the WT enzyme. We therefore generated the Asp$^{208}$ → Asn and the Asp$^{208}$ → Glu mutants to further probe the role of this residue in enzyme activity. Both mutants lacked any detectable activity above the assay background, underscoring the critical nature of this residue to catalysis. Assessment of the biological activity of the Asp$^{208}$ mutants was also supportive of an essential role in catalysis with kanamycin A MIC levels indistinguishable from controls containing no APH(3')-IIIa (Table III).
WT enzyme is inhibited by high concentrations of Mg\(^{2+}\). We next examined the dependence of initial rates of the WT and Asn\(^{195}\)→Ala enzymes at fixed concentrations of ATP and kanamycin, on both Mg\(^{2+}\) and Mn\(^{2+}\) ion concentration (Fig. 4). The results obtained for the WT enzyme clearly indicate that increasing concentrations of both these metal ions are in fact inhibitory, with the effect being more pronounced when Mn\(^{2+}\) is used as the divalent cation. On the other hand, the lack of inhibition at higher concentrations of divalent cation is striking compared with the Asn\(^{195}\)→Ala mutant.

The observation that the Asn\(^{195}\)→Ala mutant is not significantly inhibited by high concentrations of Mg\(^{2+}\) prompted an investigation of the Mg\(^{2+}\) dependences of the other active site mutants examined in this study. The results from this analysis are presented graphically in Fig. 5 and quantitatively in Table V.

What is most evident from Fig. 5 is the extent to which the WT enzyme is inhibited by high concentrations of Mg\(^{2+}\) (\(K_i = 0.57 \pm 0.23\) mM), in comparison to the active site mutants described here. The Mg\(^{2+}\) dependence of the Phe\(^{264}\)→Ala mutant (17) was included as a negative control to show that a mutation far from the catalytic center of APH(3')-IIIa does not have as great an impact on the Mg\(^{2+}\) dependence as do those residues that make direct or indirect contact with either Mg1 or Mg2.

**Solvent Isotope and Solvent Viscosity Effects**

The dramatic change in the Mn\(^{2+}\) dependence of the Asn\(^{195}\)→Ala mutant, suggested that the rate-limiting step for this mutant was no longer exclusively of ADP release as it is with the WT enzyme (14). Therefore, to examine whether or not proton abstraction was now contributing to the rate, we measured solvent isotope effects (SIEs) for the Asn\(^{195}\)→Ala mutant by performing kinetic experiments in D\(_2\)O, in the presence of either 10 mM MgCl\(_2\) or 15 mM MnCl\(_2\) (Table VI). The SIEs on both \(k_{cat}/K_m\) and \(k_{cat}/K_{cat}\) were decreased compared with WT, and there was a significant inverse effect on \(k_{cat}/K_m\) (Table VI).

In a further effort to compare the rate-limiting step of the Asn\(^{195}\)→Ala mutant to that of the WT enzyme, we examined the solvent viscosity effect (SVE\(^5\)), using the microviscogen glycerol. Macroviscogen controls using PEG 8000 were also performed and showed no effect as previously reported for the WT enzyme (14). The SVE of APH(3')-IIIa, on both \(k_{cat}/K_m\) and \(k_{cat}/K_{cat}\), is -1 (14), whereas the SVE on \(k_{cat}/K_{cat}\) of the Asn\(^{195}\)→Ala mutant, is 0.20 ± 0.06. The SIE and the SVE for the Glu\(^{60}\)→Ala mutant were also plotted using the left Y axis, whereas the initial rates obtained for the Phe264→Ala mutant was plotted using the right Y axis.

**TABLE IV**

| M\(^{2+}\) Substrate | 6× | \(K_m\) | \(k_{cat}\) |
|---------------------|----|---------|----------|
| MgCl\(_2\) (10 mM) ATP\(^{\gamma}\) | 114 ± 10.4 | 1.70 ± 0.06 |
| MgCl\(_2\) (10 mM) Kanamycin A\(^{\gamma}\) | 15.6 ± 2.7 | 2.03 ± 0.07 |
| MnCl\(_2\) (15 mM) ATP\(^{\gamma}\) | 12.0 ± 1.4 | 0.33 ± 0.02 |
| MnCl\(_2\) (15 mM) Kanamycin A\(^{\gamma}\) | 34.3 ± 2.4 | 0.44 ± 0.01 |

\(\gamma\) Kanamycin A was held at 100 \(\mu\)M.

\(\gamma\) ATP was held at 1 mM.

**TABLE V**

| Enzyme | \(K_m\) | \(k_{cat}\) |
|--------|---------|----------|
| WT | 52.2 ± 25.4 | 7.24 ± 1.80 | 0.57 ± 0.2 |
| Glu\(^{60}\)→Ala | 304 ± 41 | 0.075 ± 0.004 | 21.5 ± 5.9 |
| Asp\(^{190}\)→Glutamic acid | 211 ± 16 | 0.048 ± 0.002 | 21.5 ± 3.9 |
| Asn\(^{195}\)→Ala | 523 ± 252 | 1.71 ± 0.32 | 28.7 ± 27.3 |
| Phe264→Ala* | 136 ± 23 | 2.73 ± 0.22 | 2.7 ± 0.5 |

* Described in Ref. 17.

\(\gamma\) SVE is defined as the slope of the relative rate \(k_{cat}/k_{cat}\) versus the relative viscosity of the assay solution.

**Fig. 4.** Dependence of APH(3')-IIIa activity on M\(^{2+}\) ions. Magnesium ion dependence of APH(3')-IIIa (solid line; ○) and the Asn\(^{195}\)→Ala mutant (dashed line; ●). Heavy dotted line, manganese ion dependence of APH(3')-IIIa (light dotted line; □) and the Asn\(^{195}\)→Ala mutant (dashed and dotted line; ■). Initial rates were plotted against the free M\(^{2+}\) concentration or the total Mn\(^{2+}\) concentration.

**Fig. 5.** Magnesium ion dependences of APH(3')-IIIa and mutant proteins. Initial rates determined at various Mg\(^{2+}\) concentrations for wild type (○), Glu\(^{60}\)→Ala (●), Asp\(^{190}\)→Glu (□), Asn\(^{195}\)→Ala (■), and Phe264→Ala (▲) APH(3')-IIIa enzymes. The initial rates obtained for APH(3')-IIIa, the Glu\(^{60}\)→Ala mutant, the Asp\(^{190}\)→Glu mutant, and the Asn\(^{195}\)→Ala mutant were plotted using the left Y axis, whereas the initial rates obtained for the Phe264→Ala mutant was plotted using the right Y axis.
Elucidation of the mechanism of phosphoryl transfer catalyzed by aminoglycoside kinases and the contribution of specific active site residues is central to efforts to understand antibiotic resistance at the molecular level. The remarkable three-dimensional similarity between Ser/Thr/Tyr protein kinases and APH(3′)-IIa suggested the possibility that these enzymes may catalyze phosphoryl transfer in a conserved fashion, and, indeed, of the five invariant amino acids between protein and aminoglycoside kinases, all are located within the active site. In an effort to understand the contribution of APH(3′)-IIa active site residues to rate enhancement, we have analyzed the effect of mutagenesis of Glu60, Asp190, Asn195, and Glu208, which represent four of the active site amino acids conserved between protein and aminoglycoside kinases. The effects of mutation of the invariant Lys and its role in ATP binding have been reported (10).

Phosphoryl transfer can occur through two extreme mechanisms in which nucleophilic attack either precedes (associative mechanism) or occurs after (dissociative mechanism) leaving group bond breakage (Scheme 1). The very practical importance of the determination of the mechanism of phosphoryl transfer in aminoglycoside kinase enzymes lies in developing a molecular understanding of transition state structure, which has utility in the design of specific inhibitors. This requires distinguishing between a minimal reaction coordinate distance with overall charge of −3 for a fully associative mechanism and larger reaction coordinate distance with overall charge of −1 for a full metaphosphate-generating dissociative mechanism. However, as has been previously noted (21), a continuum of mechanisms is possible and most enzymes likely do not adopt either fully associative or dissociative mechanisms but stabilize transition states in which bond breakage or formation predominate but are not complete. One of the goals of these mutagenesis studies was to provide supportive evidence to describe the phosphoryl transfer mechanism of APH(3′)-IIa.

Glu60—In a ternary structure model of APH(3′) in complex with ATP and the aminoglycoside ribostamycin (17), the carboxylate of Glu60 is positioned at least 10 Å away from the phosphate-accepting hydroxyl group of the aminoglycoside. Glu60 is however interacting with the ATP ligand Lys44 in an arrangement that is conserved in protein kinases (Fig. 2). Therefore, it was predicted that mutation of this residue to Ala was unlikely to exert a direct effect upon aminoglycoside affinity, but that ATP binding could be affected. However, this mutant was not dramatically impaired in substrate affinity, catalysis, or MIC. The SVE on kcat/Km for ATP again reflects the altered optimal ATP free energy of activation (14), which can be interpreted as an impact of the viscosities of glucose matching the viscosity of D2O (−9%) were not sufficient to match the observed inverse SIE. There are no active site thiols in APH(3′)-IIa, eliminating the possibility of contribution of exchange of a Cys SH group. Medium effects are expected to be global and common to all the studies performed here on WT and mutant enzymes and are not likely to be the source of the inverse effect in the Asn195 → Ala mutant alone. Asn195 is an Mg2 ligand, and therefore, loss of the amide-Mg bond will result in altered metal binding at the active site and constellation of requisite ligands. Therefore, the inverse SIE could in fact reflect a dissociation of a metal-OD− interaction in this mutant. Similarly, it is not possible to rule out that the inverse effect is due to restricted torsional effects upon productive substrate capture. The large SIE in the presence of Mn2+ determined for the Asn195 → Ala mutant only in kcat/Km for ATP again likely reflects the alteration in optimal metal ligand availability and indicated that, for the larger manganese, there is a significant barrier to productive ATP capture in D2O, quite the opposite of the situation with magnesium where this rate is enhanced in D2O. The molecular
Asp190 carboxylate contributes at least two orders of magnitude to rate but not position, whereas the Asn mutant is isosteric but predicted to play a central role (32, 33). An associative mechanism where base catalysis would be prenated analogues, and Br- pH, are not improved substrates for the enzyme over fluorinated Tyr analogues, which are deprotonated at assay substrate studies using the protein Tyr kinase CsK revealed that critical role in substrate deprotonation. Supporting evidence has contributed to the suggestion that this invariant residue acts as an active site base, deprotonating the incoming hydroxyl group and thus increasing its nucleophilicity. The suggestion that this Asp residue is an active site base is however challenged by the magnitude of rate reduction upon mutation to Ala, which is not generally consistent with a critical role in substrate deprotonation. Supporting evidence has also arisen in the protein kinase field where alternate substrate studies using the protein Tyr kinase CsK revealed that fluorinated Tyr analogues, which are deprotonated at assay pH, are not improved substrates for the enzyme over protonated analogues, and Brønsted values are not consistent with an associative mechanism where base catalysis would be predicted to play a central role (32, 33).

To further characterize the importance of Asp190 in aminoglycoside antibiotic phosphorylation, we generated the Asp190 → Glu and Asp190 → Asn mutants, where Glu conserves charge but not position, whereas the Asn mutant is isosteric but without appreciable charge. The Asp190 → Glu mutant was markedly impaired in its ability to phosphorylate a variety of aminoglycoside substrates with a resulting 1100-fold decrease in $k_{cat}/K_m$. Comparison of the catalytic rates of the Asp190 → Ala to Asp190 → Glu mutants indicates that positioning of the carboxylate contributes at least two orders of magnitude to rate enhancement. A similar amount of catalytic power has been attributed to the rate enhancement afforded by the correct positioning of His195 in the catalytic mechanism of nucleoside diphosphate kinase (34).

The Asp190 → Asn mutant was significantly impaired in catalytic capacity but maintained demonstrable phosphoryl transfer activity above the Asp190 → Ala mutant. The $p_K$ of the Asn amide is less than 0 and is therefore unlikely to act as a general base. These results are therefore not consistent with a role for Asp190 as a general base. Rather, the carboxylate may participate in orienting the incoming aminoglycoside hydroxyl for optimal attack upon the γ-phosphate of ATP, but that phosphate transfer likely precedes substrate deprotonation, which is supportive of a dissociative phosphoryl transfer mechanism.

Asp208—Asp208 is both an Mg1 and Mg2 ligand (Fig. 2), and we have previously reported the importance of Asp208 to the protein kinase activity of APH(3′)-IIIa (12). The Asp208 → Ala mutant lacks any detectable aminoglycoside kinase activity, thereby indicating that this residue is absolutely critical for the catalytic mechanism of APH(3′)-IIIa. This result parallels those obtained when the corresponding residue of yeast PKA was mutated to Ala resulting in inviable cells (30). Mutation of APH(3′)-IIIa Asp208 to Glu or Asn generated mutants without detectable kinase activity. Because the isosteric Asn is also a potential Mg2+ ligand, these results suggest that charge neutralization of Mg is important for generating and or stabilizing the transition state, although Mg2+ ligation by Asn will admittedly likely be different than Asp. Thus Asp208 and Mg2+ (likely Mg1), and not Asp190, are critical factors required for transition state formation. Given that a role for Asp190 as a catalytic base is not required for APH(3′)-IIIa catalysis argues against an associative transition state and therefore favors a dissociative mechanism with a metaphosphate-like intermediate.

Roles of $M^{2+}$—In an effort to better understand the role that metal ions play in the catalytic mechanism of APH(3′)-IIIa, we examined the concentration dependence of free Mg2+ on the initial rates of reaction, and as well on the steady-state kinetic parameters. APH(3′)-IIIa-catalyzed aminoglycoside phosphorylation was both activated and inhibited by increased Mg2+ concentration, demonstrating potent substrate inhibition with an optimal concentration of ~1 mM free Mg2+, which resulted in a roughly 2-fold decrease in $k_{cat}$ from 1–10 mM. The Glu195 → Ala mutant was less affected by Mg2+ concentration, but required 3-fold more metal ion for maximal activity. Although the altered metal ion dependence of this mutant enzyme may reflect a change in the rate-limiting step, it is more likely that the loss of the indirect interactions that the Glu60 carboxylate maintains with Mg1 is responsible for these changes. Such an interaction occurs between the carboxylate of Glu60 and the α-amino group of Lys44 (10). Lys44 interacts with the non-bridging oxygens of the α- and β-phosphates, and is important for nucleotide binding (10). Thus, this interaction would clearly be important for holding ADP in place after phosphoryl transfer has occurred. Therefore, the loss of binding energy albeit indirect, would be expected to affect the inhibition caused by increasing concentrations of magnesium. If this is the case, then it indicates that increases in solvent viscosity would inhibit a conformational change that is required for phosphate transfer. Because the presence of Glu60 normally prevents this dramatic solvent viscosity effect, the pocket, created by the removal of this functional group, could become filled with the viscoegen, which would prevent the diffusion-controlled movement of residues lining this region of the active site.

Mg2+ inhibition is virtually non-existent in the Asn195 → Ala mutant. This result, in combination with the effects noted on the affinity of ATP for this mutant enzyme, indicates that Mg2 is required for the optimal binding of ATP. However, neither Mg2 nor Asn195 is essential for catalysis. This is remarkable
when one considers that the loss of the Asp\(^{200}\) carboxylate, and in effect the coordination site(s) on Mg1, results in the complete loss of any detectable activity. These results taken together suggest a role for Mg2 in facilitating nucleotide triphosphate binding and that Asn\(^{195}\) plays a critical role in making ADP release the rate-limiting step for APH(3\'-)IIIa.

The observation that the Asp\(^{190}\) → Glu mutant has an altered Mg\(^{2+}\) dependence suggests that this residue may also play a role in Mg\(^{2+}\) binding. In the APH(3\'-)IIIa:ADP crystal structure the carboxylate of Asp\(^{190}\) is 3.9 Å from Mg1 at its closest approach. Although this distance is long in the available ground state structures of APH(3\'-)IIIa, it is possible that Mg1 could move closer to this functional group in the transition state. Thus the change in the apparent dissociation constant could move closer to this functional group in the transition ground state structures of APH(3\'-)IIIa.

The role of Mg\(^{2+}\) and charge of Asp208 are critical to the catalytic mechanism of this enzyme. These results, combined with solvent isotope and solvent viscosity effects, favor a dissociative-like transition state. These results also attempt to explain the roles of the two magnesium ions that are required for catalysis to occur. Mg2 is important for nucleotide binding, whereas Mg1 is the catalytic magnesium ion that directs phosphoryl transfer and could thereby aid the formation of a meta-phosphoryl-like intermediate.

The results also describe the importance of a number of residues that are absolutely conserved among APHs and protein kinases. The results described herein confirm that these residues are not only conserved at the primary and tertiary structure levels but are also functionally conserved. This finding has profound implications for the evolution of enzymes when one considers that sequence conservation among the APH and protein kinase families of enzymes is less than 5%, and that only five absolutely conserved residues are shared between these two superfamilies of enzymes. Interestingly, these residues all make up the catalytic core of the enzyme and are involved in nucleotide binding, metal binding, or the promotion of phosphate transfer. Thus it appears likely that these two families of enzymes evolved from a common progenitor, i.e.,

as these enzymes evolved, the architecture of the active site was maintained, while residues responsible for substrate binding were replaced to fulfill the unique substrate binding requirements of each individual enzyme.

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