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AMINOGLYCOSIDE PHOSPHOTRANSFERASES: PROTEINS, STRUCTURE, AND MECHANISM

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1. ABSTRACT

Aminoglycoside antibiotics constitute an important class of clinically useful drugs which are imperiled by the emergence of resistant organisms. Aminoglycoside resistance in the clinics is primarily due to the presence of modifying enzymes which *N*-acetylate, *O*-adenylate or *O*-phosphorylate the antibiotics. The latter family of enzymes are termed the aminoglycoside phosphotransferases or kinases and are the subject of this review. There are seven classes of aminoglycoside phosphotransferases (APH(3'), APH(2"), APH(3"), APH(6), APH(9), APH(4), APH(7")) and many isozymes in each class, and although there is very little overall general sequence homology among these enzymes, certain signature residues and sequences are common. The recent determination of the three-dimensional structure of the broad spectrum aminoglycoside kinase APH(3')-IIIa complexed with the product ADP, in addition to mechanistic and mutagenic studies on this and related enzymes, has added a great deal to our understanding of this class of antibiotic resistance enzyme. In particular, the revelation of structural and mechanistic similarities between APHs and Ser/Thr and Tyr kinases has set the stage for future inhibition studies which could prove important in reversing aminoglycoside resistance.

2. INTRODUCTION

The aminoglycoside/aminoclyclitol antibiotics include many clinically important drugs such as gentamicin, amikacin, tobramycin, and streptomycin which find extensive use in the treatment of infections caused by many bacteria. These antibiotics constitute a large family of amino-compounds which exhibit broad antibacterial and antiprotozoal activity and have found clinical use since their discovery in the mid-1940s. The aminoglycosides target the bacterial ribosome, and in particular footprint to the 16S rRNA where they are thought to interfere with translation, often resulting in incorrect reading of the mRNA, which results in a variety of downstream effects. Unlike other antibiotics which interfere with bacterial translation such as tetracycline and chloramphenicol, most aminoglycosides are bactericidal rather than bacteriostatic. This property makes aminoglycosides highly desirable anti-infective agents.

One of the more significant consequences associated with translational errors caused by many aminoglycosides is membrane damage (1). This results in a breach of membrane integrity and a disruption of ion gradients which precipitates cell death. Aminoglycosides which bind to the ribosome but do not bring about mistranslation, such as hygromycin or spectinomycin, do not result in membrane damage and as a consequence are bacteriostatic (2).

Aminoglycosides all contain a six-membered aminocyclitol ring (a cyclohexane group to which are attached amino and hydroxyl groups) and carbohydrate moieties, many of which are aminosugars. Thus aminoglycosides are water soluble, basic in nature, and generally positively charged at physiological pH. The aminocyclitol ring is generally derived from glucose-6-phosphate either through the synthesis of *myo*-inositol followed by oxidation and transamination to give *scyllo*-

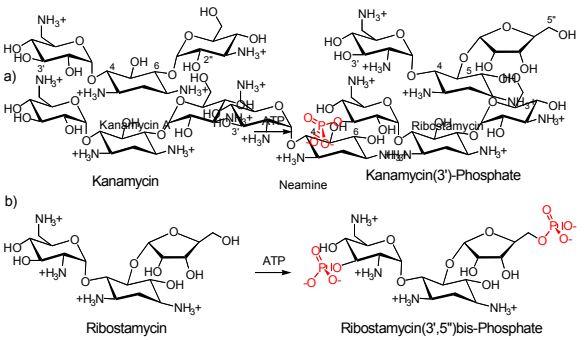

Figure 1. Structures of some aminoglycoside antibiotics

Figure 2. Reactions catalyzed by APH(3'). a) 3'-Phosphorylation of kanamycin A; b) 5"-Phosphorylation of ribostamycin (not all APH(3')s have been shown to have 5"-phosphorylation activity).

inosamine in the case of streptamine containing aminoglycosides e.g. streptomycin, or through 2-deoxy-scyllo-inosamine required for the synthesis of 2-deoxystreptamine containing aminoglycosides such as kanamycin, gentamicin, and neomycin (reviewed in (3)). The aminocyclitol ring is numbered simply, and attached carbohydrates are designated with prime (') or double prime (") superscripts (figure 1).

Resistance to the aminoglycoside antibiotics can manifest itself in three fashions: 1) mutations in target ribosomal RNA or proteins, 2) altered uptake of the molecules, or 3) the expression of resistance enzymes. Ribosomal mutations are relatively rare in the clinic, though streptomycin resistance in *Mycobacterium tuberculosis* is the exception (4). Similarly, altered uptake resistance mechanisms are not common though general resistance exhibited by anaerobes (5) and organisms such as *Pseudomonas aeruginosa* (6) fall into this class. Enzymatic resistance is found in two forms: enzymes which modify the target rRNA, and enzymes which modify the aminoglycosides directly. Several aminoglycoside antibiotic producing organisms express base-specific rRNA methyltransferases which confer high-level resistance to aminoglycosides e.g. Grm in *Micromonospora purpurea* and *Micromonospora rosea* (7), but thus far these enzymes have not been found in bacterial clinical isolates. On the other hand, the synthesis of aminoglycoside modifying enzymes is the primary mode of resistance in most clinical isolates. Modifying enzymes include acetyl CoA-dependent *N*-acetyltransferases, ATP-dependent *O*-adenyltransferases, and ATP-dependent *O*-phosphoryltransferases. Modified aminoglycosides lose their capacity to bind ribosomes in a fashion which impairs their biological activity, and thus the cells exhibit a drug-resistance phenotype. The aminoglycoside phosphotransferases, or kinases, generally abbreviated APHs, are the topic of this review.

3. AMINOGLYCOSIDE KINASES (APHS)

3.1. Nomenclature

The APH family of enzymes includes several members which are differentiated on the basis of three criteria: 1) substrate specificity or resistance phenotype, 2) regiospecificity of phosphoryl transfer, and 3) protein/gene sequence. In the past, aminoglycoside modifying enzymes were named based on the site of phosphorylation, e.g. genes encoding enzymes which modified the 3'-hydroxyl were termed *aphA*, followed by a number to designate a distinct enzyme. Shaw and colleagues have proposed an alternate nomenclature in which the regiospecificity of group transfer is explicitly included in the name followed by a roman numeral designating phenotype and a letter to differentiate different genes, e.g APH(3')-Ia (8). This review will use the latter nomenclature. A recent appeal for a centralized database for aminoglycoside modifying enzymes is echoed here (9).

3.2 APH(3')

The largest family of APHs are the enzymes which modify kanamycin and related compounds at the 3'-hydroxyl group (figure 2a). Thus compounds such as the gentamicin Cs and tobramycin which lack this functionality, are not substrates for these enzymes. The exception to this rule is lividomycin which is phosphorylated by several APH(3')s. Here phosphoryl transfer is directed to the 5"-hydroxyl group of the pentose ring. Other aminoglycosides which incorporate a pentose linked to position 5 of the 2-deoxystreptamine ring also have the potential to be phosphorylated at this position (figure 2b).

Since aminoglycoside therapy is primarily administered in a clinical setting, most aminoglycoside resistance determinants have been isolated from nosocomial pathogens. In particular, APH(3') enzymes have been identified in both Gram-negative and Gram-positive pathogens over the past 30 years. The emergence of these enzymes has effectively removed aminoglycosides such as kanamycin and neomycin from clinical use. Resistance to other aminoglycosides such as amikacin, isepamicin, butirosin, and lividomycin serve as the basis for classification into seven distinct classes (I-VII) (table 1).

3.2.1. APH(3') from clinical isolates

Kanamycin and neomycin resistance in clinical isolates of *Enterobacteriaceae* was determined to be caused by ATP-dependent phosphotransferase activity in 1967 (22) and the site of modification was later found to be the 3'-hydroxyl group (23). A similar resistance mechanism was subsequently detected in the Gram-positive bacterium *Staphylococcus aureus* (24) and over the past 30 years, a number of genes encoding these APH(3') enzymes have been cloned from pathogenic bacteria.

The APH(3')-I class of enzymes are broadly distributed among Gram-negative bacteria. The most widely distributed among these is encoded by *aph(3')-Ia* which is frequently found on transposable elements e.g. Tn*903* (10). The APH(3')-Ia enzyme has been

Table 1. APH(3') isozymes

Class	Subtype	Resistance Profile	Organism	Reference

I	a	Kan, Neo, Paro, Rib, Liv, Gent B	*Escherichia coli*	(10)
	b		*E. coli*	(11)
	c		*Klebsiella pneumonia*	(12)
II	a	Kan, Neo, Paro, Rib, But, Gent B	*E. coli*	(13)
III		Kan, Amk, Isep, Neo, Paro, Rib, Liv, But, Gent B	*Enterococcus and Staphylococcus*	(14, 15)
IV	a	Kan, Neo, Paro, Rib, But	*Bacillus cirulans*	(16)
V	a	Kan Neo, Paro, Rib	*Streptomyces fradiae*	(17)
	b		*Streptomyces ribosidificus*	(18)
	c		*Micromonospora chalcea*	(19)
VI	a	Kan, Neo, Paro, Rib, But, Gent B	*Acinetobacter baumani*	(20)
VII	a	Kan, Amk, Isep, Neo, Paro, Rib, Liv, But, Gent B	*Campylobacter jejuni*	(21)

Kan, kanamycin; Amk, amikacin; Isep, isepamicin; Neo, neomycin; Paro, paromomycin; Rib, ribostamycin; Liv, lividomycin; But, butirosin; Gent B, gentamicin B.

overexpressed in *E. coli*, purified and enzymatically characterized by the group of S. Mobashery (25). The enzyme has a monomer molecular mass of 31 kDa, a feature common to most APH(3')s. In a fashion analogous to APH(3')-IIIa (*vide infra*), APH(3')-Ia can be isolated as a DTT sensitive dimer, indicative of intermolecular disulfide bonds. Steady state kinetic analysis demonstrated a broad aminoglycoside substrate specificity as predicted by the resistance phenotype with k_{cat}/K_m between 10^6 and 10^8 M^{-1} s^{-1} (25), values approaching the diffusion limit for small molecules in solution, thus APH(3')-Ia is a highly evolved catalyst.

Two other APH(3')-I isozymes have recently been described. The first, designated APH(3')-St, is present on the *Salmonella typhimurium* plasmid NTP16 and encoded by transposon derived sequences and is virtually identical (>95%) to APH(3')-Ia (26). The second, which we have designated APH(3')-Id based on its homology to other type I APH(3')s, is most closely related to APH(3')-Ib and is encoded on the bacterial, incompatibility group Q, plasmid pIE693 (27).

The APH(3')-II class of enzymes find frequent use as a tool in molecular biology. In particular, the *neo* gene derived from Tn*5* (13) is a general antibiotic resistance marker in wide use for both prokaryotic (kanamycin and neomycin resistance) and eukaryotic (geneticin resistance) studies. The corresponding enzyme, APH(3')-IIa, has been overexpressed in *E. coli*, purified and characterized (28). The roles of specific amino acid residues in APH(3')-IIa has been studied by site directed mutagenesis, in particular His188 which is invariant in all APHs, was determined to be important by virtue of an increase in aminoglycoside antibiotic minimal inhibitory concentration (MIC) (29, 30) and decrease in enzyme activity (30). Mutation of Tyr218 to Ser, Asp or Phe resulted in a change in aminoglycoside recognition but not of ATP (31), and Arg211 to His, Lys, and Pro mutations were determined to alter ATP binding (32). The specific roles for these amino acids can now be inferred using the three-dimensional structure of APH(3')-IIIa.

Interestingly, a chromosomally encoded APH(3')-IIb has recently been described in *Pseudomonas aeruginosa* (33). APH(3')-IIb is highly homologous to APH(3')-IIa (approximately 52% identity) and its chromosomal location in *P. aeruginosa* may contribute to the low level resistance to aminoglycoside antibiotics intrinsically associated with *Pseudomonas* strains.

The gene encoding APH(3')-IIIa has been cloned from *Enterococcus faecalis* (15) and *Staphylococcus aureus* (14). In *E. faecalis*, the gene is located on the multi-resistance plasmid pJH1 along with streptomycin and macrolide resistance determinants. The gene has also been found on plasmid pIP1433 in the Gram-negative organism *Camplylobacter coli* (34). APH(3')-IIIa has been overexpressed in *E. coli*, purified and characterized (35). Like APH(3')-Ia, the enzyme can be isolated as a monomer or a kinetically indistinguishable disulfide bridged dimer. The enzyme exhibits a very broad aminoglycoside substrate range with specificity constants (k_{cat}/K_m) generally on the order of 10^6 M-1s-1. Steady state kinetic analysis of the enzyme revealed a special case of an ordered BiBi mechanism termed Theorell-Chance with ATP binding first followed by the aminoglycoside (figure 3) (36). Release of the phosphorylated aminoglycoside then precedes release of ADP. The specific case of a Theorell-Chance mechanism indicates that under steady state conditions, the chemical conversion of the ternary complex: (ATP•Aminoglycoside•Enz↔ADP•Phospho-aminoglycoside• Enz), does not contribute to the observed maximal rate, k_{cat}. Generally, this implies that second product release is rate-limiting, in this case ADP. The kinetic mechanism was validated and rate-limiting release of ADP demonstrated through a series of experiments including solvent isotope effects, ATPgammaS thio effect and viscosity effects (37).

In addition, the regiospecificity of phospho-transfer by APH(3')-IIIa was definitively established by purification of phosphorylated aminoglycosides followed by detailed analysis by a variety of NMR and mass spectral techniques (35, 38). Based on these experiments, it was determined that the enzyme phosphorylates 4,6-disubstituted-2-deoxystreptamine aminoglycosides such as kanamycin and amikacin exclusively at the 3'-hydroxyl. On the other hand, 4,5-disubstituted-2-deoxystreptamine such as lividomycin which lacks a 3'-hydroxyl are substrates, but phosphorylation occurs at the 5"-hydroxyl group of the pentose ring. Aminoglycosides with both a 3'-

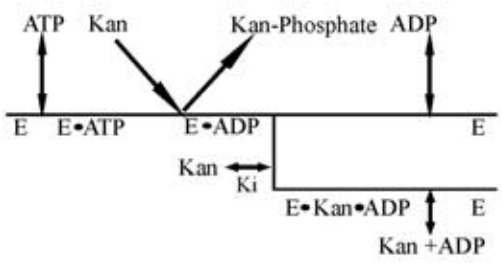

Figure 3. Kinetic Mechanism of APH(3')-IIIa.

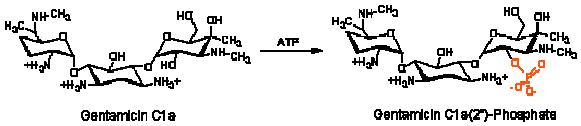

Figure 4. Reaction catalyzed by APH(2").

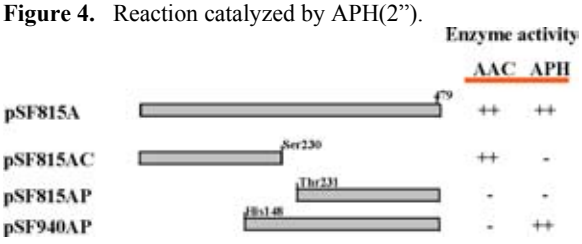

Figure 5. Domain structure of bifunctional AAC(6')-APH(2").

and 5"-hydroxyl group can be efficiently di-phosphorylated (38). APH(3')-VI and APH(3')-VII encoding genes have been cloned from *Acinetobacter baumani* and *Campylobacter jejuni* respectively (20, 21). These enzymes confer resistance to most common aminoglycosides and are distinguished by their ability to confer resistance to amikacin (APH(3')-VI only) and lividomycin (APH(3')-VII only).

3.2.2. APH(3') from antibiotic producing organisms

APH(3')-IV has been cloned from the butirosin producer *Bacillus circulans* (16). The enzyme has been overexpressed in *E. coli* and the product of phosphorylation of ribostamycin characterized by NMR (39). The APH(3')-V family of enzymes are expressed by aminoglycoside producing actinomycetes. Thus *aph(3')* type *Va* and *Vc* have been respectively cloned from the neomycin producers *Streptomyces fradiae* (17) and *Micromonospora chalcea* (40), and the type *Vb* gene has been cloned from the ribostamycin producer, *Streptomyces ribosidificus* (18).

3.3. Other Aminoglycoside Kinases

Aminoglycoside kinases which phosphorylate aminoglycosides at positions other than the 3'- and 5"-hydroxyls are widely distributed and some play important roles in conferring clinical resistance to these antibiotics. These are summarized in **table 2** and described below.

3.3.1. APH(2")

In Gram-positive organisms, gentamicin resistance arises primarily from the presence of a 57 kDa bifunctional enzyme with both aminoglycoside 6'-acetyltransferase and 2"-phosphotransferase activity (figure 4). The *aac(6')-aph(2")* gene has been cloned from both *E. faecalis* and *S. aureus* (41, 42) and analysis of the predicted protein sequence reveals homology to aminoglycoside acetyltransferases in the N-terminal region to aminoglycoside kinases in the C-terminus. Ferretti at al. have prepared truncated gene products and confirmed the predicted location of the two aminoglycoside modification activities (figure 5) (42).

The enzyme has been purified from *S. aureus* and *S. epidermidis* (65), and overexpressed in both *E. coli* (66) and *Bacillus subtilis* (D. Daigle & G. Wright, in press). The kinetic mechanisms of both the acetyltransferase and phospho-transferase activities have been determined to be random rapid equilibrium where both the ATP (or acetylCoA for the AAC(6') activity) and aminoglycosides substrates may bind to the enzyme first or second and the rate of dissociation of the substrates exceeds the rate of reaction to form products (67).

The product of enzymatic modification of both kanamycin (66) and arbekacin (68) by this bifunctional enzyme have been characterized by NMR and the predicted regiospecificities confirmed. In addition, both of these studies detected some product which was both *N*-6'-acetylated and *O*-2"-phosphorylated, thus the enzyme has the capacity to doubly modify target antibiotics, an observation which may impact on the remarkable ability of this enzyme to confer high level resistance to a very broad range of aminoglycosides.

Recently, an APH(2") homologue, *aph(2")-Ic*, was cloned from a veterinary isolate of *Enterococcus gallinarum* (44). The gene encodes a 306 amino acid protein with a predicted mass of 34.7 kDa which lacks acetyltransferase activity. A similar enzyme has also been cloned from an *E. coli* isolate (43). This enzyme, APH(2")-Ib, is 299 amino acids in length with a predicted mass of 33 kDa. Both these enzymes show approximately 20% identity to each other and the bifunctional AAC(6')- APH(2") enzyme. The discovery of this potent and broad specificity enzyme in Gram-negative *E. coli* is highly alarming from a clinical perspective.

3.3.2. APH(3") and APH(6)

Streptomycin resistance due to aminoglycoside phosphotransferases is the result of two classes of enzymes, the APH(3")s and the APH(6)s (figure 6). Both enzymes are found in the streptomycin producer *Streptomyces griseus* (45,47). The *aphD* gene encoding APH(6)-Ia is clustered with the streptomycin biosynthetic genes (47) while the *aphE* gene encoding APH(3")-Ia is not (45). The reason for this redundancy in resistance is not known at present. In *Streptomyces glaucescens*, another streptomycin producer, the *sph* gene encodes the self resistance enzyme APH(6)-Ib (48). The *str* gene of Tn*5* also encodes a streptomycin kinase, APH(6)-Ic in addition to APH(3')-IIa and *ble*, a bleomycin resistance determinant (49).

In Gram-negative organisms, a two-gene cassette comprised of *aph(3")-Ib* and *aph(6)-Id* (also respectively

Table 2. Other APHs

Enzyme (gene)	Resistance Profile	Organism	Reference
AAC(6')-APH(2")-Ia	Kan, Tobr, Neo, Liv, Gent C	*Staphylococci and Enterococci*	(41, 42)
APH(2")-Ib	Kan, Tobr, Neo, Liv, Gent C	*Escherichia coli*	(43)
APH(2")-Ic	Kan, Tobr, Neo, Liv, Gent C	*Enterococcus gallinarum*	(44)
APH(3")-Ia (*aphE*)	Strep	*Streptomyces griseus*	(45)
APH(3")-Ib (*strA*)	Strep	*E. coli*	(46)
APH(6)-Ia (*aphD*)	Strep	*Streptomyces griseus*	(47)
APH(6)-Ib (*sph*)	Strep	*Streptomyces glaucescens*	(48)
APH(6)-Ic (*str*)	Strep	*E. coli*	(49)
APH(6)-Id (*strB*)	Strep	*E. coli*	(46)
APH(9)-Ia	Spect	*Legionella pneumophila*	(50)
APH(9)-Ib (*spcN*)	Spect	*Streptomyces flavopersicus*	(51)
APH(4)-Ia	Hygr	*E. coli*	(52)
APH(4)-Ib (*glpA*)	Hygr, Glyphosate	*Pseudomonas pseudomallei*	(53)
APH(7")-Ia	Hygr	*Streptomyces hygroscopicus*	(54)
APH(3')-VSr	Kan, Neo	*Streptomyces rimosus*	(55)
Hydroxyurea kinase (*hur*)	hydroxyurea	*Streptomyces aureofaciens*	(56)
APH-Ll (*orf8*)	unknown	*Lactococcus lactis subsp. lactis*	(57)
APH-STRN (*strN*)	unknown	*S. griseus*	(58)
MPH-I (*mphA*, *mphk*)	erythromycin	*E. coli*	(59, 60)
MPH-II (*mphB*)	erythromycin	*E. coli*	(61)
Viomycin kinase (*vph*)	viomycin	*Streptomyces vinaceus*	(62)
Capreomycin kinase (*cph*)	capreomycin	*Streptomyces capreolus*	(63)
MtPH-I	unknown	*Mycobacterium tuberculosis*	(64)
MtPH-II	unknown	*M. tuberculosis*	(64)
MtPH-III	unknown	*M. tuberculosis*	(64)

Kan, kanamycin; Tobr, tobramycin; Neo, neomycin; Liv, lividomycin; Gent C, gentamicin C complex; Strep, streptomycin; Spect, spectinomycin; Hygr, hygromycin.

known as *strA* and *strB*) is located on broad host range plasmids e.g RSF1010 (46). A recent survey of environmental isolates has shown that the *strA-strB* genes are widely distributed in the environment (69).

3.3.3. APH(9)

Recently, two genes encoding spectinomycin kinases have been cloned from *Legionella pneumophila* (50) and the spectinomycin producer *Streptomyces flavopersicus* (51). The *L. pneumophila* enzyme has been overexpressed in *E. coli*, characterized, and the product of spectinomycin phosphorylation determined to be exclusively spectinomycin-9-phosphate by NMR methods (90). Based on these results, we propose that the enzyme be classified as APH(9)-Ia (figure 7).

The *S. flavopersicus* gene product has also been shown to phosphorylate spectinomycin, though the regiospecificity of phosphoryl transfer has not been established. As phylogenetic analysis (see section 3.4) reveals that this enzyme and APH(9)-Ia cluster together and given the demonstrated site of phospho-transfer in the latter enzyme, we predict that these enzymes will share specificities and thus the *S. flavopersicus* enzyme should be designated APH(9)-Ib.

3.3.4. APH(4)

Resistance to the aminoglycoside hygromycin is a useful genetic marker for a number of molecular biological experiments in both prokaryotes and eukaryotes. The APH(4)-Ia gene has been cloned from *E. coli* (52), and the regiospecificity of phosphorylation determined by NMR (figure 8a) (70). One other hygromycin phosphotransferase has been identified in *Pseudomonas pseudomallei,* designated here APH(4')-Ib. This APH(4)-Ia homologue confers tolerance to the herbicide glyphosate (N-phosphonomethylglycine) in *E. coli* presumably by formation of the acylphosphate which is necessary for metabolism of this inhibitor of 5-enoylpyruvylshikimate-3-phosphate synthase (figure 8b) (53).

3.3.5. APH(7")

Hygromycin resistance in the producing organism *Streptomyces hygroscopicus* is conferred by a kinase which has been demonstrated to phosphorylate the antibiotic at position 7" (figure 9) (71). The gene has been cloned (54) and the enzyme purified and characterized from an *E. coli* construct (72, 73).

3.3.6. Other aminoglycoside and miscellaneous phosphotransferases

The sequencing of various genes clusters as well as bacterial genomes have resulted in the identification of several new genes which either have been confirmed to be novel APHs, or which show significant homology to aminoglycoside kinases. Specifically, kinases which show the signature catalytic sequence $HGD(X)_4N$, where X is any amino acid, (see section 4 for discussion of the importance of this peptide sequence) have been included here.

An APH(3')-VSr has been cloned from *Streptomyces rimosus* (55) and while it mediates resistance to kanamycin and neomycin, little else is known except that

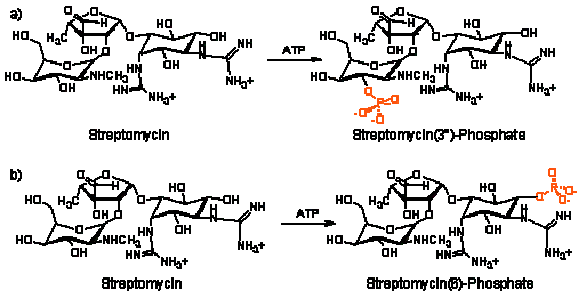

Figure 6. Reactions catalyzed by APH(3") and APH(6).

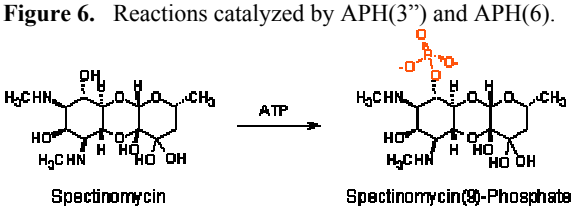

Figure 7. Reaction catalyzed by APH(9).

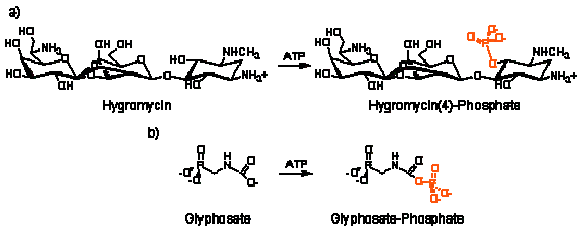

Figure 8. Reaction catalyzed by APH(4).

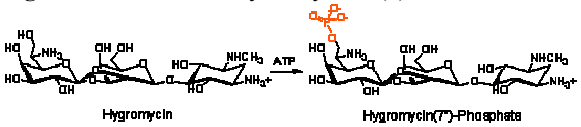

Figure 9. Reaction catalyzed by APH(7").

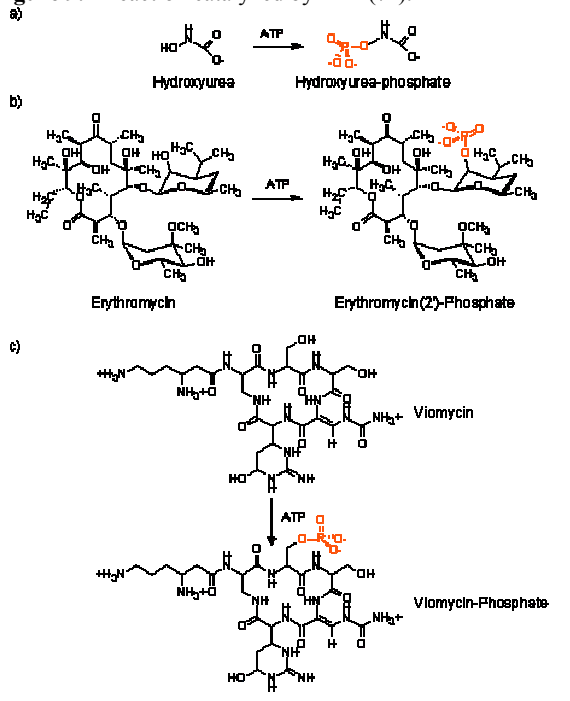

Figure 10. Reactions catalyzed by APH homologues.

APH(3')-VSr shows little homology to other type V 3'APHs. In fact it is most closely related to the hygromycin phosphotransferase APH(7")-Ia (see section 3.3.5).

The gene, *hur*, encoding a hydroxyurea resistance element has been cloned from the chlortetracycline producer *Streptomyces aureofaciens* (figure 10a) (56). Expression of the *hur* gene product in *E. coli* confers resistance to hydroxyurea, a synthetic inhibitor of DNA synthesis. Hydroxyurea kinase (HK) is a 340 amino acid protein which shows approximately 50% identity to APH(6)-Ia. Despite this significant homology to streptomycin kinase, HK does not confer resistance to either streptomycin (≤10 microg/mL) or neomycin, kanamycin and spectinomycin.

A gene encoding an APH-like enzyme (*orf8*), has been found within the His operon of *Lactococcus lactis subsp. lactis*. The function of this gene with regard to His biosynthesis is not known at present. The predicted protein, APH-Ll, while weakly homologous to APH(3') enzymes (13-21% similarity), does not confer resistance to kanamycin, tobramycin, butirosin, lividomycin, neomycin, dibekacin, amikacin, streptomycin and spectinomycin but does contain the expected catalytic sequence HGDYCLPN (57).

APH-STRN is a 35.6 kDa aminoglycoside phosphotransferase cloned from the streptomycin producer *Streptomyces griseus* (58). The regiospecificity of this enzyme has not been established, but APH-STRN is most closely related to the APH(9)s. It is thought that StrN may be involved in the control of streptomycin biosynthesis.

Erythromycin resistance in *E. coli* can be conferred by two APH-like genes that encode the macrolide phosphotransferase proteins MPH(2')-I and MPH(2')-II (figure 10b) (59-61). These proteins are somewhat different from APHs in that they incorporate a $HGD(X)_8D$ sequence rather than the APH signature $HGD(X)_4N$ motif, and thus the general similarity in terms of structure and mechanism remains to be demonstrated. MPH(2')-I is a 301 amino acid *E. coli* protein that in combination with MRX, a protein of unknown function, confers high level resistance to erythromycin. MPH(2')-II is a 302 amino acid *E. coli* protein that is 42 % identical to MPH(2')-I. The MPHs are most homologous to the 2"-aminoglycoside phosphotransferases, particularly in their C-termini.

Also related to the 2"-aminoglycoside phosphotransferases are two kinases which modify the cyclic peptide antibiotic viomycin. The *vph* gene from the viomycin producer *Streptomyces vinaceus* encodes a kinase (62), which *O*-phosphorylates viomycin and the related capreomycins IA and IIA (figure 10c). A related kinase designated CK, cloned from *Streptomyces capreolus,* has a similar substrate profile but preferentially phosphorylates capreomycin IA over viomycin (63).

3.3.7. Mycobacterial APHs

Three mycobacterial protein sequences, designated here MtPH-I, MtPH-II, and MtPH-III, were identified based on their homology to the known

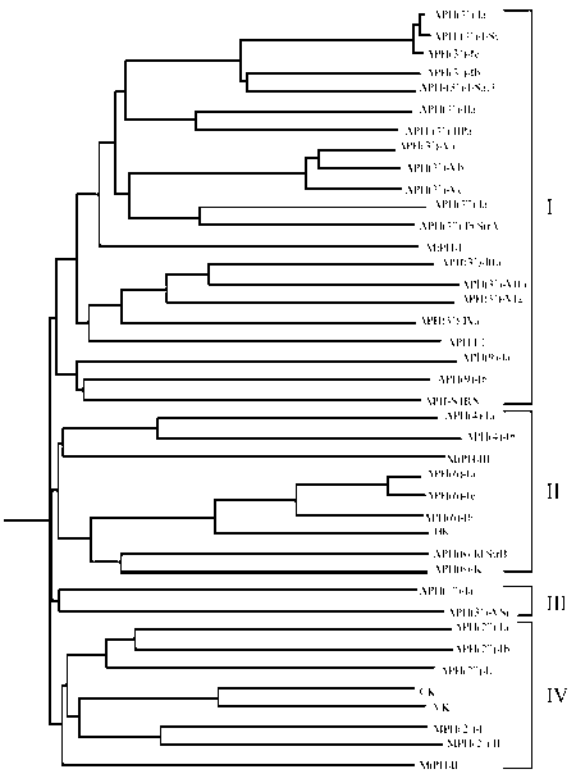

Figure 11. Phylogenetic relationships among APHs and related enzymes. Sequences were aligned using the program Clustal W (76).

aminoglycoside phosphotransferases (Genbank accession numbers Z97188, Z95120, and AL021646, respectively) during sequencing of the *M. tuberculosis* genome (64). The function of these proteins in mycobacteria is unknown, but are presumed to be kinases based on homology to both aminoglycoside and eukaryotic protein kinases. Interestingly, a family of 2'-*N*-aminoglycoside acetyltransferases has recently been identified in several species of mycobacteria and appear to be ubiquitously found in this genus (74, 75). The presence of so many aminoglycoside resistance determinants that do not appear to mediate high level aminoglycoside resistance in *M. tuberculosis* suggests that these proteins likely play a role in the normal function of the cell and also demonstrate the potential for mycobacteria to act as reservoirs for antibiotic resistance genes.

3.4. Phylogenetic relationship between APHs

The APHs and related proteins listed above were aligned in a pairwise fashion by the program Clustal W (76) and the resulting phylogenetic tree was plotted using the Phylip ver 3.5 programs Drawgram (77) (figure 11). From this analysis, 4 subfamilies emerge (I-IV). The largest grouping (family I) consists of all the APH(3')s, APH(3")s and APH(9)s, the second includes the APH(4) and APH(6) enzymes, the III consists only of APH(7") and APH(3')-VSr, and the final group includes the APH(2")s, as well as the macrolide and viomycin kinases. Most of the resistance elements found in clinical settings are clustered within the group I enzymes with the exception of a few isolated enzymes such as the bifunctional AAC(6')-APH(2") which is found in group IV. The alignment speaks to the broad dissemination of APHs and related proteins within bacterial populations, and APHs from antibiotic producing and non-producing organisms are found in all four groups. Such alignments are certainly fraught with potential pitfalls, and one must be cautious to avoid over-interpretation of the data. Nonetheless, this alignment is of useful predictive value for biochemical analysis of potential new APHs as they emerge from, for example, whole genome sequencing exercises.

4. APH(3')-IIIA STRUCTURE AND MECHANISM

The broad spectrum aminoglycoside kinase from enterococci and staphylococci, APH(3')-IIIa, is the only member of the aminoglycoside kinases for which a three-dimensional structure is currently known. The structure of APH(3')-IIIa complexed with ADP has been determined to 2.2 Å resolution by x-ray crystallographic methods (78). The structure of the enzyme was determined using the technique of multi-wavelength anomalous dispersion (MAD) from a single crystal of the enzyme enriched with Se-methionine at all 6 Met sites in the monomeric enzyme.

The enzyme crystallized as a head to tail covalent dimer linked by two disulfide bonds between Cys19 and Cys156. As noted above, the enzyme can be purified as a monomer or dimer and both forms show equal ability to phosphorylate aminoglycosides, thus the dimeric nature of the crystallized form of APH(3')-IIIa is not relevant to the mechanism of antibiotic resistance.

The monomer is a bilobal enzyme with a beta-sheet rich N-terminus and alpha-helix rich C-terminus (Fig 12). The enzyme active site lies a the junction of these two domains. The most striking aspect of the structure which immediately came apparent is the dramatic similarity to eukaryotic Ser/Thr and Tyr kinases (figure 13). This structural similarity is even more extraordinary as the amino acid similarity between APHs and Ser/Th/Tyr protein kinases is less than 10%, with only key active site residues conserved (described below).

The N-terminal lobe consists of five anti-parallel beta-sheets which are linked through a twelve amino acid tethering region to the C-terminal lobe. This portion of the enzyme is structurally dominated by six alpha-helices. The C-terminal Phe264 is unconventionally well defined in the crystal structure and may serve a role in aminoglycoside substrate recognition.

The ADP is bound within a pocket composed of a tethering region between the N- and C-terminal domains. This region of the enzyme shares several amino acids residues with Ser/Thr/Tyr kinases including Lys44, Glu60, Asp190, Asn195, Asp208 (table 3). The purine ring is stabilized by a hydrogen bond (H-bond) between Ser90 and the N6-amino group, as well as an H-bond between N1 and the amide hydrogen of Ala93. A water molecule also

Figure 12. Structure of APH(3')-IIIa monomer. Structure was prepared using the program RasMol (89).

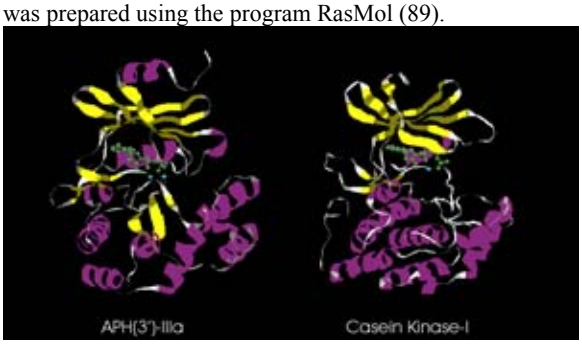

Figure 13. Comparison of APH(3')-IIIa and casein kinase-I (pdb accession no. 1CSN). Structures were drawn using the program RasMol (89).

Table 3. Common amino acid residues between APHs and Ser/Thr and Tyr protein kinases

Residue[1]	Role
Lys44	ATP binding
Glu60	Orientation of Lys44 for ATP binding
Asp190	Catalysis, possible active site base
Asn195	Mg^{2+} binding
Asp208	Mg^{2+} binding

[1] APH(3')-IIIa numbering

bridges N7 and the alpha-phosphate and provides a connection to the main chain amide of Asp208 via another water molecule. The purine ring also stacks with the aromatic side chain of Tyr42. The 2'-hydroxyl group of the ribose is within H-bonding distance to the carbonyl of Ser194 and is linked to the alpha–beta-phosphate bridging Mg^{2+} ion via an intermediary water molecule. The alpha– and beta-phosphate groups are also linked to Lys44, a residue which is conserved in all APH(3')s. Lys44 and Lys33 had been predicted to line the ATP binding site through affinity labeling studies with 5'[*p*-fluorosulfonyl)benzoyl]adenosine (79). Lys33 is in the vicinity of the ADP binding site, but does not directly contact the nucleotide. Consistent with these observations, a Lys33Ala site mutant did not have significant effect on the steady state kinetic parameters of the enzyme, but a Lys44Ala and a Lys33Ala-Lys44Ala double mutant resulted in a >27 fold increase in K_m for ATP, but had no effect on k_{cat} or the K_m for the aminoglycoside substrate kanamycin (78). Glu60, also an invariant residue in APH(3')s, appears to interact with Lys44, positioning it for the interaction with the phosphate groups.

There are two Mg^{2+} atoms which directly interact with the enzyme, the first (Mg1), shows typical octahedral geometry with ligands derived from the alpha– and beta-phosphates, as well the invariant APH residues Asn195 and Asp208. The additional ligands are contributed by water molecules. Mg2 shows a distorted octahedral geometry with ligands derived from the beta-phosphate of ADP, Asp208 and four water molecules, one of which is also interacting with Asp190.

Previous work had demonstrated that the mechanism of phosphoryl transfer was consistent with a direct attack of the substrate hydroxyl group upon the γ-phosphate of ATP and that an enzyme-bound phosphate was not involved (36, 80). There had been some suggestion that an invariant His at position 188 could be a followed at both the gamma and beta-phosphate positions using ^{31}P NMR as the presence of ^{18}O results in an upfield shift of 0.02 ppm of the ^{31}P signal (81). No exchange was observed either with APH(3')-IIIa + ATP alone, or in the presence of the 3'deoxy aminoglycoside inhibitor of the enzyme, tobramycin (80). These results thus support a direct attack mechanism in which phosphate transfer to the aminoglycoside hydroxyl group proceeds without a phospho-enzyme intermediate.

The three-dimensional structure and the relationship with protein kinases has suggested the importance of the invariant Asp190 in catalysis. In protein kinases, this residue has been suggested to be a general base which assists in deprotonating the target substrate hydroxyl group thus increasing it's nucleophilicity (82). This proposed role for Asp has not gone unchallenged and at present the precise role has not been effectively defined (83, 84). It is evident however, that based on mutagenesis studies that this residue is critical for catalysis. Mutagenesis of Asp190 to Ala in APH(3')-IIIa essentially results in completely inactive enzyme, supporting a role in catalysis (78). Thus a possible mechanism of phosphoryl transfer from ATP to kanamycin in which Asp190 directly participates in hydroxyl group deprotonation is shown in figure 15. Additional studies including mutagenesis, structural and kinetic approaches are ongoing and required to elucidate the mechanism of phosphoryl transfer which is required for accurate design of APH(3') inhibitors.

5. INHIBITION OF APHS

Inhibition studies with aminoglycoside resistance enzymes have generally been limited. Aminoglycosides

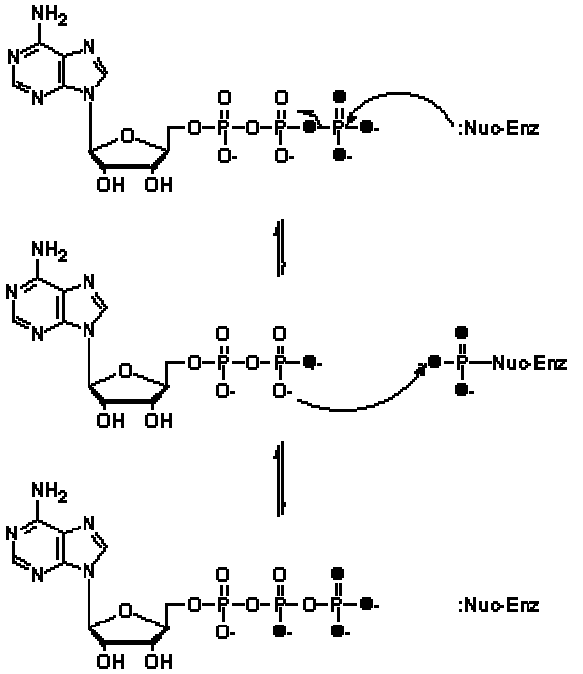

Figure 14. Positional isotope exchange experiment. Scrambling of the ^{18}O from the bridge to non-bridge position can be monitored by ^{31}P NMR.

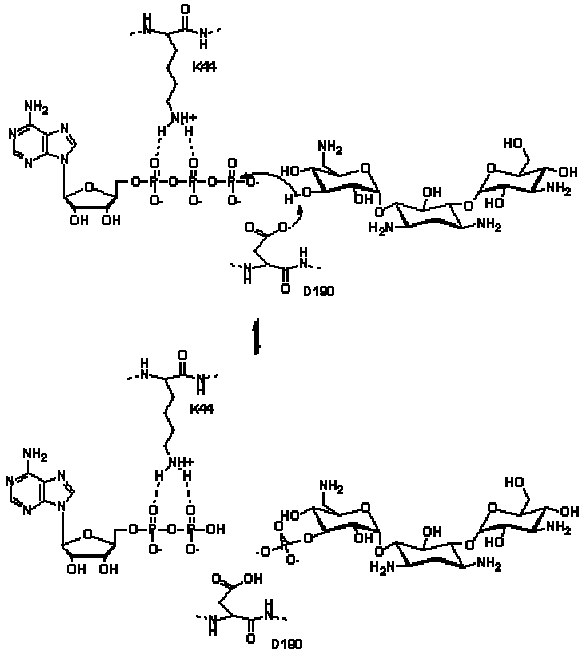

Figure 15. Proposed mechanism of phosphoryl transfer catalyzed by APH(3')-IIIa.

which lack the target of modification have been shown to inhibit inactivating enzymes in a competitive fashion. For example, tobramycin, a homologue of kanamycin B which lacks the 3'-hydroxyl group is a competitive inhibitor of aminoglycoside binding to APH(3')-IIIa with a K_i of 0.5 microM and a non-competitive inhibitor of ATP (K_i 0.6 microM) (36). Therefore, aminoglycosides which lack sites of enzymatic modification have proven quite useful in the clinic.

Mobashery and colleagues have described an elegant inactivation of APH(3')-Ia and APH(3')-IIa using mechanism-based enzyme inactivators consisting of analogues of neamine and kanamycin A which incorporate nitro groups at position 2' (85). Phosphorylation of the 3'-hydroxyl group by APH(3') generates an intermediate which can readily undergo spontaneous elimination of phosphate to form a nitroalkene. This compound can then react with an enzyme-derived nucleophile in a Michael addition to yield covalently inactivated enzyme. The partition ratio for an enzyme inactivator is a measure of the ratio of enzymatic turnovers (k_{cat}) to inactivation (k_{inact}). For the neamine derived compounds, partition ratios were approximately 2 for APH(3')-Ia and 4100 for APH(3')-IIa, while for the kanamycin derived compounds, the partition ratios were not measurable, suggesting stoichiometric inactivation. Unfortunately, these compounds, while highly potent with purified enzymes, did not reverse aminoglycoside resistance *in vivo*, nonetheless this study provides proof of concept for future design and synthesis of mechanism based inhibitors.

Mobashery's group has also synthesized a number of deaminated derivatives of neamine and kanamycin which are very poor substrates for both APH(3')-Ia and APH(3')-IIa (k_{cat}/K_m ~10^{1-2} $M^{-1}s^{-1}$ compared to 10^7 $M^{-1}s^{-1}$ for fully aminated compounds) (86). Though these compounds were not evaluated directly for their ability to inhibit the enzymes, these studies are highly important as some of the compounds retained their antimicrobial activity even against organisms harboring APH(3')-Ia or APH(3')-IIa. On the other hand, when evaluated with purified APH(3')-IIIa, an enzyme with more liberal substrate tolerance, most of these compounds were relatively good substrates with k_{cat}/K_m ~10^5 $M^{-1}s^{-1}$ with the exception of compounds which lack a 6'-amino group (87). These latter compounds were poorer enzyme substrates with k_{cat}/K_m ~10^{2-3} $M^{-1}s^{-1}$. These studies open the potential for additional synthesis of aminoglycoside antibiotics which are not readily inactivated by at least the APH(3') class of modifying enzymes.

The discovery of the structural relationship between protein kinases and APHs has led to the evaluation of known inhibitors of the former enzymes with APHs (88). Several protein kinase inhibitors such as the flavanoids quercetin and genistein, as well as the isoquinoline sulfonamides such as H-9 and CKI-7 were good competitive inhibitors of ATP and non-competitive inhibitors of kanamycin for both APH(3')-IIIa and the APH(2") activity of the bifunctional enzyme AAC(6')-APH(2"), with K_is in the sub-mM range. On the other hand staurosporine, an inhibitor of many Ser/Thr protein kinases as well as erbstatin and analogues of tyrphostin, potent inhibitors of protein Tyr kinases, were not inhibitors of APH(3')-IIIa or APH(2"). This successful initial pass at evaluating protein kinase inhibitors for inhibition of APHs augurs well for future work in this area.

6. EVOLUTION OF APHS

Aminoglycoside resistance determinants must have either been preexisting or co-evolved with aminoglycoside biosynthesis in antibiotic producing organisms. The three-dimensional structure of APH(3')-IIIa has presented the possibility that protein kinases and APHs share a common evolutionary origin. Furthermore, the fact that protein kinases and APHs share similar catalytic strategies also supports such a link. We have recently demonstrated that APHs under certain conditions can indeed act as Ser protein kinases (91). Aminoglycosides are produced primarily by actinomycetes or bacilli. In recent years, members of both of these families of organisms have been shown to encode eukaryotic-like Ser/Thr kinases. The coexistence of both APHs and protein kinases in antibiotic producing organisms places both these genes in the same context and provides a suggestive link between them. It is therefore not unreasonable to suggest that APHs and protein kinases evolved from a common ancestor despite the low overall amino acid sequence homology.

In addition, it is clear that based on the sequences of other antibiotic detoxifying enzymes such as viomycin and hydroxyurea kinases and their similarity to APHs (and thus protein kinases), that the kinase fold and catalytic mechanism (inferred) is one which exhibits broad general application in biology. The sequencing of whole bacterial genomes has already provided a wealth of new data on potential new aminoglycoside resistance proteins e.g. in mycobacteria, and APHs in particular. Current work on establishing the role(s) of some of these cryptic genes in bacterial metabolism will shed light not only on the evolution of the genes currently found in the clinics, but also will be of value in the prediction of the emergence of new resistance determinants in the future.

7. ACKNOWLEDGMENTS

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