CHARACTERIZATION OF AAC(6')-APH(2"), A BIFUNCTIONAL AMINOGLYCOSIDE MODIFYING ENZYME

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

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Doctor of Philosophy (2003)
(Biochemistry)

McMaster University
Hamilton, Ontario

TITLE: Characterization of AAC(6')-APH(2"), a bifunctional aminoglycoside-modifying enzyme

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Number of pages: xix, 187

ABSTRACT

AAC(6')-APH(2") is a bifunctional enzyme which catalyzes the inactivation of aminoglycoside antibiotics by ATP-dependent *O*-phosphorylation and acetyl coenzyme A-dependent *N*- and *O*-acetylation. It is the most prominent aminoglycoside-modifying enzyme found in gentamicin-resistant clinical isolates of Enterococci and Staphylococci. Although capable of inactivating all 2-deoxystreptamine aminoglycosides, gentamicin is the most clinically important substrate commonly administered in combination therapies to treat nosocomial Enterococcal infections. Gentamicin-resistant clinical isolates carrying *aac(6')-aph(2")* are characterized by high level resistance to gentamicins and other aminoglycosides.

In an attempt to obtain a more thorough understanding of it's functionalities and develop strategies to inhibit its' function *in vivo*, studies were initiated to characterize the substrate specificities, the regiospecificities of inactivation, and the potential for generation of lead compounds with inhibitory action towards AAC(6')-APH(2"). The studies reported herein, identify: i) two novel activities exhibited by AAC(6')-APH(2") (a Serine protein kinase activity exhibited by the phosphotransferase APH(2")-Ia and an O-acetyltransfer activity exhibited by the acetyltransferase AAC(6')-Ie, ii) the substrate structural requirements for enzyme/substrate interactions, iii) an unanticipated regiospecificity of inactivation of the 4,5-disubstituted aminoglycosides and iv) inhibition of the APH(2")-Ia by isoquinolinesulfonamide protein kinase inhibitors.

Acknowledgements:

I would like to thank my supervisor Dr. Gerard Wright for his guidance and his patience over the years and especially as it pertains to the writing of this thesis. I would also like to acknowledge the members of my supervisory committee, Dr. John Capone and especially Dr. David Andrews who was instrumental in my choice of graduate program and supervisor.

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ABBREVIATIONS

AAC	Aminoglycoside Acetyltransferase
ADP	Adenosine Diphosphate
AGAC	Aminoglycoside-Aminocyclitol
AGRP	Aminoglycoside Resistance Profile
ANT	Aminoglycoside Nucleotidyltransferase
APH	Aminoglycoside Phosphotransferase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CIP	Calf Intestinal Phosphatase
CKI-7	*N*-(2-aminoethyl)-5-chloroisoquinoline-8 Sulfonamide
CKI-8	1-(5-chloroisoquinoline-8-sulfonyl) Piperazine
Csk	C-Terminal Src Kinase
Da	Dalton
D-ala-D-ala	D-alanyl D-alanine
DEPC	Diethylpyrocarbonate
DHF	Dihydrofolate
DHFR	Dihydrofolate Reductase
DMS	Dimethyl Sulfate

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPG	*N*-dibenzylphospho-*N'*-3-(2,6-dichlorophenyl)-propylguanidine
DTDP	4,4'-dithiodipyridine
DTT	Dithiothreitol
EDP	Energy-dependent Phase
EDTA	*N,N,N',N'* Ethylenediaminetetraacetic Acid
EPK (or EPKs)	Eukaryotic Protein Kinase(s)
ESI-MS	Electrospray Ionization Mass Spectrometry
FPLC	Fast Protein Liquid Chromatography
H-7	1-(5-isoquinolinesulfonyl)-2-Methylpiperazine
H-9	*N*-(2-aminoehtyl)-5-isoquinoline Sulfonamide
HA-1004	*N*-(2guanidinoethyl)-5-isoquinoline Sulfonamide
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic Acid
HPLC	High Pressure Liquid Chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
k_{cat}/K_m	Specificity Constant
k_{cat}	Catalytic Constant
kDa	kiloDaltons

K_I	Substrate Inhibition Constant
K_{ii}	Intercept Inhibition Constant
Kis	Slope Inhibition Constant
K_m	Michaelis-Menten Constant
IgE	Immunoglobulin E
LDH	Lactate Dehydrogenase
LB	Luria Bertani Broth
MAPK	Mitogen-activated Protein Kinase
MARCKS K	Myristolated Alanine-Rich Protein Kinase-C Substrate (Lysine Rich)
MARCKS R	Myristolated Alanine-Rich Protein Kinase-C Substrate (Arginine Rich)
MBP	Myelin Basic Protein
MIC	Minimal Inhibitory Concentration
MOPS	3-Morpholinopropanesulfonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (Reduced form)
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PABA	para-Aminobenzoic Acid

PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PK	Pyruvate Kinase
PKA	cAMP-dependent Protein Kinase
PKC	Protein Kinase C
PKG	cGMP-dependent Protein Kinase
PMSF	Phenylmethylsulfonylfluoride
PSI	Pounds Per Square Inch
RBS	Ribosome Binding Site
RTFs	Resistance Transfer Factors
SAT	Serine Acetyltransferase
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel Electrophoresis
Spp.	Species
THF	Tetrahydrofolate
TLC	Thin-Layer Chromatography
Tris	Tris-(hydroxymethyl)aminomethane
U	Unit
V_m	Maximal Velocity
VRC	Vanadyl Ribonucleoside Complex

VRE	Vancomycin-resistant Enterococci

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PROTEIN DATABASE CODES

PDB Code	PDB Description	Reference
1CSN	Casein kinase-1 from *S. pombe* in complex with Mg^{2+}-ATP.	27
2CSN	Casein kinase-1 from *S. pombe* bound by the isoquinolinesulfonamide inhibitor CKI-7.	27
1J7L	APH(3')-IIIa from *E. faecalis* and *S. aureus* in complex with Mg^{2+}-ADP.	89
1L8T	APH(3')-IIIa from *E. faecalis* and *S. aureus* in complex with Mg^{2+}-ADP and kanamycin A.	57
1B87	AAC(6')-Ii from *E. faecium* in complex with acetyl coenzyme A.	217
1KNY	Kanamycin nucleotidyltransferase (ANT(4')-Ia) from *S. aureus* in complex with AMP-PCP and kanamycin A.	172
1BYT	Gentamicin C1a bound to a 27 nucleotide RNA molecule encompassing the 16S ribosomal RNA A-site.	220
1J7T	Paromomycin bound to a 27 nucleotide RNA molecule encompassing the 16S ribosomal RNA A-site.	58

PERSONAL PUBLICATIONS

1. Daigle, D.M., McKay, G.A. and Wright, G.D. (1997) Inhibition of Aminoglycoside Antibiotic Resistance Enzymes by Protein Kinase Inhibitors. *J. Biol. Chem.* **272(40)**: 24755-24758.

2. Daigle, D.M., McKay, G.A., Thompson, P.R. and Wright, G.D. (1999) Aminoglycoside Antibiotic Phosphotransferases are also Protein Kinases. *Chemistry and Biology* **6(1)**: 11-18.

3. Daigle, D.M., Hughes, D.W. and Wright, G.D. (1999) Prodigious Substrate Specificity of AAC(6')-APH(2"), an Aminoglycoside Antibiotic Resistance Determinant in Enterococci and Staphylococci. *Chemistry and Biology* **6(2)**: 99-110.

Chapter 1

Introduction

Chapter 1 - Introduction

1.1 Perspective

No one event in history exemplifies the human threat waged by bacteria more than the Black Death. Otherwise known as: "The Pestilence" or "The Great Mortality", this epidemic was caused by a bacterium, *Yersinia pestis*. In the space of four years, from 1346 to 1350, the bubonic plague raged across the Mediterranean, western and northern Europe killing an estimated twenty million people which represented nearly one third of the world population. Although not the first report of such an epidemic, this outbreak was historically amongst the most devastating. Since then, medical breakthroughs such as the development of aseptic surgical procedures and undoubtedly the discovery and use of antibiotics, have broadened the arsenal of therapeutic tools and subsequently prolonged human life. It only remains to be seen whether humans can keep the microbial world at bay.

1.2 The Discovery of the Microbial World

Even though bacteria were first seen by Anton van Leeuwenhoeck in 1674, it wasn't until 1840 that a German histologist Friedrich Gustav Jacob Henle published the theory that living micro-organisms caused infectious diseases [169]. Later, in 1850, a French parasitologist by the name of Casimir-Joseph Davaine, was the first to observe blood-borne organisms in diseased patients while showing communicable anthrax bacilli in the blood of inoculated sheep [184]. It wasn't however, until 1863, that French

microbiologist Louis Pasteur demonstrated that bacteria could cause disease. As a result, a modern medical revolution began which led to the discovery and development of antibiotics.

1.3 The Discovery and Development of Antibiotics

Early research on antibacterials focused primarily on synthetic compound libraries as sources for the "magic bullet", a term coined by Paul Ehrlich, which describes a drug capable of killing bacteria while having no effect on the human host. His work on arsenical compounds led to the discovery of the chemotherapeutic antibacterial drug salvarsan (Figure 1.1a) in 1909 [59]. Aside from the synthetic arsenicals, sulfonamides were introduced as chemotherapeutic agents in 1935 by Dogmak [59]. One of these compounds, prontosil (Figure 1.1b), was a dye which had antibacterial activity against infections caused by beta-hemolytic Streptococci [59]. It was later discovered that sulfanilamide (Figure 1.1c), a modification of prontosil formed in the body, was the active component of the drug [59]. Subsequent chemical modifications of the sulfonilamides gave compounds with even higher and broader antibacterial activity [59].

The antibiotic era encompasses only a very short period of history. In 1929, Alexander Fleming discovered a compound produced by the common mold *Penicillium notatum* called penicillin (Figure 1.1d) [59]. Unfortunately, his discovery remained undeveloped for more than 10 years, due to difficulties in purifying the compound. It wasn't until war raged in Europe in 1939 that two scientists, Ernst Chain and Howard Florey developed commercial purification scale-up methods to isolate penicillin [59,101].

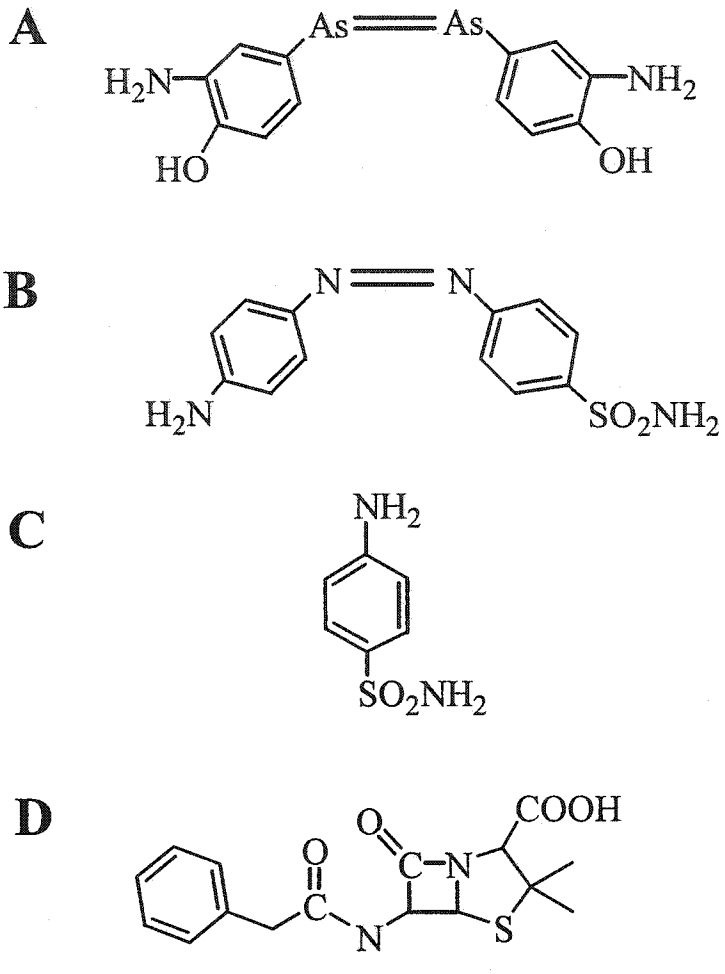


Figure 1.1: Structures of the first chemotherapeutic agents. **A**- salvarsan, **B**- prontosil, **C**- sulfanilamide the active molecule of prontosil and **D**- penicillin G.

All three scientists shared the Nobel Prize in Physiology or Medicine for this work in 1945.

1.4 Natural Products as Sources of Antibiotics

Antibiotics are biologically active bacterial metabolites [59]. It is not known why microorganisms produce antibiotics but it must be that these compounds provide a nutritional advantage in their habitat by antagonizing other organisms, or have an indirect action as a hormone or signaling molecule associated with cellular processes such as sporulation, dormancy or germination. Antibiotics are produced at the same time as cells begin the sporulation processes and tend to be rather large complicated organic molecules with molecular weights ranging from 300 to 1500 Da. These compounds can necessitate a multitude of enzymatic steps to synthesize requiring a substantial component of the bacterial genome for their synthesis. This would suggest that their production is important, if not essential, for the survival of these organisms in their environment.

The phenomenal success of penicillin led scientists away from synthetic compound screening and to search for other natural products from antibiotic-producing micro-organisms. One of the early successes was the discovery of streptomycin (Fig 1.12) from the soil actinomycete *Streptomyces griseus* [62]. While fungi do produce antibiotics, the vast majority of drugs are produced by Gram-Positive sporulating bacteria such as *Streptomyces*, *Micomonospora* and certain *Bacillus* species. Apart from penicillin, the most important antibiotics from fungi are the cephalosporins, amphotericin B and griseofulvin (Figure 1.2) (from *Penicillium griseofulvum* and related species), the

latter of which is used to treat athlete's foot and related fungal infections of the skin. The vast majority of clinically useful antibiotics have been obtained from *Streptomyces* species.

1.5 Classes of Antimicrobial Agents and their Modes of Action

All antibiotics target one of five processes: i) metabolism, ii) nucleic acid synthesis, iii) cell membrane integrity, iv) cell wall biosynthesis and v) the protein synthesis machinery. The majority of clinically effective compounds target the former two processes (Figure 1.3).

1.5.1 Drugs that Target Metabolism, Nucleic Acid Synthesis and Cell Membrane Integrity

Drugs which target bacterial metabolic enzymes are mostly synthetic chemotherapeutic agents. They all target metabolic enzymes in the pathway leading to the production of tetrahydrofolate (THF).

Both the sulfonamides (Figure 1.1c) [3] and trimethoprim (Figure 1.4a) [24] are inhibitors of bacterial enzymes required for the synthesis of THF, the vitamin form of folic acid which is essential for 1-carbon transfer reactions. Sulfonamides are structurally similar to para-aminobenzoic acid (PABA) (Figure 1.4b), the substrate of dihydropteroate synthase in the THF pathway, and competitively inhibit this enzyme [3]. Trimethoprim is structurally similar to dihydrofolate (DHF) (Figure 1.4c) and competitively inhibits the second step in THF synthesis mediated by dihydrofolate reductase (DHFR) [24].

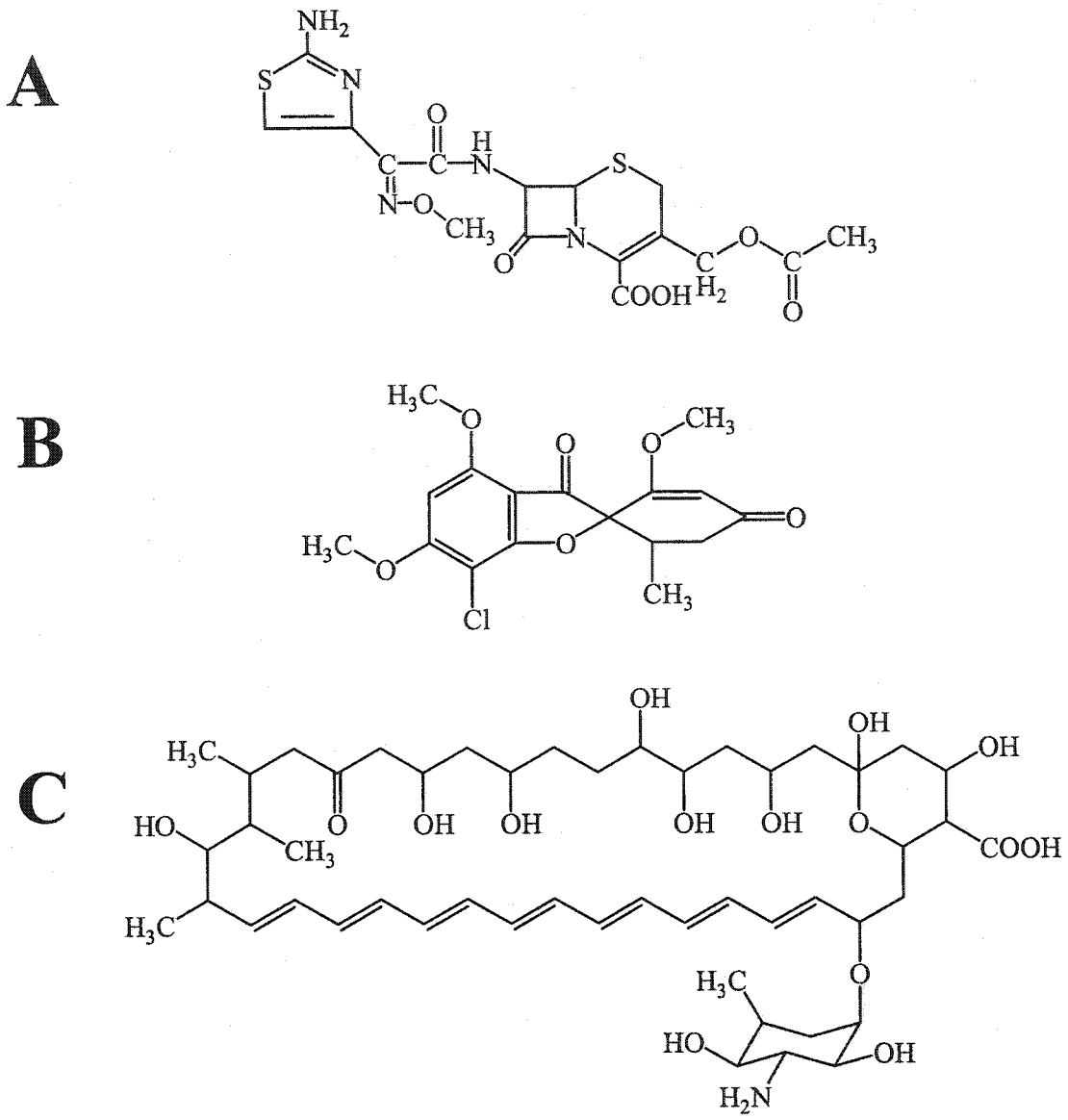


Figure 1.2: Structures of antibiotics from fungi. **A**- cefotaxime (semi-synthetic cephalosporin), **B**- griseofulvin, and **C**- amphotericin B.

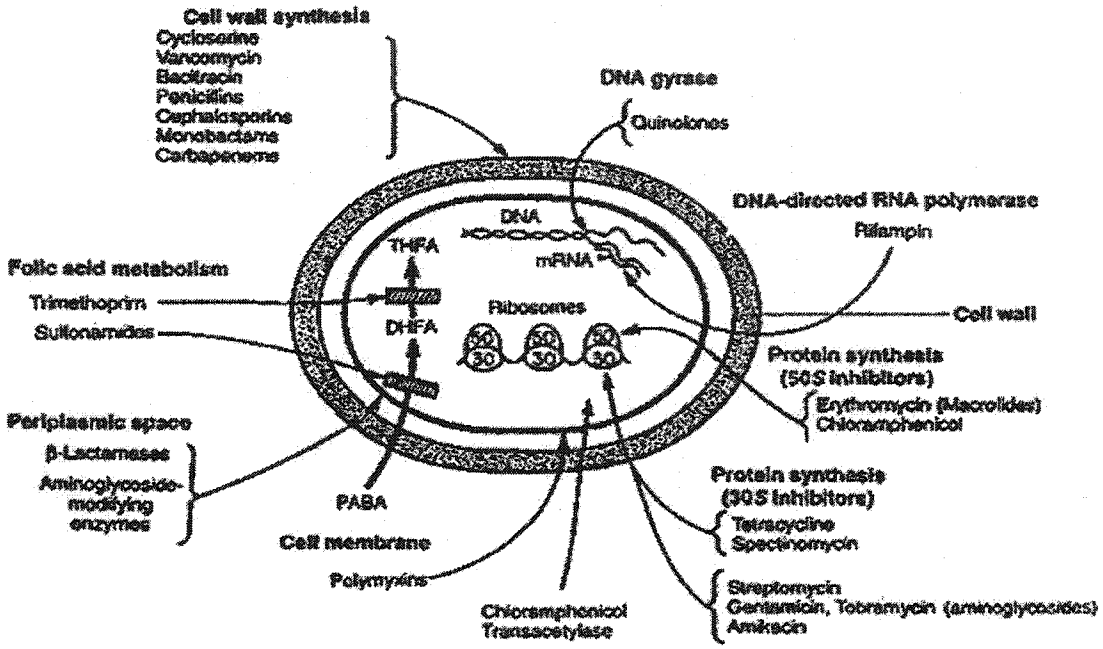


Figure 1.3: Targets of various antibiotics. Figure copied with copyright permission from [147].

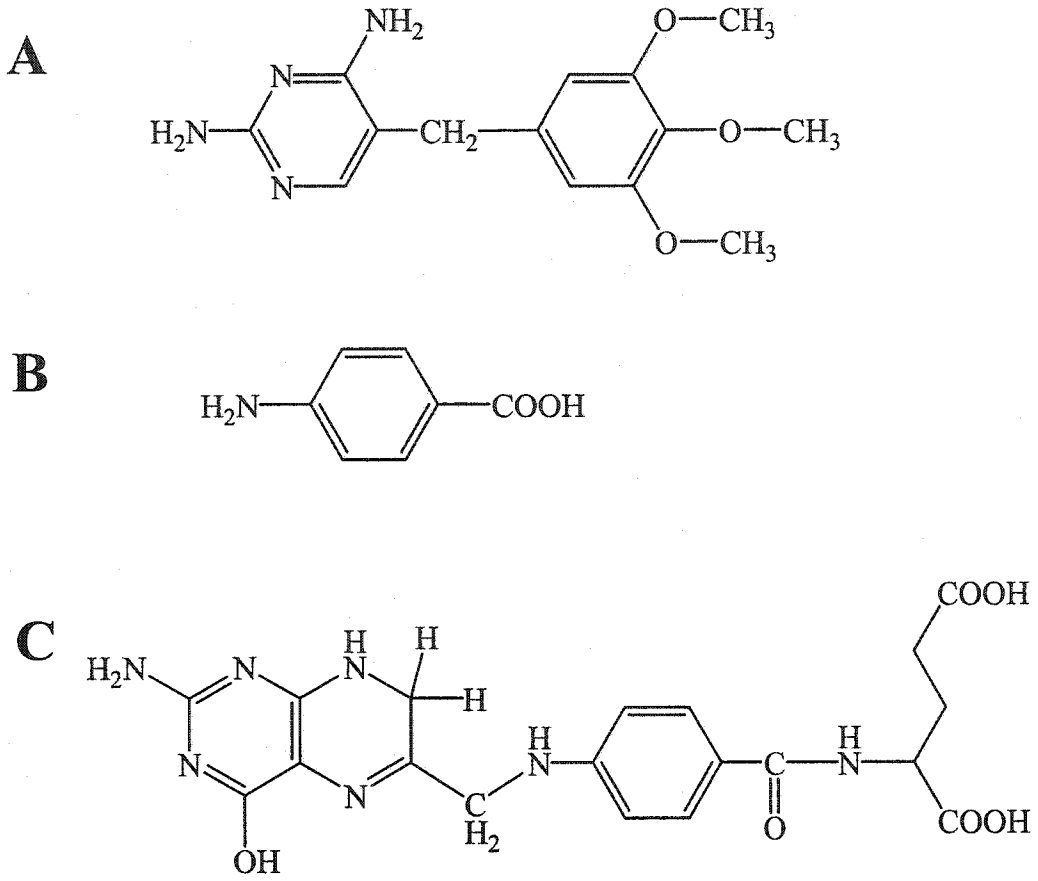


Figure 1.4: Structures of **A**- trimethoprim, **B**- para-aminobenzoic acid (PABA), and **C**- dihydrofolic acid.

As animal cells do not synthesize their own folic acid, these compounds are selective for bacteria.

Some chemotherapeutic agents affect the synthesis of or bind to DNA or RNA and inhibit nucleic acid synthesis. These drugs are generally non-selective and thus have little therapeutic application since they affect both eukaryotic and prokaryotic cells. Two nucleic acid synthesis inhibitors which have selective activity against prokaryotes and some clinical usefulness are nalidixic acid [74,189] and rifamycins [207].

Nalidixic acid (Figure 1.5a) is a narrow spectrum synthetic antibiotic which has activity mainly against Gram-negative bacteria [74]. Although no longer in clinical use because of bacterial resistance, naladixic acid has been replaced by several new fluoroquinolones, one of which is ciprofloxacin (Figure 1.5c). These compounds exhibit their bactericidal action by inhibiting the DNA gyrase enzyme (topoisomerase II), essential for DNA replication [189].

The rifamycins are products of *Streptomyces*. Rifampicin (Figure 1.5b), the best known rifamycin, is a semisynthetic derivative that is active against some Gram-negative bacteria and Gram-positives including *Mycobacterium tuberculosis* [207]. It acts specifically on bacterial RNA polymerase by binding to the beta-subunit and blocking entry of the first nucleotide inhibiting mRNA synthesis [207].

Some antibiotics disrupt the structural integrity of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital for the survival of bacteria. Similarities in the phospholipid content of eubacteria and eukaryotic membranes results

in limited specificity of these compounds. The only effective antibiotic of clinical importance that acts by this mechanism is polymyxin (Figure 1.5d). Produced by *Bacillus polymyxis*, polymyxin is mainly effective against Gram-negative bacteria and is usually restricted to topical usage [110]. The drug affects the structure and function of membranes and cell integrity by binding to membrane phospholipids [110]. As a result of low specificity, there is high human toxicity associated with this antibiotic [110].

Finally, gramicidin (Figure 1.5e), a non-ribosomally synthesized oligopeptide antibiotic secreted by *Bacillus brevis*, was among the first antibiotics commercially manufactured [107]. The drug forms a transmembrane ion channel through the outer membrane of susceptible bacteria which alters cytoplasmic membrane function [107]. It is particularly effective against Gram-positive organisms but due to high toxicity, it has been limited to topical applications [107].

1.5.2 Drugs that Target Cell Wall Biosynthesis

Antibiotics that target cell wall synthesis include beta-lactams, bacitracin, cycloserine and glycopeptides. Cell wall synthesis inhibitors generally inhibit a step in the synthesis of peptidoglycan and usually show selective toxicity against eubacteria.

The Beta-lactams are composed of a 4-membered beta-lactam ring. The penicillins (Figure 1.1d and Figure 1.6) and cephalosporins (Figure 1.2a) are two classes of beta-lactams produced by two groups of fungi, *Penicillium* and *Cephalosporium* molds respectively [60]. The basis for their inhibitory activity lies in the fact that beta-lactams are stereochemically related to D-alanyl-D-alanine which is a substrate for the

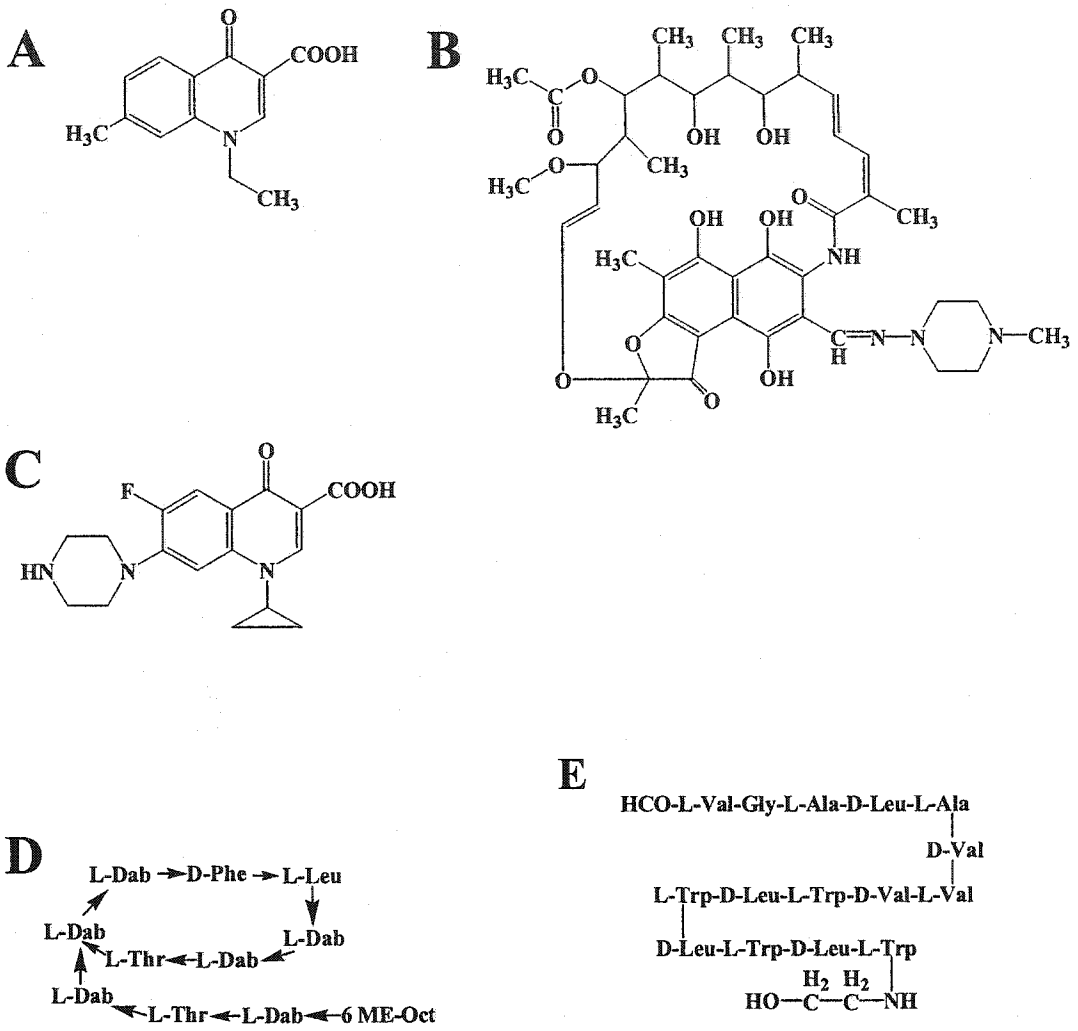


Figure 1.5: Structures of drugs that target nucleic acid synthesis and membrane integrity. **A**- naladixic acid, **B**- rifampicin, **C**- ciprofloxacin, **D**- polymyxin and **E**- gramicidin.

last step in peptidoglycan biosynthesis [102]. This enables beta-lactams to bind to and inhibit the carboxypeptidase and transpeptidase enzymes that are required for the synthesis of peptidoglycan [102]. These drugs are normally bactericidal and require that cells be actively growing in order to exert their toxicity [102].

The advent of semisynthetic penicillins in 1959 broadened the therapeutic applications of cell wall antibiotics, converting the natural penicillins from narrow spectrum drugs (effective against few bacterial species), to broad spectrum antibiotics. Amoxicillin and ampicillin are active against Gram-negatives while methicillin was designed as a penicillinase-resistant semisynthetic beta-lactam (Figure 1.6) [104]. Clavulanic acid (Figure 1.6d), a naturally occurring inhibitor of beta-lactamases, is often added to these semisynthetic penicillin preparations known as clavamox or augmentin [105]. Although non-toxic, penicillins have been known to generate deadly allergic responses in certain patients [103]. It has been shown that in allergic individuals, the beta-lactam molecule attaches to a serum protein which initiates an IgE-mediated inflammatory response [Reviewed in 7].

Cephalosporins are broad spectrum beta-lactam antibiotics with a similar mode of action to penicillins [106]. While they are subject to degradation by some bacterial beta-lactamases, they tend to be resistant to beta-lactamases from *Staphylococcus aureus* [106]. There are two other classes of beta-lactams: i) the carbapenems (Figure 1.7a) and ii) the monobactams (Figure 1.7b) [106]. The latter are particularly useful for the

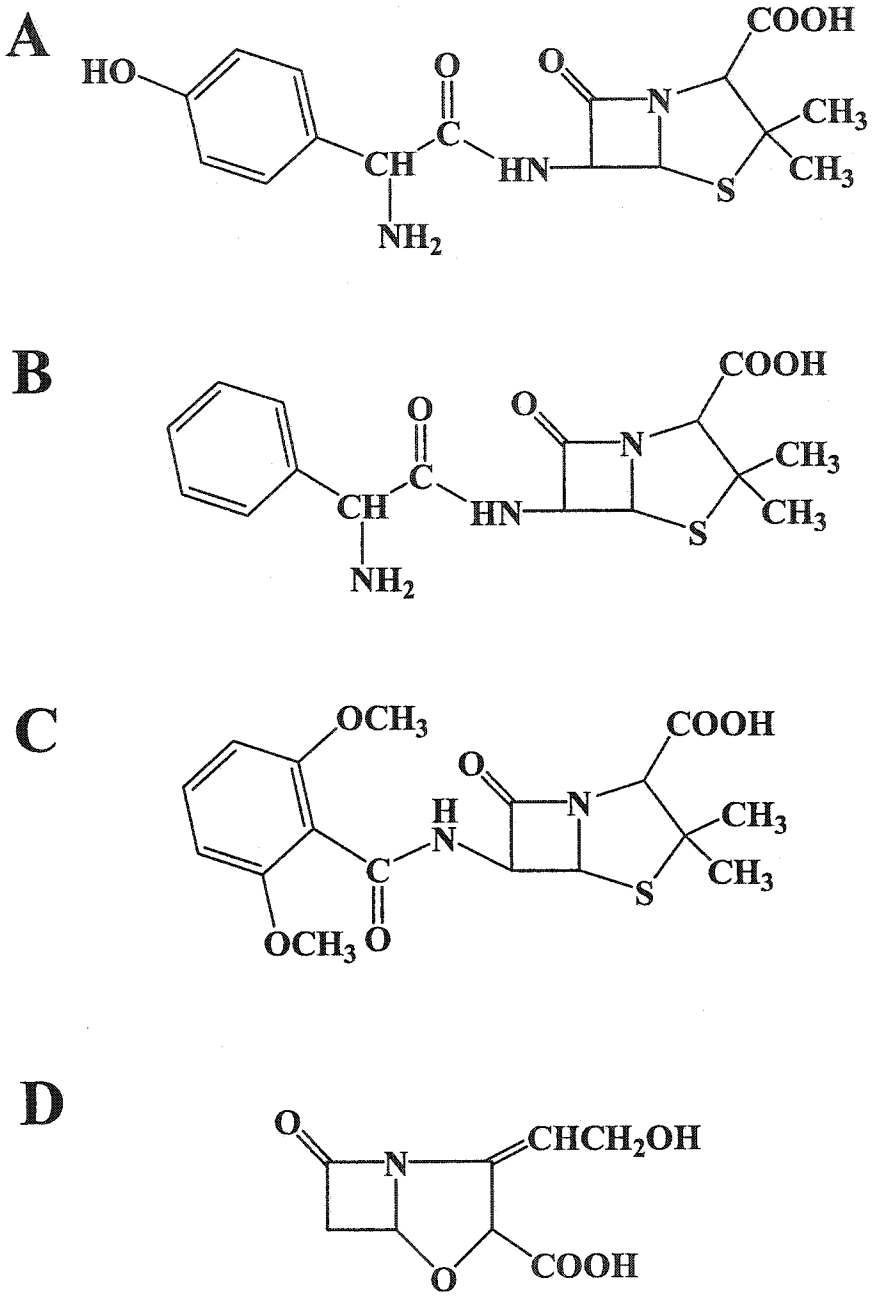


Figure 1.6: Structures of Beta-lactam antiobiotics targeting cell wall biosynthesis and a Beta-lactamase inhibitor. **A**- amoxicillin, **B**- ampicillin, **C**- methicillin, and **D**- clavulanic acid (specific inhibitor of bacterial Beta-lactamases).

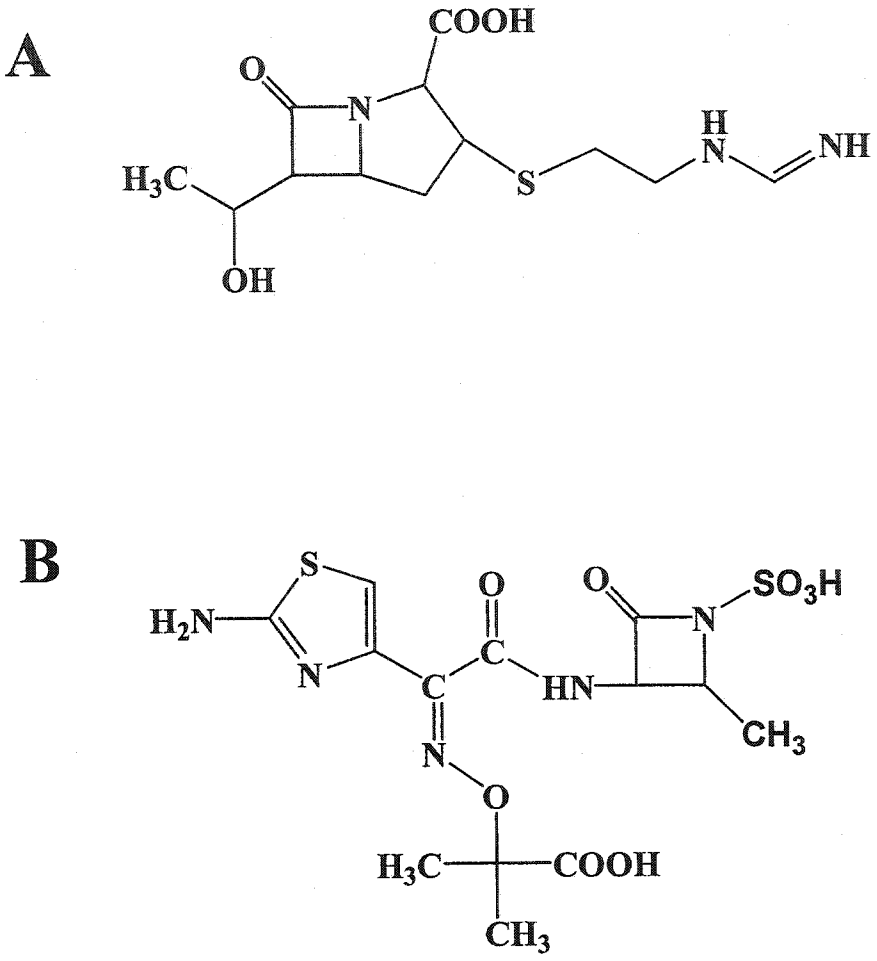


Figure 1.7: Structures of a monobactam and a carbapenem that target cell wall synthesis. **A**- imipenem, a carbapenem, and **B**- aztreonam, a monobactam antibiotic.

treatment of allergic individuals [106].

Bacitracin (Figure 1.8a) is a polypeptide antibiotic produced by *Bacillus* species. It prevents cell wall growth by inhibiting the release of the muropeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane [107]. Teichoic acid biosynthesis in Gram-positive bacteria is also inhibited because it requires the same carrier [107]. Bacitracin is present in many topical antibiotic preparations due in part to its high toxicity.

Cycloserine (Figure 1.8b) inhibits the early stages of murein synthesis when D-alanyl-D-alanine is added to the growing peptide chain on peptidoglycan [112]. The antibiotic resembles D-alanine and competitively inhibits both the racemase reaction that converts L-alanine to D-alanine and the synthetase reaction that joins two D-alanine molecules together [112]. Even though cycloserine is actively transported and its affinity for these enzymes is about a hundred times greater than that of D-alanine, it is quite toxic resulting in its limited use [112].

Glycopeptides, such as vancomycin (Figure 1.8c), form complexes with D-alanyl D-alanine and inhibit both transglycosylation and transpeptidation reactions during peptidoglycan synthesis [111]. Vancomycin, while ineffective against Gram-negative bacteria, has become an important clinical drug for treatment of infections by strains of *Staphylococcus aureus* that are resistant to virtually all other antibiotics [111].

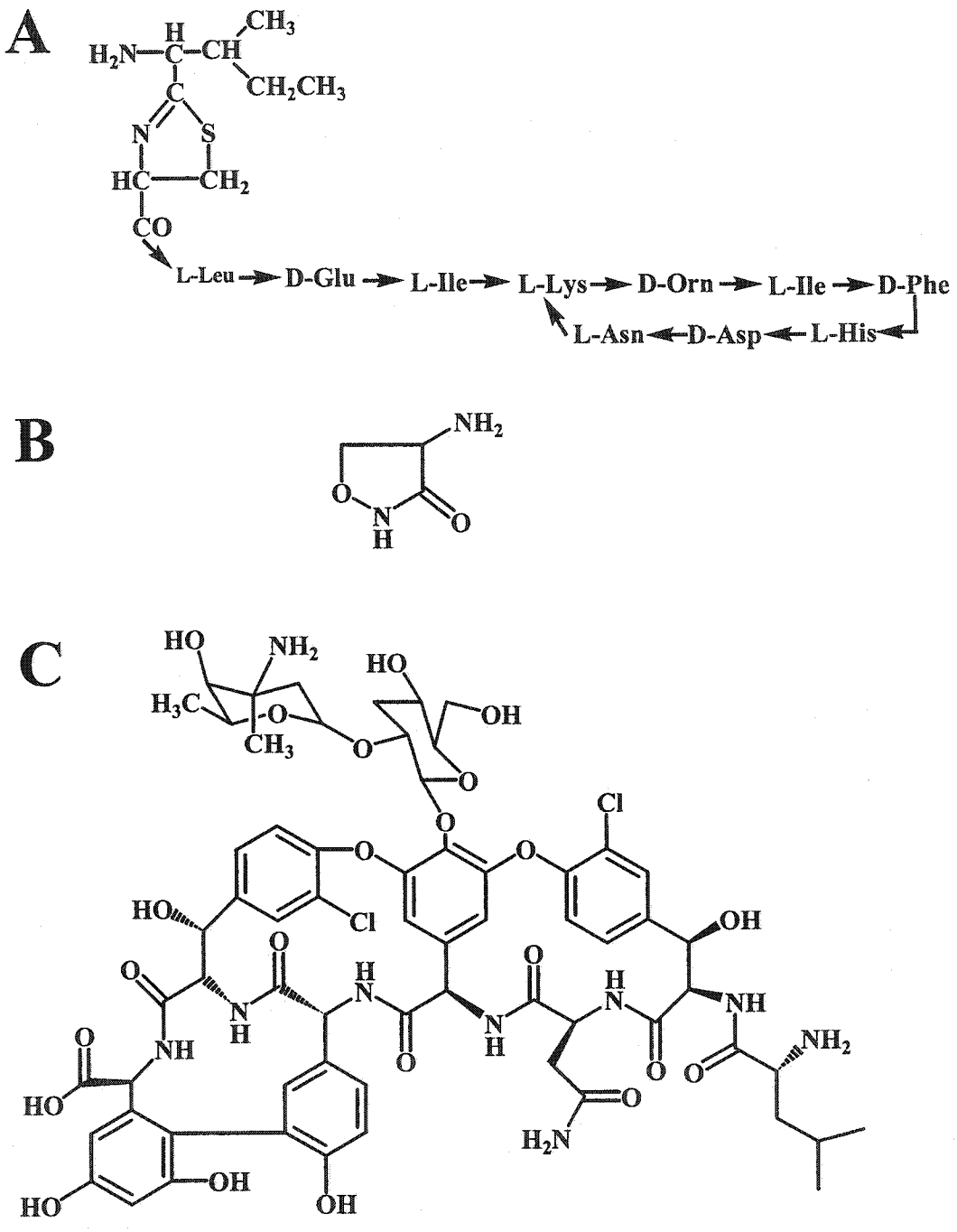


Figure 1.8: Structures of other antibiotics that target cell wall biosynthesis. A- bacitracin, B- cycloserine, and C- vancomycin.

1.5.3 Drugs that Target the Protein Synthesis Machinery

Many clinically useful antibiotics inhibit a step in protein synthesis. Most have an affinity for prokaryotic 70S (as opposed to eukaryotic 80S) ribosomes, and achieve their selective toxicity in this manner. The most important antibiotics in this class are the tetracyclines, chloramphenicol, the macrolides and the aminoglycosides (discussed in section 1.6).

The tetracyclines are almost all natural products of *Streptomyces* species with the exception of semi-synthetic derivatives. Tetracycline (Figure 1.9a) and doxycycline are the best known and are broad-spectrum antibiotics with a wide range of activity against both Gram-positive and Gram-negative bacteria [63]. They inhibit protein synthesis by blocking the binding of aminoacyl tRNA to the A site on ribosomes [17]. The low toxicity and broad spectrum of activity of tetracyclines led to overuse of these drugs. Today, resistance to tetracylines is wide-spread [168].

Chloramphenicol (Figure 1.9b) was originally discovered and purified from the fermentation of *Streptomyces venezuelae* [108]. It is a bacteriostatic broad spectrum antibiotic that binds to the 50S ribosomal subunit where it prevents docking of the aminoacyl tRNA inhibiting the peptidyl transferase and preventing growth of the polypeptide chain [108].

The macrolide antibiotics are composed of large lactone rings linked through glycosidic bonds with amino sugars. The best-known macrolide is erythromycin (Figure

1.9c). Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit, and inhibiting elongation by the peptidyl transferase [109].

1.6 Aminoglycoside-aminocyclitols

One of the first classes of antibiotics discovered were the aminoglycoside-aminocyclitols (AGAC). The first of which, streptomycin (Figure 1.12), discovered in 1943 by Waksman, was introduced in clinics in 1944 [203]. This was the first antibiotic used to treat patients afflicted with tuberculosis. What followed was a rapid succession of discovery and application of many other clinically useful aminoglycosides such as kanamycin (Figure 1.10), isolated in 1957 from *Streptomyces kanamyceticus* [61,85], the aminoglycoside of choice in the late 1950's. In 1964, gentamicins (Figure 1.10) were isolated from *Micromonospora purpurea* [2], and have since found extensive use for the treatment of both Gram-positive and Gram-negative nosocomial infections. These were followed by tobramycin in 1968 from *Streptomyces tenebraeius* [49], amikacin in 1972, a semisynthetic derivative of kanamycin A [162], and netilmicin in 1976, a semisynthetic derivative of sisomicin (Figure 1.10) [97]. Most aminoglycosides originate from *Streptomyces* species, however, some like the gentamicins and sisomicin are from *Micromonospora* species and for this reason, there is a difference in spelling from –ycin in the case of kanamycin and tobramycin, to –icin for gentamicins and sisomicin. This characteristic is also observed for butirosin, an aminoglycoside which originates from *Bacillus brevis* [61].

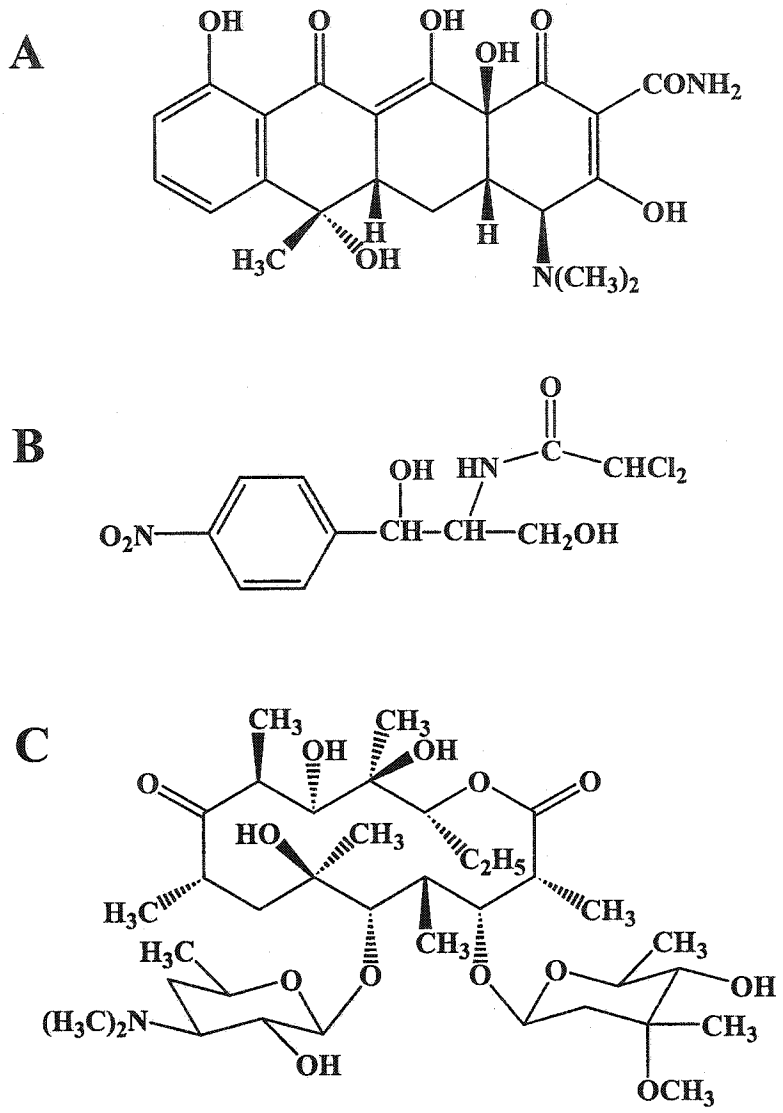


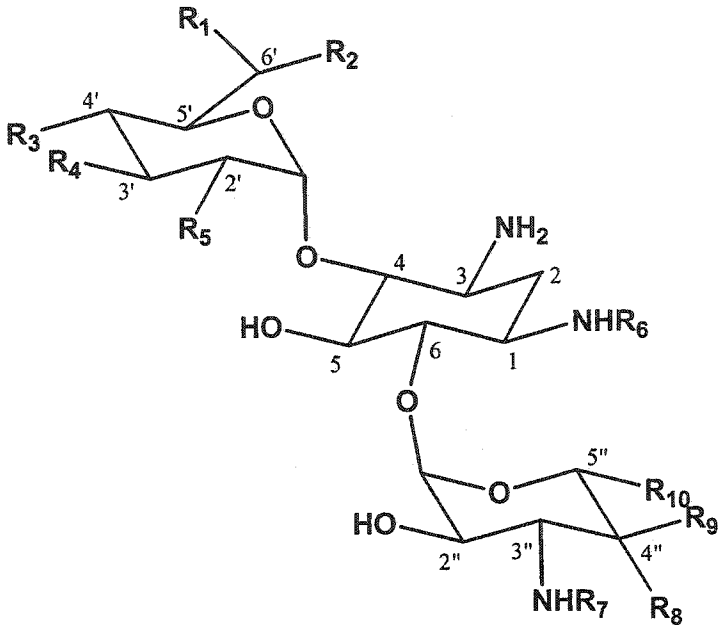
Figure 1.9: Structures of well-known antibiotics which target protein synthesis. **A**- tetracycline, **B**- chloramphenicol, and **C**- erythromycin.

Gentamicin (a mixture of 3 components, C1, C1a and C2) is active against many strains of Gram-positive and Gram-negative bacteria, including some strains of *Pseudomonas aeruginosa* [61]. Kanamycin is active at low concentrations against many Gram-positive bacteria, including penicillin-resistant Staphylococci [61].

These antibiotics are highly potent, broad-spectrum drugs consisting of a central aminocyclitol ring (hexose nucleus - ring B) bridging two amino sugars (rings A and C) [61]. The aminocyclitol ring in most clinically relevant aminoglycosides is 2-deoxystreptamine, whereas in streptomycin it is streptidine [61]. The aminosugars are linked by glycosidic bonds and ring positioning is defined by an established numbering system in which ring A is denoted in positions 1' through 6', ring B is 1 through 5, while ring C is 1" through 5". Additional rings take on an added prime denotation such as 1''' and 1'''' (Figure 1.10 and 1.11) [61].

There are three classes of AGAC's, i) the 4,6-disubstituted deoxystreptamine aminoglycosides which include: amikacin, gentamicin and dibekacin (Figure 1.10); ii) the 4,5-disubstituted deoxystreptamine aminoglycoside antibiotics which include: neomycin, and butirosin (Figure 1.11); and a third class including: streptomycin, spectinomycin apramycin, and hygromycin B (Figure 1.12). The 4,6- and 4,5- classification denotes the ring substitutions on the central 2-deoxystreptamine ring, while the third class contains drugs which are excluded from the first two classifications.

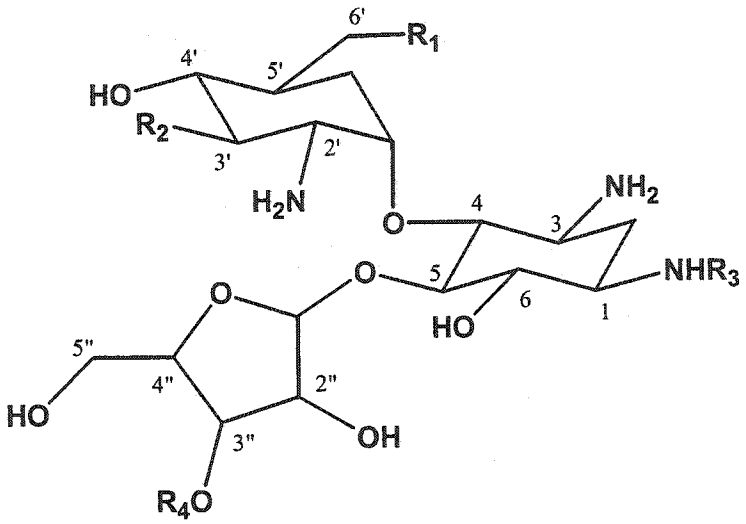
The toxicity of many antibiotics including aminoglycosides has long been known to be a problem. Numerous compounds demonstrate antibiotic activity, but only a limited few are clinically useful due in most part to human toxicity. Prolonged use of



	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
gentamicin C1	CH_3	$NHCH_3$	H	H	NH_2	H	CH_3	OH	CH_3	H
gentamicin C1a	H	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
gentamicin C2	CH_3	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
kanamycin A	H	NH_2	OH	OH	OH	H	H	H	OH	CH_2OH
kanamycin B	H	NH_2	OH	OH	NH_2	H	H	H	OH	CH_2OH
tobramycin	H	NH_2	H	OH	NH_2	H	H	H	OH	CH_2OH
dibekacin	H	NH_2	H	H	NH_2	H	H	H	OH	CH_2OH
netilmicin	H	NH_2	H	H	NH_2	X	CH_3	OH	CH_3	H
amikacin	H	NH_2	OH	OH	OH	Y	H	H	OH	CH_2OH

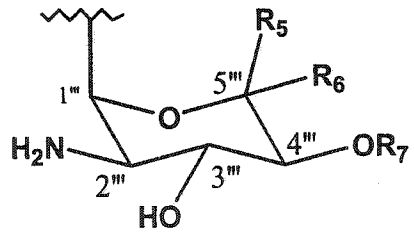
$X = CH_2CH_3$ $Y = COCH(OH)\text{-}CH_2CH_2NH_2$

Figure 1.10: Structures and annotation of the 4,6-deoxystreptamine aminoglycoside antibiotics.



	R1	R2	R3	R4	R5	R6	R7
neomycin B	NH_2	OH	H	X	H	CH_2OH	H
neomycin C	NH_2	OH	H	X	CH_2NH_2	H	H
paromomycin	OH	OH	H	X	H	CH_2NH_2	H
lividomycin A	OH	H	H	X	H	CH_2NH_2	H
lividomycin B	OH	H	H	X	H	CH_2NH_2	mannose
butirosin	NH_2	OH	Y	H	absent	absent	absent
ribostamicin	NH_2	OH	H	H	absent	absent	absent

X =



$Y = COCH(OH)CH_2CH_2NH_2$

Figure 1.11: Structures and annotation of the 4,5-deoxystreptamine aminoglycoside antibiotics.

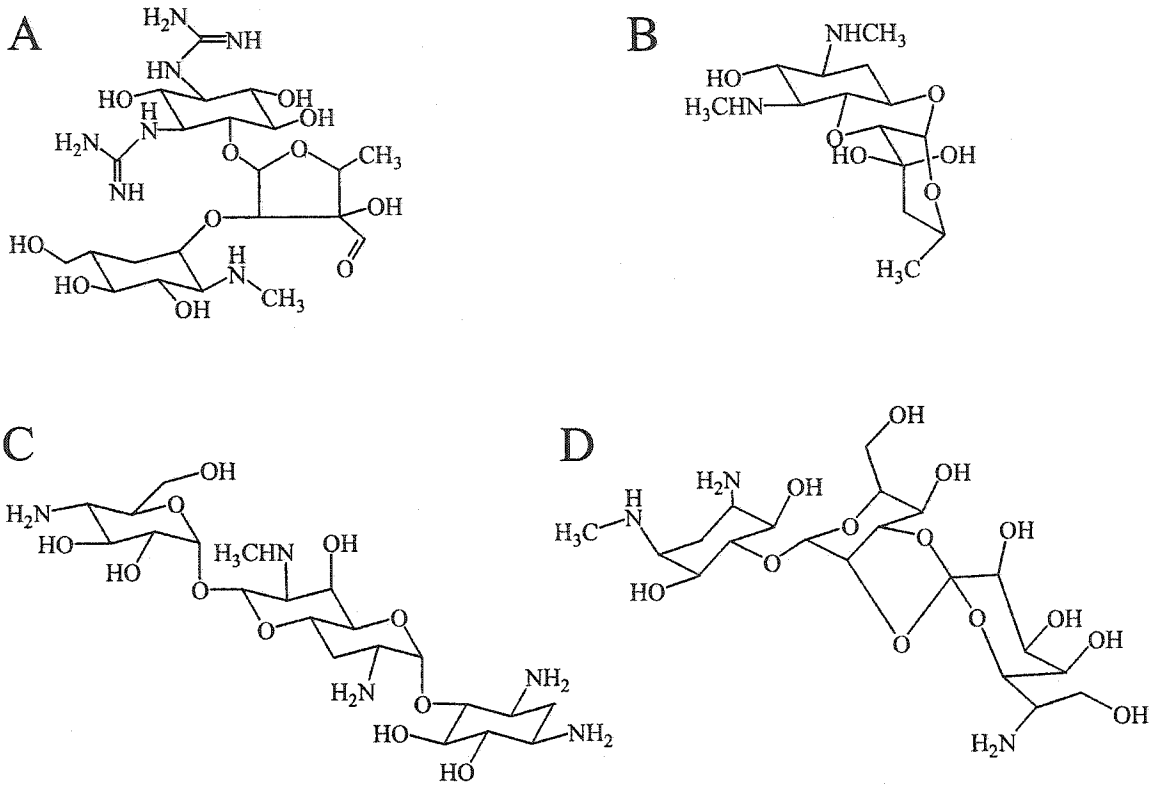


Figure 1.12: Structures of other aminoglycosides. **A**- streptomycin, **B**- spectinomycin, **C**- apramycin, and **D**- hygromycin.

aminoglycosides is known to impair kidney function (nephrotoxic) [178,204] and cause damage to the auditory nerves (ototoxic) leading to deafness [93]. Despite this toxicity, aminoglycosides are still highly employed in combination therapies against serious nosocomial Enterococcal and Staphylococcal infections [222].

1.7 Aminoglycoside Entry into Cells and Mode of Action

Once administered, these small highly cationic molecules must traverse the negatively charged cell wall to enter the bacterial cells and exert their bactericidal action. Observations in *E. coli* [21,79], *P. aeruginosa* [21] and *S. aureus* [70,140] with streptomycin and gentamicin, have demonstrated that aminoglycosides have a three-stage entry process into bacterial cells. The first step is rapid adsorption to cell surface Mg^{2+}-binding sites on lipopolysaccharides by ionic and non-covalent interactions in a drug concentration-dependent manner [79]. This is followed by a slow and irreversible uptake of small amounts of drug in an energy-dependent fashion driven by the proton motive force (proton gradient) across the membrane termed energy-dependent phase I (EDP-I) [20,79]. EDP-I is the rate-limiting step in the transport process and is reversibly blocked by divalent cations [8,22], hyperosmolarity and electron transport inhibitors [22,140]. The final step, termed energy-dependent phase II (EDP-II), is characterized by an increased level of entry of the drug into cells [20,79]. As in the case of EDP-I, drug entry during EDP-II is irreversible, however EDP-II is also characterized by inhibition of protein synthesis and loss of cell viability [20]. Comparison of aminoglycoside effects on resistant and sensitive strains demonstrated that membrane integrity was altered in the former [22]. This finding

remained controversial as most believed that the effects on the ribosome resulted in the bacteriocidal action of these drugs. The contribution of membrane damage to the bacteriocidal action of aminoglycosides was not fully appreciated until it was coupled to mistranslation on the ribosome.

Aminoglycosides have two principle effects on bacterial cells. The first is to induce errors in translation during the EDP-I stage when small amounts of drug enter the cells and reduce translational fidelity resulting in the production of aberrant and mistranslated proteins [45]. The aberrant mistranslated proteins are believed to insert into the cell membrane and contribute to membrane damage as well as further drug entry leading to EDP-II [26]. Evidence for this phenomenon comes from experiments where aminoglycoside-sensitive cells treated with aminoglycosides failed to export alkaline phosphatase and had the protein localized in the membrane fraction [46]. The second effect of aminoglycosides coincides with EDP-II when an increased level of drug enters the cell and reaches a threshold concentration which causes total inhibition of protein synthesis [45]. Unlike other drugs which act on bacterial ribosomes such as tetracycline and chloramphenicol, aminoglycosides are bactericidal [45]. The precise bactericidal action exhibited by aminoglycosides has not been established yet and remains a controversial topic, although bacterial membrane damage does appear to be essential. Studies performed with puromycin, a nucleoside antibiotic that causes premature chain termination by acting as an analog of the 3' terminal end of aminoacyl-tRNA, have demonstrated that EDP-I can be bypassed by increased membrane damage induced by this compound, thus implicating the

importance of a membrane damage component to the bactericidal action of aminoglycosides [70].

As suspected very early on, aminoglycosides act on bacterial ribosomes. Using *in vivo* ^{35}S-methionine labeling, bacterial cells treated with aminoglycosides have been shown to have reduced protein levels, suggesting an impact on protein synthesis [46]. While early reports suggested that these drugs interacted with ribosomal proteins, it was later discovered through chemical footprinting [143,211], structural studies by NMR of aminoglycosides bound to synthetic RNA molecules [58,220], and an X-ray structure of paromomycin bound to 30S ribosomes [28], that they specifically bind to 16S rRNA in the 30S ribosomal subunit A-site. These drugs interfere with bacterial protein translation by causing misreading of the genetic code and inhibiting translocation [45]. The proofreading errors are believed to occur because the drugs decrease the dissociation rates of aminoacyl tRNA's from the ribosomal A-site [220].

Details of the specific binding site of aminoglycosides on 16S ribosomal RNA was obtained by NMR solution structures of paromomycin and gentamicin C1a bound to a synthetic 27 nucleotide oligomer encompassing the A-site decoding region of bacterial 30S ribosomal subunits (Figure 1.13) [58,220]. The drugs bind to the synthetic RNA in the major groove of the model A-site formed by the combination of an adenosine-adenosine base pair (A^{1408}-A^{1493}) and a bulged adenosine (A^{1492}) and appear to be maintained by a combination of proper conformation and electrostatic interactions (Figure 1.13) [58,220]. RNA/drug interactions for both aminoglycosides are driven by rings I and II of the drugs

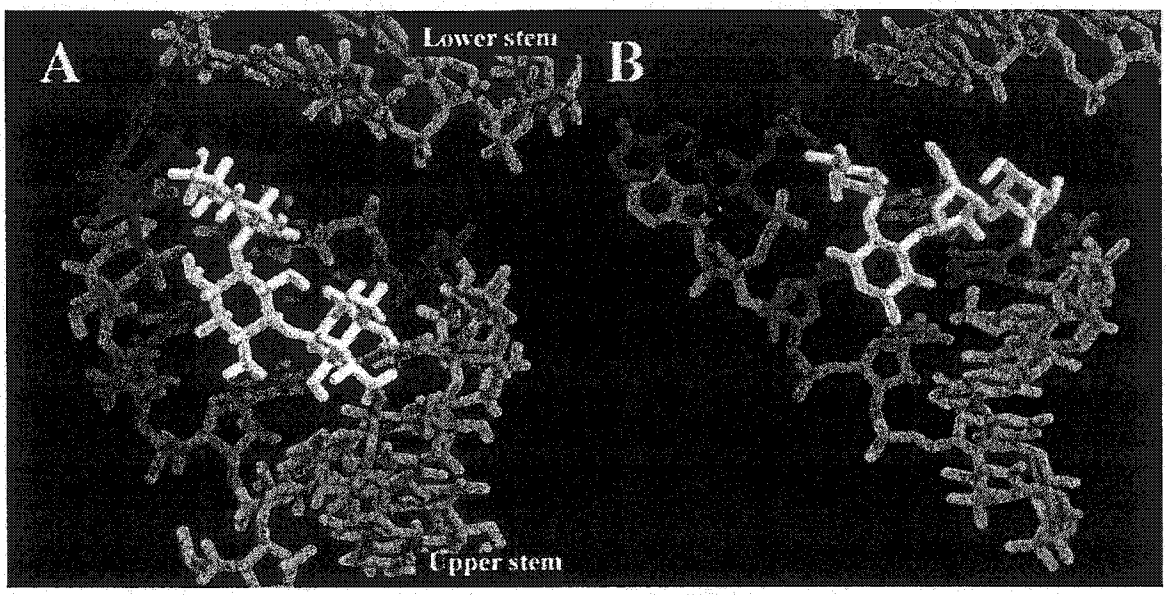


Figure 1.13: Structures of gentamicin C1a and paromomycin bound to a 27 nucleotide RNA molecule encompassing the 16S ribosomal RNA A-site. **A**- gentamicin C1a-RNA complex. **B**- paromomycin-RNA complex. Highlighted for both structures are: i) the aminoglycoside in white, ii) the bulged adenosine A^{1492} is red, iii) the A^{1408}-A^{1493} base pair is green, iv) the C^{1407}-G^{1494} base pair is purple, v) the C^{1409}-G^{1491} base pair is blue, the G^{1405}-C^{1496} base pair is cyan, and vi) U^{1495} is red-orange. The upper and lower stems of the modeled 16S ribosomal RNA A-site are labeled. (PDB code 1BYT for the gentamicin-RNA complex and 1J7T for the paromomycin-RNA complex. The image was created using RasMol v2.6 [175].

which are similarly oriented in the structures and use an analogous hydrogen bonding network [58,220]. One striking difference between the two structures is the fact that ring III of gentamicin C1a directs specific interactions with the upper stem of the RNA A-site whereas with paromomycin, rings III and IV are oriented differently due to a different position of the ring III substitution and only weakly contribute to binding of the aminoglycoside to the lower stem of the RNA A-site by non-specific interactions (Figure 1.13) [58,220]. This unique feature may explain the increased clinical effectiveness of the 4,6-disubstituted class of aminoglycosides. Even though the binding of rings III and IV of paromomycin is different from that of ring III of gentamicin C1a, both drugs protect the same bases from chemical modification by dimethyl sulfate (DMS) [220].

Critical nucleotides in the A-site include the C^{1407}•G^{1494} base pair, the A^{1408}-A^{1493} base pair, the G^{1405}-C^{1496} base pair and U^{1495} (Figure 1.13) [58,220]. The orientation of ring I is facilitated by a drug-drug hydrogen bond between ring I and ring III [58]. Rings II, III and IV of paromomycin have a linear arrangement that lines the major groove [58]. The specificity of aminoglycosides for bacterial 70S versus eukaryotic 80S ribosomes is based on the fact that the C^{1407}-G^{1494} base pair and the A^{1408}-A^{1493} base pairs are required for aminoglycoside binding as methylation of G^{1494} or A^{1408} prevents this interaction and base changing A^{1408} to G^{1408} which is found in eukaryotic ribosomes results in a 15-fold lowering of sensitivity to the drugs [127]. Adding to the G^{1408} modification, there is a base pair between residues 1409 and 1491 (Figure 1.13) that forms the lower support for the antibiotic binding pocket which is mispaired in higher eukaryotes and contributes to the bacterial specificity of aminoglycosides [127]. The additional rings past ring II appear to contribute

to the binding affinity of the drug for the ribosomal RNA as well as in assisting in the proper orientation of rings I and II through the formation of drug-RNA and/or drug-drug interactions [220].

Changes in the conformation of ribosomal RNA induced by aminoglycoside binding has led to the suggestion that the aminoglycoside-RNA complexes, which cause miscoding, are in a high affinity state for mRNA-tRNA recognition in the A-site, explaining the decrease in dissociation rates of aminoacyl-tRNAs in the A-site which have previously been observed.

1.8 Emergence of Bacterial Resistance

Antibiotic resistance is not a recent phenomenon. The period from the late 1940s to the early 1950s was characterized by the discovery and introduction of a plethora of natural compound antibiotics which ushered in the age of antibiotic chemotherapy. However, contrary to early expectations, antibiotics were not as robust as originally believed. Clinical antibiotic resistance emerged soon after introduction of the drugs. In fact, one year after the first applications of streptomycin, within two years of penicillin use, and one year after the introduction of methicillin, resistance had become apparent [203]. Fluoroquinolones, which were extensively used to treat nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA), had lost most of their clinical effectiveness within a year of use [158]. The situation in some cases has become alarming, with the emergence of pathogenic strains of Gram-positive cocci, which are major sources of nosocomial infections [196], that are highly resistant to several clinically important drugs

such as gentamicins and vancomycin. One of the most important examples is multiple-resistant strains of MRSA in hospitals. Some of these strains are resistant to virtually all clinically useful antibiotics, including methicillin, cephalosporins, macrolides, and aminoglycosides. The only compound that can be used effectively against these is vancomycin, and resistance to this drug has already emerged in some strains of Enterococci (vancomycin-resistant Enterococci (VRE)) [120]. Our challenge now is to maintain the clinical usefulness of existing drugs by combating antibiotic resistance in an effort to stay one step ahead of bacterial evolution.

1.9 The Sources of Bacterial Resistance to Antibiotics

Following the first appearances of bacterial antibiotic resistance, much work went into understanding the biochemical basis for drug resistance. It was subsequently discovered that bacteria could be inherently resistant to the effects of an antibiotic or acquire resistance to the drugs.

Inherent bacterial antibiotic resistance can occur by: i) a bacteria establishing a permeability barrier against the drug, ii) a drug-producing organism possessing a gene responsible for resistance to its own antibiotic, iii) a bacteria lacking a transport system for the antibiotic or iv) an organism which lacks the target that is affected by the drug.

Some bacteria can develop resistance to antibiotics. This type of resistance results from changes in the bacterial genome or in associated genetic material. Acquired resistance is driven by two genetic processes in bacteria: i) mutation followed by

selection and ii) exchange of genes between strains and species. In the first case, a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population. Under selective pressure by the antibiotic, the wild type cells are killed and the resistant mutant strain can survive. In the second case, a gene encoding for antibiotic resistance in one bacteria is transferred into other bacteria.

There are several processes for genetic exchange that exist in bacteria: conjugation, transduction, and transformation. Conjugation involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient. In fact, it is common for DNA to be transferred as plasmids between mating bacteria. Transduction is characterized by virus-induced transfer of genes between bacteria. In transformation, DNA is acquired directly from the environment, having been released from other cells.

Since bacteria usually develop their genes for drug resistance on plasmids (called resistance transfer factors, RTFs or R-plasmids), they are able to spread drug resistance to other strains and species during genetic exchange processes. For example, many of the antibiotic-resistance genes in Staphylococci are carried on plasmids that can be exchanged with *Bacillus* spp. and *Streptococcus* spp. [91], providing the means for acquiring additional genes and gene combinations. Most of these plasmids contain transposable genetic elements which are segments of DNA that can exist either in the chromosome or on plasmids. Through genetic recombination, these mobile genetic elements can lead to the emergence of new genotypes. As a result, genetic transfers can account for the wide-spread dissemination of resistance genes to all bacterial genotypes.

Genetic transfer of resistance genes between soil microorganisms [73] and transfer of genes encoding tetracycline efflux pumps between *M. tuberculosis* and *Streptomyces* spp. have also been observed [153]. It was originally believed that a barrier existed between Gram-positives and Gram-negatives which prevented genetic transfers, however this was refuted by the finding of gene transfer in a clinical setting between *Streptococcus* and *Campylobacter* [16]. As a result, scientists began to realize that bacteria could pass genes for multiple drug resistance between strains and even between species. This discovery was alarming because resistance carried by the relatively harmless gut bacterium *E. coli* could be transferred to other bacterial species more threatening to man.

There are many hypotheses about the origins of resistance genes. Some believe that they have evolved from drug-producing organisms [10,11,153], while others believe that they may have evolved from ancestral proteins implicated in signalling and/or metabolism [36,166,180]. There is some evidence supporting the idea of drug producers being sources of resistance genes [42,192]. Both theories are not mutually exclusive as these ancestral proteins may have been the source of resistance genes found in the drug-producing organisms [131]. Finally, adding to the dissemination of resistance determinants in nature is the finding of contaminating DNA from drug-producing organisms in antibiotic preparations [206].

The combination of fast growth, genetic processes of mutation and selection, and the ability to exchange genes, account for the astonishing rates of adaptation and evolution that can be observed in bacteria. As a result of intense evolutionary pressure

imposed by overprescription, misdiagnosis and misuse of antibiotics, antibiotic-resistant strains have emerged. The problem has become an international health crisis [12,147].

1.10 Types of Acquired Antibiotic Resistance

Acquired bacterial antibiotic resistance results from: i) altered membrane permeability ii) efflux pumps, iii) altered targets or iv) the production of enzymes which covalently modify the drugs (see section 1.11). While non-enzymatic resistance mechanisms do occur, they are generally less frequent. Target modification is exemplified by mutations in ribosomal proteins which lead to streptomycin resistance in *Mycobacterium tuberculosis* [13,56,113, reviewed in 145] or mutations in RNA polymerase which cause fluoroquinolone resistance [82]. Efflux pumps on the other hand, are the most common route of resistance to tetracyclines [reviewed in 176].

1.11 Aminoglycoside-modifying Enzymes

1.11.1 General Background

A common route of aminoglycoside resistance is the bacterial production of modifying enzymes capable of detoxifying the drugs and rendering them inoffensive to the host organism [10,43,44,77,179,214]. The first description of enzymatic modification of an aminoglycoside was the phosphorylation of kanamycin in *S. aureus* [51], in *P. aeruginosa* [50] and in *E. coli* [198].

The genes encoding aminoglycoside modifying enzymes are most often found outside the chromosome on R-plasmids and transposons [9,44,198], which explains their rapid spread among the bacterial population. Surveys using DNA hybridization techniques show that typical aminoglycoside-resistant isolates carry more than one resistance gene [179]. AGAC resistance genes can in some cases be located on chromosomes as in the case of AAC(2')-Ia, from *Providencia stuartii* [155], AAC(6')-Ii from *Enterococcus faecium* [215] and AAC(3)-Ic from *Serratia marcescens* [30]. Regardless of their location, all aminoglycoside resistance genes appear to be constituitively expressed in the resistant organisms [44]. Finally, enterococci, which are opportunistic nosocomial pathogens, frequently exhibit high level aminoglycoside resistance, a problem which has been recognized since the mid 1970's in clinical settings [52,91,151,223].

1.11.2 Types and Classification of AGAC Resistance Enzymes

There are three types of aminoglycoside-modifying enzymes: i) phosphotransferases (APH), ii) acetyltransferases (AAC) and iii) nucleotidyltransferases (ANT) (Figure 1.14) [179]. A single aminoglycoside modification impairs its ability to bind to ribosomes [41]. Found in both Gram positive and Gram negative pathogens as well as in drug producing organisms, the nomenclature of this broad class of modifying enzymes encompassing more than 50 different proteins has been fully defined by Shaw [reviewed in 179]. The classification of these resistance proteins is based on their regiospecificity (i.e. the site of modification) which is written in parentheses, the subfamily which is based on resistance profiles to different aminoglycosides termed AGRP (aminoglycoside resistance profile)

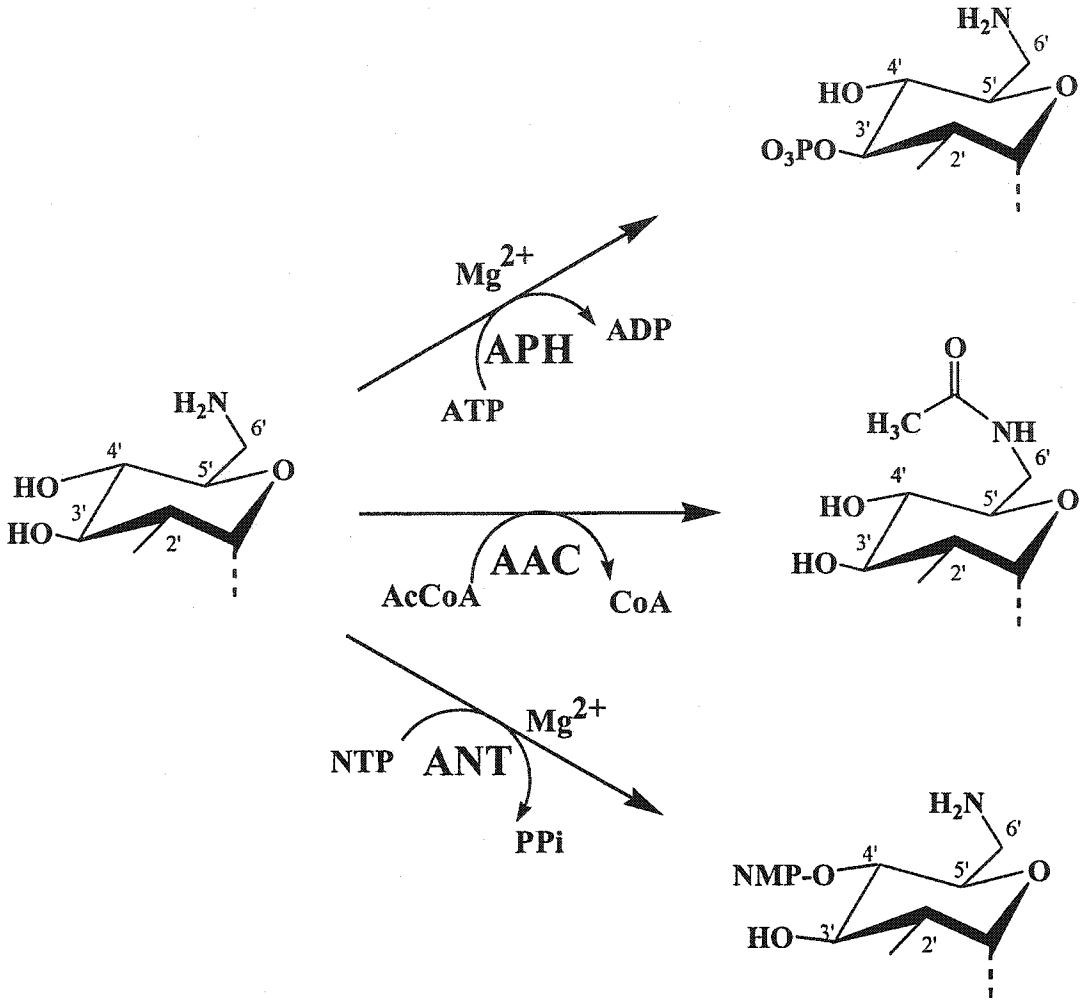


Figure 1.14: Aminoglycosides can be inactivated by three different covalent modifications, *O*-adenylation, *O*-phosphorylation and *N*-acetylation.

depicted with a roman numeral and the specific gene which is given a letter code. In the case of AAC(6')-APH(2"), the acetyltransferase is termed AAC(6')-Ie, because acetylation occurs on the 6'-amino group, the resistance profile is type I of two possible profiles for AAC(6') enzymes, and the gene designation is the letter (e). The phosphotransferase on the other hand is termed APH(2")-Ia, because the phosphorylation event occurs on the 2" hydroxyl with a type I resistance profile and the gene designation is the letter (a) [179].

1.11.3 Aminoglycoside Nucleotidyltransferases (ANTs)

Aminoglycoside nucleotidyltransferases (ANTs) catalyze the transfer of a nucleoside monophosphoryl group from a nucleotide (ATP or others) to an aminoglycoside substrate. There are seven different ANT enzymes found in both Gram positive and Gram negative bacteria which are capable of inactivating aminoglycosides on the 6, 9, 2", 3" and 4'-hydroxyl groups [179]. The best characterized enzymes of this class include ANT(2")-Ia from gentamicin-resistant *Klebsiella pneumoniae* [64-66,200] and ANT(4')-Ia from *S. aureus* which was the first aminoglycoside resistance protein to be solved by X-ray crystallography in the apoenzyme form [98,172] and in the ternary complex of AMP-PCP and kanamycin (Figure 1.15) [156]. ANT(4')-Ia modifies the 4'-hydroxyl groups of aminoglycosides such as kanamycin [68] and is formed by two structural domains [172]. The N-terminus is composed of a five stranded mixed β-pleated sheet while the C-terminal domain is composed entirely of α-helices (Figure 1.15) [172]. The structures displayed a dimer where both the nucleotide and aminoglycoside binding pockets are exposed to solvent and created by residues contributed by both individual monomers [156].

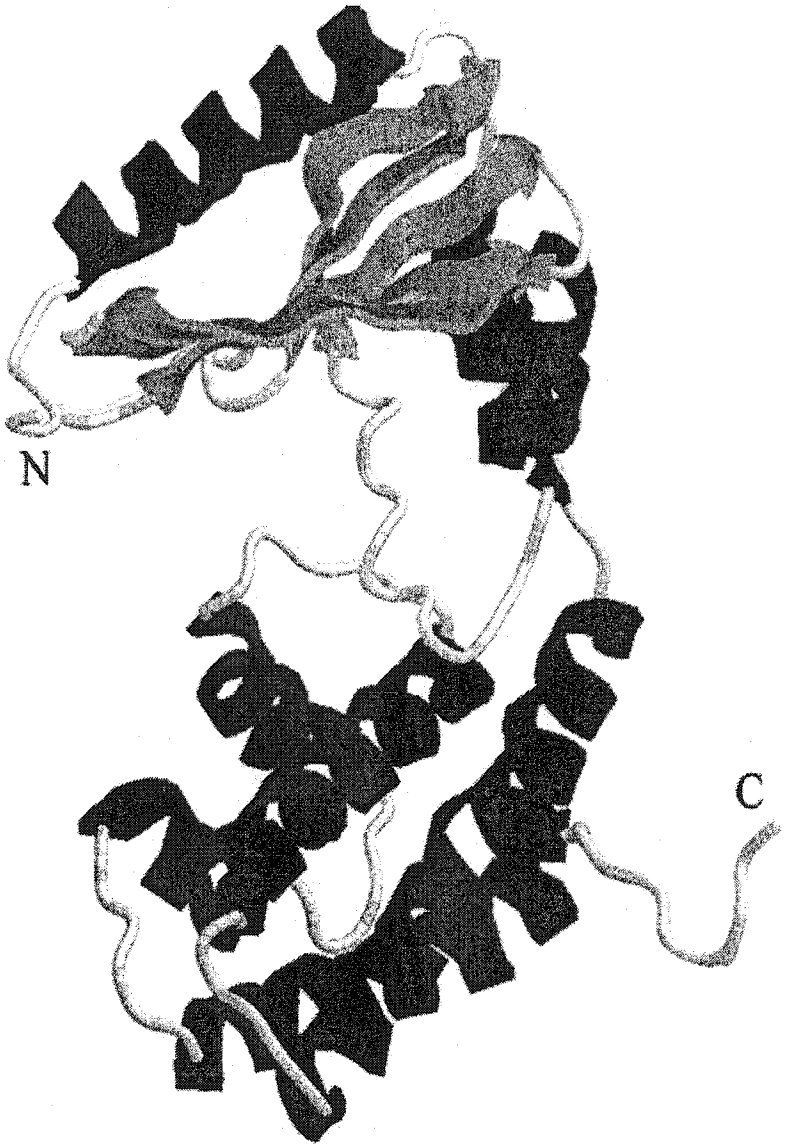


Figure 1.15: Structure of kanamycin nucleotidyltransferase (ANT(4')-Ia). Structure is of the monomer displaying the two domains. The N-terminus is composed largely of a β-pleated sheet and the C-terminus is entirely α-helical (PDB code 1KNY). The image was created using RasMol v2.6 [175].

1.11.4 Aminoglycoside Acetyltransferases (AACs)

Aminoglycoside acetyltransferases (AACs) catalyze the acetylCoA-dependent transfer of an acetyl group to an aminoglycoside substrate [179]. AACs are characterized by a diverse class of over 20 different AGAC acetyltransferases which can acetylate the drugs on the 1, 3, 2' and 6' amino groups. Found in both Gram positive and Gram negative bacteria, both on plasmids as in the case of AAC(6')-Ib [163] and integrated into the chromosomes of resistant organisms [81,167,215], these enzymes display very little sequence homology [179]. Three members of this family have been studied structurally. AAC(6')-Ii from *Enterococcus faecium* was co-crystallized with acetyl coenzyme A (Figure 1.16) [217], AAC(3)-Ia from *Serratia marcesens* bound by coenzyme A [210], and AAC(2")-Ic from Mycobacterium tuberculosis in the ternary complexe of coenzyme A with multiple aminoglycoside substrates [201]. Investigation of these structures revealed that AAC enzymes belong to a GCN5 superfamily of *N*-acetyltransferases typified by GCN5 and the yeast protein HAT1 [210,217]. Another acetyltransferase, AAC(2')-Ia from *Providencia stuartii*, is not only characterized by its ability to acetylate aminoglycoside antibiotics but has also been shown to *O*-acetylate peptidoglycan suggesting that aminoglycoside detoxification may be secondary to a role in metabolism of peptidoglycan [155]. It is also worth mentioning that overexpression of AAC(2')-Ia causes high-level aminoglycoside resistance [166].

Figure 1.16: Structure of AAC(6')-Ii from *E. faecium*, a member of the GCN5 superfamily of N-acetyltransferases. Acetyl coenzyme A, seen in green, is bound in a cleft forming the active site (PDB code 1B87). The image was created using RasMol v2.6 [175].

1.11.5 Aminoglycoside phosphoryltransferases (APHs)

Aminoglycoside phosphoryltransferases (APHs) catalyze the ATP-dependent transfer of the γ-phosphate of ATP to an aminoglycoside substrate [179]. APHs are found in a variety of Gram positive and Gram negative organisms and are capable of phosphorylating aminoglycoside substrates on 4, 6, 9, 3', 2", and 3" hydroxyls [179]. This class of proteins is composed of over 20 enzymes which do present three somewhat conserved functional domains in the C-terminus [131]. Motif I is composed of a VxxHGDxxxxN which is involved in phosphate transfer [131]. Motif II is the P-loop structure characterized by the sequence pattern GxxDxGRxG which is involved in binding the phosphate backbone of ATP [131]. Motif III, which is characterized by the sequence pattern DxxR/KxxF/YxxxLDE, is found in both ANTs and APHs and is believed to be involved in either nucleotide hydrolysis or conformational changes in the proteins [131].

One APH which has been extensively studied both biochemically [134-136] and structurally [89] is APH(3')-IIIa from *E. faecalis* and *S. aureus*. Determination of the Theorell-Chance kinetic mechanism [136], the catalytic mechanism [137], the regiospecificity of inactivations to both 4,5- and 4,6-aminoglycosides [193], as well as active site mapping using 5'-[p-(fluorosulfonyl)benzoyl]adenosine (FSBA) [134] and site-directed mutagenesis [194] were completed prior to the first structural determination of the ADP bound form of the enzyme by X-ray crystallography (Figure 1.17) [89]. It was revealed from the structure that APH(3')-IIIa shares structural homology with Ser/Thr and Tyr protein kinases [89]. The advent of multiple structures including: i) the apoenzyme [25],

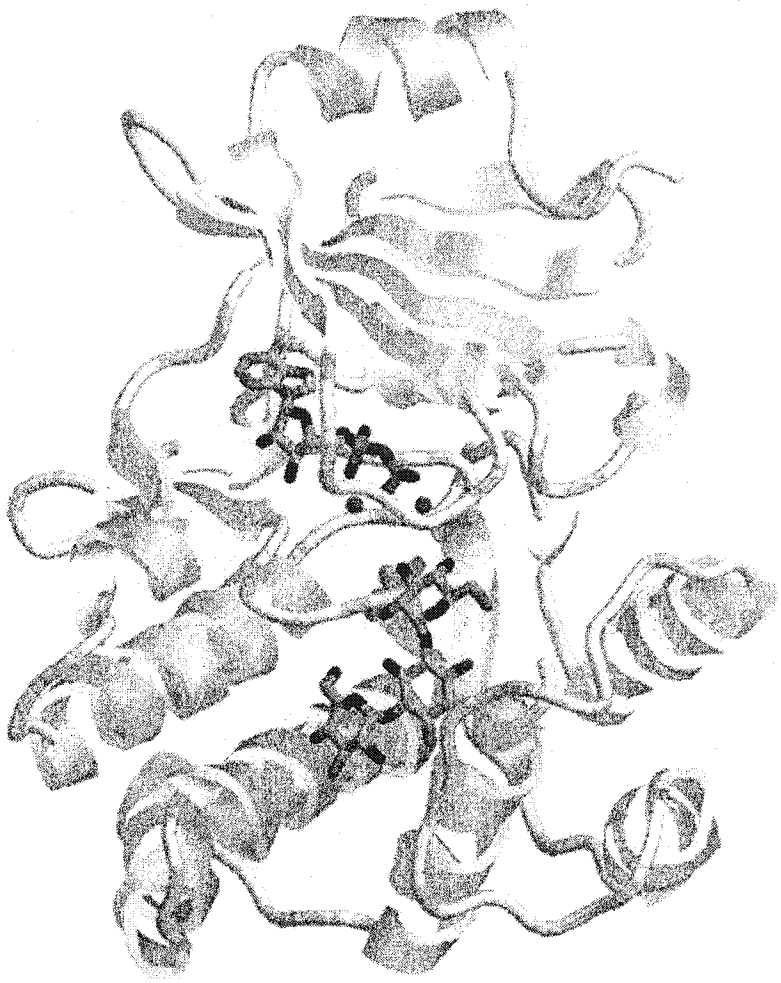


Figure 1.17: Structure of APH(3')-IIIa in complex with Mg^{2+}-ADP and kanamycin A. The magnesium atoms are green. The structure reveals homology to eukaryotic protein kinases (PDB code 1L8T). The image was created using RasMol v2.6 [175].

the enzyme bound by ADP [89], AMP-PNP [25], and both ternary complex structures of APH(3')-IIIa bound by ADP and kanamycin A (Figure 1.18) or neomycin B [57] have enabled the dissection and further understanding of conserved motifs involved in substrate binding and catalysis.

1.11.6 Target Mimicry Employed by Aminoglycoside Modifying Enzymes

The aminoglycoside modifying enzymes are vastly under characterized. Those that have been studied are highly efficient at detoxifying aminoglycosides with k_{cat}/K_m values in the 10^6-10^8 M^{-1} s^{-1} range. Not only is there limited functional characterization of these enzymes, but there are even less structural studies performed with these proteins. The structures have been very informative in deciphering mechanistic aspects of enzymatic function, and thus are crucial to the proper understanding of each class of AGAC-modifying enzymes. In fact, information obtained from recent structures of AGAC-modifying enzymes bound by aminoglycosides have uncovered what appears to be a common mechanism of target mimicry which is employed by the proteins to impart specificity for the aminoglycoside substrates in their active sites [57,152]. Comparisons of bound aminoglycosides to ribosomal RNA or modifying enzymes show that rings I and II of aminoglycosides adopt a conformation which is recognized by both 16S ribosomal RNA and their resistance determinants [37,57,152].

1.12 AAC(6')-APH(2")

The gene encoding the AAC(6')-APH(2") is found on mobile genetic elements such as Tn*4001* in *S. aureus* [126], Tn5281 in *E. faecalis* [88], and Tn*4031* found in *S. epidermidis* [191] explaining the increasing frequency of gentamicin-resistant strains. In 1977, some gentamicin-resistant strains of *S. aureus* were found to possess two plasmid-born aminoglycoside modifying enzymes, a 6'-acetyltransferase (AAC(6')) and a new phosphotransferase specific for 2"-OH containing aminoglycosides such as the clinically important gentamicins [121]. It wasn't until 1983, that the same group discovered that the activities observed were linked to the product of a single polypeptide and the only known bifunctional aminoglycoside modifying enzyme AAC(6')-APH(2") [55,130,197].

The N-terminal AAC(6')-Ie of the bifunctional enzyme, which is only found in Gram positive bacteria, is part of a sub-family of 6'-aminoglycoside acetyltransferases formed by AAC(6')-Ib, AAC(6')-IIa and AAC(6')-IIb which are found exclusively in Gram-negative bacteria [179]. While there is a more than 80 % amino acid similarity between these last proteins, the AAC(6')-Ie is only distantly related with 52 % amino acid similarity to other sub-family members [179]. Amino acid sequence alignments of the AAC(6')-Ie with other 6'-acetyltransferases reveals little homology as no defined conserved sequences are obtained [179].

The APH(2")-Ia as well shows low homology with other aminoglycoside phosphotransferases, however, it does share conserved sequence motifs in the C-terminal region albeit weakly (Figure 1.18) [131]. Sequence alignments performed on motif I, II and III residues shows poor conservation except for the P-loop region (motif II) (Figure 1.18).

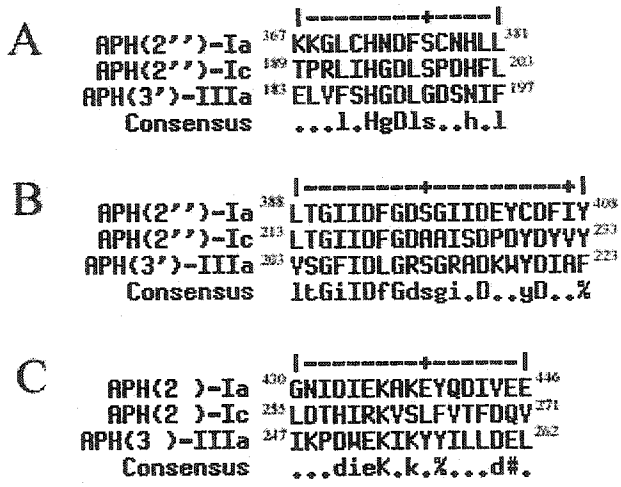


Figure 1.18: Primary sequence alignment of AAC(6')-APH(2") and other APH-family members. Residues in red are absolutely conserved while those in blue are partially conserved. Depicted are: **A**- alignment of sequences in motif I, **B**- alignment of sequences in motif II, and **C**- alignment of sequences in motif III. Enzymes are: APH(2")-Ia from *E. faecalis* and *S. aureus*; APH(2")-Ic from *E. gallinarium* and *S. aureus*; APH(3')-IIIa from *E. faecalis* and *S. aureus*. Multiple sequence alignments were constructed using the MultAlin method with hierarchical clustering and employing a BLOSUM62 score matrix [35].

Martel and colleagues further characterized both the acetyltransferase and the phosphoryltransferase by using a combination of initial velocity, and product and dead-end inhibition studies [130]. Both enzymes followed rapid equilibrium random kinetic mechanisms [130].

The spread of *aac(6')-aph(2")* was detected early on as the bifunctional protein was subsequently purified from aminoglycoside-resistant strains of *S. epidermidis* [197] and *E. faecalis* [55]. The gene coding for AAC(6')-APH(2") was first isolated from the *E. faecalis* plasmid pIP800 and cloned into an *E. coli*-based expression system (pUC8) to form plasmid pSF815A [55]. It possesses an open reading frame of 1,437 base pairs coding for a protein of 479 amino acids with an expected molecular weight of 56,993 Da [55]. The amino acid sequence revealed two partial homologies. In the N-terminal portion of the protein, there was homology (13.9 % identity, 28.9 % similarity) to a chloramphenicol acetyltransferase from *Bacillus pumilis*, while in the C-terminus, there was homology (19.4 % identity, 32.8 % similarity) to an aminoglycoside phosphotransferase from *Streptomyces fradiae* [55]. Subcloning experiments to delineate the functionalities of AAC(6')-APH(2") revealed the bifunctional enzyme consisted of separable enzymatic activities, leading to the suggestion that *aac(6')-aph(2")* resulted from a gene fusion event of two individual resistance determinants [55]. Aminoglycoside resistance profiles exhibited by the two separate enzymatic activities revealed that the N-terminus possessed an AAC(6`)-I profile, while a LacZ-fusion to the C-terminus lacking the first 137 amino acids specified only APH(2")-Ia activity [55].

Finally, an interesting observation is that drug producing organisms often have the presence of both an APH and an AAC and both determinants are required for high level resistance to the host drug [157]. The unique bifunctionality of AAC(6')-APH(2") renders it capable of inactivating all 2-deoxystreptamine aminoglycosides and the presence of this protein in both Enterococci and Staphylococci [52,151] is associated not only with aminoglycoside resistance, but high level resistance to clinically important aminoglycosides such as gentamicin and netilmicin. The presence of this resistance determinant is a serious clinical problem, as nearly half of clinical isolates of Staphylococci and Enterococci possess high-level aminoglycoside resistance due to the presence of *aac(6')-aph(2")* [190]. This is of some concern especially for the treatment of infections caused by MRSA.

1.13 Goals and Strategies

The goals for this project were: i) to overexpress and purify AAC(6')-APH(2") to homogeneity, ii) to define the substrate specificities by obtaining the kinetic parameters for all known substrates for both activities, iii) to determine the regiospecificity of acetyltransfer and phosphoryltransfer to both classes of AGACs (4,5-disubstituted and 4,6-disubstituted), iv) to screen for inhibitors of both the acetyltransferase and the phosphoryltransferase, and v) to perform structural studies useful for site-directed mutagenesis and in structure-based drug design.

Chapter 2

Overexpression of *aac(6')-aph(2")* in *E. coli*

Based on unpublished work

Chapter 2

2.1 Abstract

It became quickly apparent that attempts to overexpress the bifunctional protein in *E. coli* cells were problematic. Very little protein was produced as determined by small-scale protein expression experiments or by measuring active protein in lysates or partially purified samples. Multiple *E. coli* overexpression systems utilizing distinct ribosome binding sites, promoters, in addition to the use of different cell types, culturing conditions and induction methods were tried to improve the protein yield. Unfortunately, none of these modifications were successful.

Efforts were subsequently directed to elucidate the basis for the low protein yields. With this objective in mind, a Northern blot analysis was performed to identify the source of the protein expression problem. The analysis pointed towards a problem at the level of translation, as messenger RNA levels were found to be dramatically induced by isopropyl β-D-thiogalactopyranoside (IPTG). However, the true source of the problem was only revealed once attempts were made to purify whatever amount of AAC(6)-APH(2") was produced from *E. coli* W3110/pBF-9.

A three-step purification yielded two proteins possessing aminoglycoside phosphotransferase activity. One was of 56.9 kDa, the expected molecular weight for AAC(6')-APH(2"), while the other was approximately 36 kDa in size based on its mobility on 11 % SDS-polyacrylamide gels. A combination of N-terminal amino acid

sequencing, electrospray mass spectrometry and kinetic substrate profiling confirmed that this 36 kDa aminoglycoside kinase was in fact a C-terminal phosphotransferase active fragment of the bifunctional enzyme starting at Met175. Investigation of the DNA upstream of this start codon showed significant homology to many *E. coli* ribosome binding sites (GAA GAT compared to the consensus GAA GGA ahead of full-length *aac(6')-aph(2")*); which, in combination with the fact that no AAC(6')-Ie protein was detected, lead to speculation that this 36 kDa phosphotransferase was the result of an internal translational initiation.

2.2 Introduction

To obtain any structural or mechanistic information on AAC(6')-APH(2"), large amounts of protein would have to be produced and purified to homogeneity. Based on these requirements and the bacterial source of the gene, initial attempts to overproduce AAC(6')-APH(2") were explored in *E. coli*.

E. coli expression systems are most often the first choice for small (i.e. smaller than 50 kDa) bacterial cytosolic proteins that do not require translational modifications for activity [34]. The most frequent problem associated with overexpression of proteins in *E. coli* is the production of the recombinant protein in inclusion bodies [34]. Mass production of recombinant proteins in *E. coli* frequently results in insoluble protein aggregates generally formed by improper disulfide bond formation and associated protein folding. Overwhelming the protein folding machinery and the intracellular reducing

environment caused by the presence of large amounts of reduced glutathione are generally responsible for these aggregates [34].

This chapter describes our attempts to overexpress and purify AAC(6')-APH(2") by conventional means in *E. coli*, our attempted modifications to improve protein yield and finally investigations of the source of the expression problems which led to the discovery of a potential internal translational initiation start site in *E. coli* upstream of the codon encoding Met175.

2.3 Results and Discussion

2.3.1 Attempts at Overexpression in *E. coli*

The original source of the bifunctional gene *aac(6')-aph(2")*, was the plasmid pSF815A, a kind gift of Dr. Elioupoulous of the Deaconess Hospital (Boston MA) (Figure 2.1). The gene was originally derived from the R-plasmid pIP800 found in *E. faecalis* and was cloned in an *E. coli* expression system (pUC8) to form pSF815A [55]. Prior to undertaking this project, attempts by others in the Wright laboratory, to overexpress the bifunctional enzyme using this expression system in *E. coli* were unsuccessful. Consequently, one of the first tasks was to construct a successful overexpression system to overproduce AAC(6')-APH(2"). The use of highly developed pET expression vectors (Novagen, Madison, WI), based on the T7 promoter/operator and (DE3)-cell encoded T7 RNA polymerase was generally very successful with other proteins, and led us to sub-clone *aac(6')-aph(2")* into plasmid pET22b(+) [149], placing the gene under the control of the

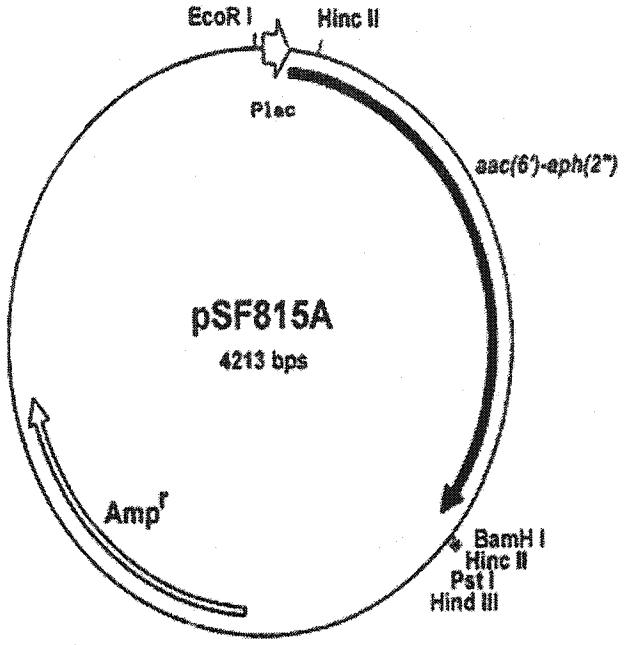


Figure 2.1: Map of *aac(6')-aph(2")* source plasmid pSF815A [55]. The backbone of pSF815A is pUC8. Indicated are the P_{lac} promoter and ampicillin resistance marker.

tightly regulated T7 promoter, generating plasmids pBF-7 and pBF-8 which differ by an internal *HpaI* to *HindIII* replacement from pSF815A to avoid sequencing the entire gene (described in Materials and Methods) (Table 2.1). Competent *E. coli* BL21(DE3) cells which possess a chromosomal copy of the T7 RNA polymerase gene were transformed with pBF-8. Transformants were screened for gentamicin resistance and the positive clones were assayed for protein expression. IPTG-induced expression of AAC(6')-APH(2") in strains carrying pBF-7 and pBF-8 gave rise to no observable overexpression as assessed by small scale expression experiments (boil preps) followed by SDS-polyacrylamide gel electrophoresis. Further attempts to find a successful over-expression construct were needed as the negligible levels of expression from the T7-based system were not satisfactory.

The next strategy employed was based on plasmid pKK223-3 [174]. This plasmid utilized a Tac promoter/Lac operator combination. Modifications were made to enable cloning and optimize expression such as creating unique restriction sites for cloning the gene as well as inserting an optimal *E. coli* ribosome binding site from gene 10 of bacteriophage T7 (see Materials and Methods for details). This system had previously been used with great success to overexpress D-ala-D-ala ligase B (DdlB) from *E. coli* [150], yielding over 100 mg of pure protein per liter of culture. It was hoped that expression of AAC(6')-APH(2") would be greatly improved by the use of such a system. The gene was cloned into pKK223-3** to give plasmid pBF-9 (Figure 2.2 and Table 2.1). *E. coli* strains carrying pBF-9 were screened for gentamicin resistance. Positive clones were assayed for protein production by induction with 1 mM IPTG.

Table 2.1: Summary of *E. coli* overexpression constructs made in this study.

Plasmid	Promoter	Operator	Plasmid backbone	Special features
pSF-815A	Lac	Lac	pUC8	none
pBF-7	T7	Lac	pET22(b)+	T7 RNA pol.
pBF-8[a]	T7	Lac	pET-22(b)+	T7 RNA pol.
pBF-9	Tac	Lac	pKK223-3	RBS[1]
pBF-10	T7	Lac	pET-15b	T7 RNA polymerase
pBF-11	λ	λ	pDOC55	cII
pBF-12	Lac	Lac	pUC19	RBS[b]
pBF-13	λ	λ	pSKF301	cII

[a] 1377 bp internal replacement from pSF815A to avoid sequencing entire gene.
[b] Ribosome binding site from gene 10 of bacteriophage T7.

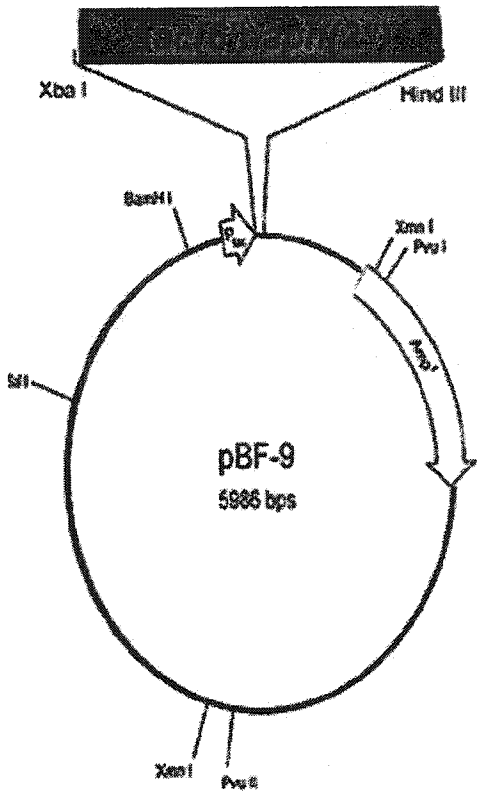


Figure 2.2: Map of AAC(6')-APH(2") expression plasmid pBF-9. Indicated are the P_{Tac} promoter and the ampicillin resistance marker.

Once again, no observable overexpression was achieved.

Although there appeared to be no overexpression of the 57 kDa bifunctional protein, a specific APH enzyme activity assay (described in Materials and Methods) showed that lysates from induced cultures of *E. coli* W3110/pBF-9 displayed increased levels of phosphotransferase activity as compared to lysates from *E. coli* carrying either pSF815A or pBF-8, suggesting that pBF-9 produced the highest level of active protein (Figure 2.3). The observed activity obtained with *E. coli* strains carrying pBF-9 appeared to be unresponsive to IPTG induction indicating that the high levels of active AAC(6')-APH(2") are due in most part to leaky protein expression from the Tac promoter in this system (Figure 2.4).

2.3.2 Use of *E. coli* TOPP Cells to Improve Protein Production

Since expression levels were still unsatisfactory, additional attempts were made to overexpress the enzyme from the pBF-9 plasmid in *E. coli* TOPP cells [187]. These cells are non-K12 derivatives of *E. coli* that are compatible with conventional expression vectors and are often used to express proteins which prove intractable to overexpression in typical *E. coli* K12 strains. Transformation of TOPP cell strains 1 through 6 with pBF-9 followed by small scale expression experiments showed some variability in protein expression but, failed to increase expression levels by any significant margin as assessed by enzymatic assay or by boil preps followed by SDS-polyacrylamide gel electrophoresis (Figure 2.5 and 2.6).

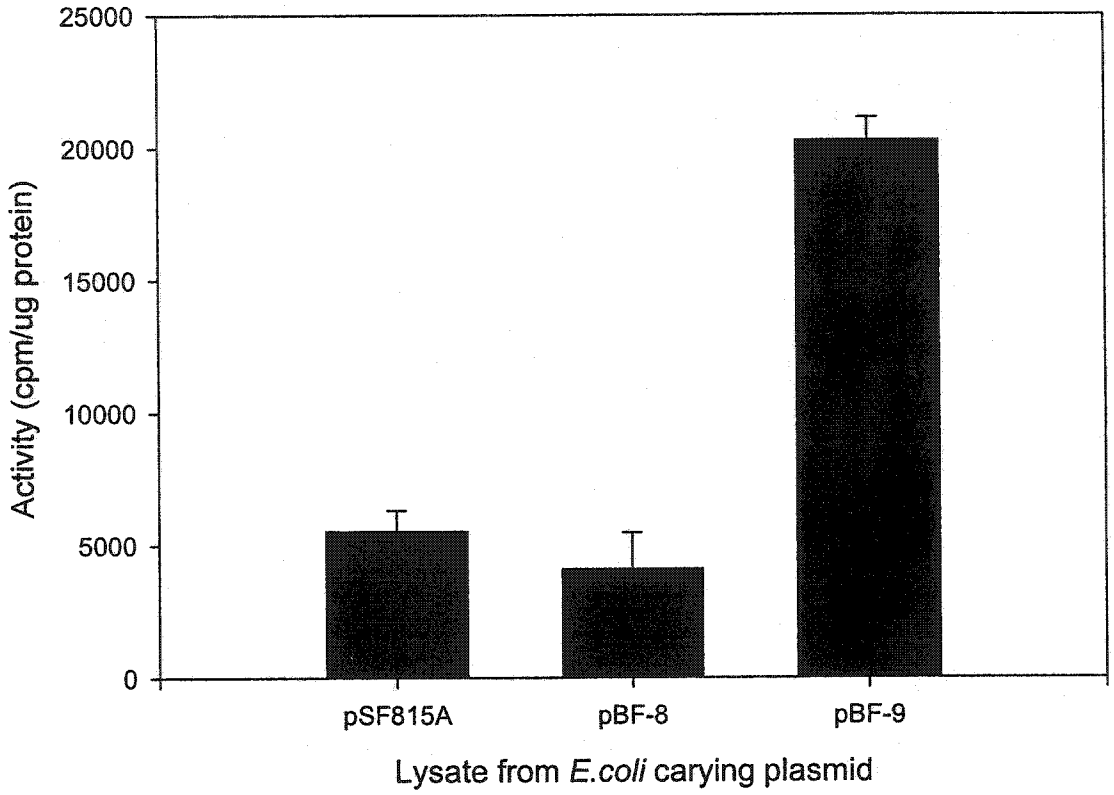


Figure 2.3: Expression levels of AAC(6')-APH(2") from *E. coli* carrying pSF815A, pBF-8 or pBF-9 as related to activity observed per μg of protein. Cell cultures were induced and harvested at similar OD_{600} readings and total protein was quantitated by a Bradford assay [15]. Activity was monitored using the phosphocellulose binding assay with γ^{32}P-ATP (see Materials and Methods).

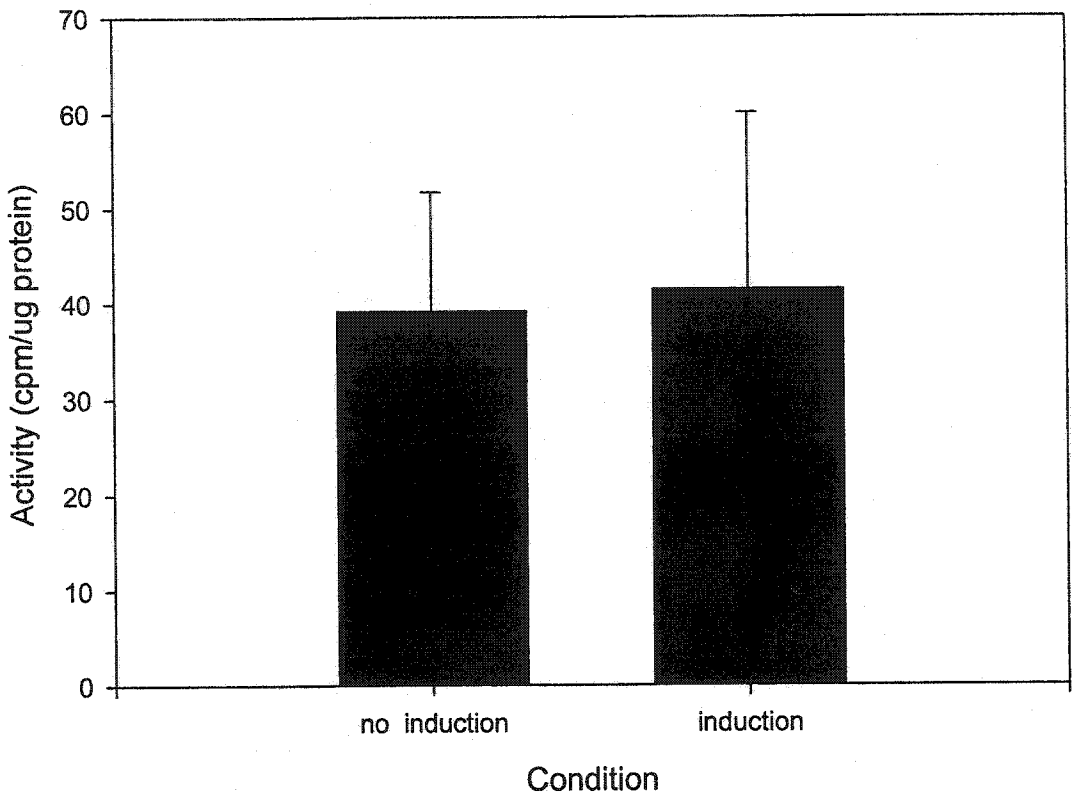


Figure 2.4: Unresponsiveness of the pBF-9 expression system to IPTG. Effect of IPTG induction as it relates to phosphotransferase activity observed in lysates of *E. coli* W3110/pBF-9. Cells were grown under identical conditions with the exception of the induction with 1 mM IPTG at OD_{600} of 0.5. Cells were harvested had OD_{600} of 1, lysed and assayed for APH activity by the phosphocellulose binding assay with γ^{32}P-ATP.

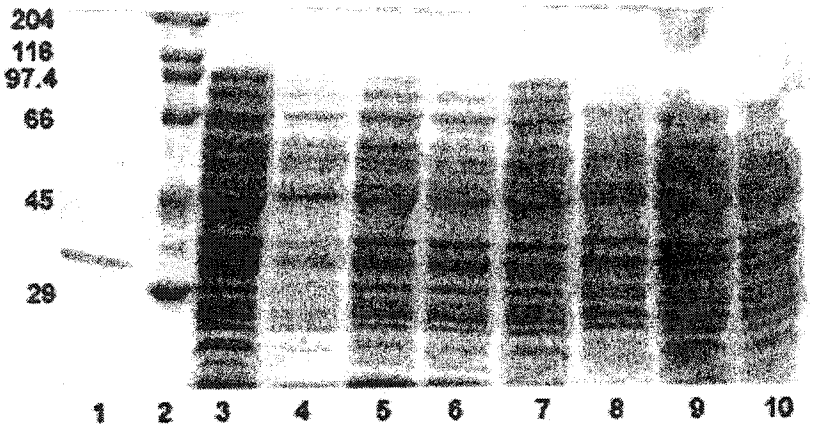


Figure 2.5: 11 % SDS-Polyacrylamide gel of lysates from induced cultures of *E. coli* TOPP cells carrying pBF-9. Equivalent protein was loaded for all TOPP cell lysates. Protein were stained with coomassie brilliant blue R-250. Lanes are: 1- APH(3')-IIIa for molecular weight identification, 2- Molecular weight marker (from top to bottom: 212 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 29 kDa), 3- *E. coli* W3110/pBF-9 no induction, 4- *E. coli* W3110/pBF-9 with IPTG induction, 5 through 10, *E. coli* TOPP 1 through 6 carrying plasmid pBF-9 and induced with IPTG. Lane 6 – *E. coli* TOPP3/pBF-9 appears to have the best expression of the TOPP cells tried.

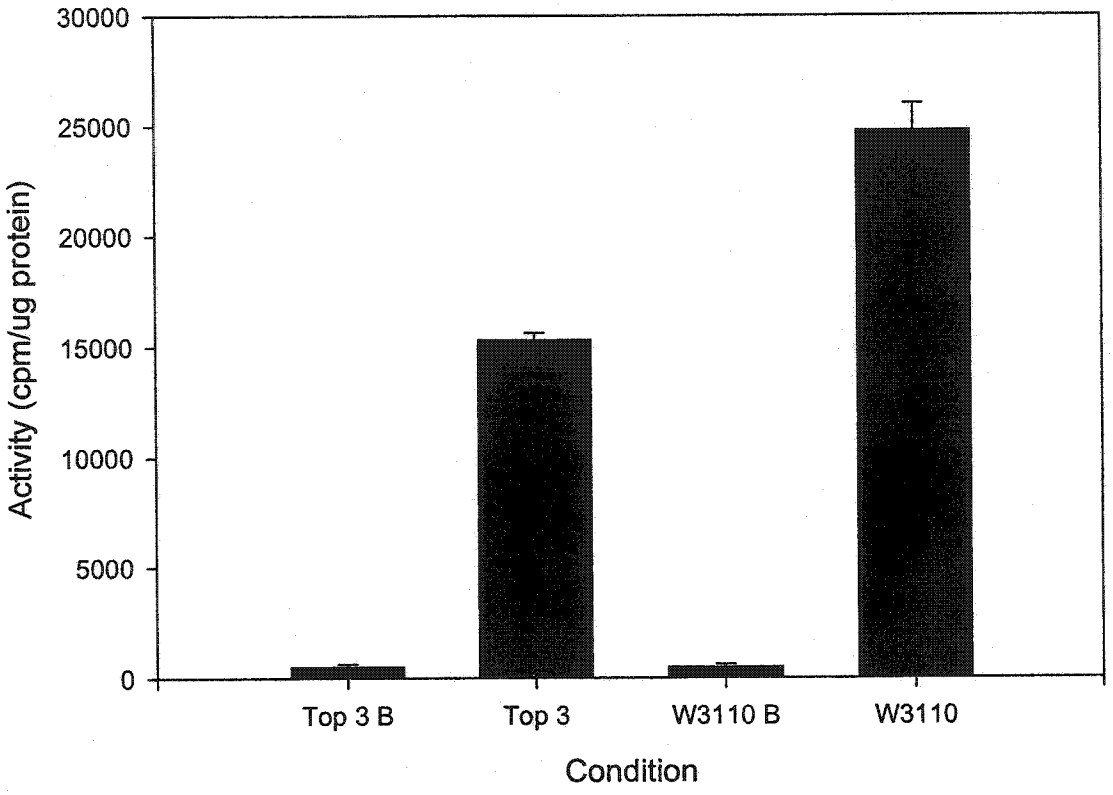


Figure 2.6: The pBF-9 construct produces higher levels of active AAC(6')-APH(2") in *E. coli* K12-derived W3110 cells. **B** indicates samples were boiled prior to assay as a control. Cell cultures were treated identically and total protein was quantitated by Bradford assay [15]. APH activity assay was performed using the phosphocellulose binding assay with γ^{32}P-ATP (described in Materials and Methods).

2.3.3 Modifications to Growth Conditions

It was becoming apparent that there was an underlying problem at the source of our failures to overexpress this protein. Using the APH activity assay as a quantitative measure of protein expression, an experiment to see if different culture temperatures and media affected protein production was performed. Results yielded little helpful information as maximal protein production in *E. coli* was obtained at the optimal growth temperature of 37 °C and in the conventional media Luria-Bertani Broth (LB). To further complement this study, different concentrations of IPTG (0.5, 1 and 2 mM final concentration) as well as different culture volumes and flask configuration were tested to optimize aeration and agitation without any improvement in protein levels or enzymatic activity. To rule out the possibility that the expressed protein was being exported from the cells, activity assays were performed on cell culture supernatants, however as anticipated no aminoglycoside phosphotransferase activity was observed.

2.3.4 *aac(6')-aph(2")* from Aminoglycoside-resistant Clinical Isolates

To exclude the source of the DNA as the root of the problem, nine gentamicin resistant clinical isolates including strains of *E. faecalis*, *S. aureus*, *B. cepacia*, *P. aeruginosa* and *E. coli* were obtained from the Clinical Microbiology Laboratory (Chedoke-McMaster Hospital, McMaster University Health Sciences Centre, Hamilton, ON) and screened for the presence of *aac(6')-aph(2")* by colony PCR. A 1.5 kb amplification product corresponding to *aac(6')-aph(2")* was obtained for two strains of *S. aureus* (MRSA) and one strain of *E. faecalis* as seen on a 1 % agarose gel (Figure 2.7). The gene

from the *E. faecalis* strain ATCC 49383 was PCR amplified and cloned into the pKK223-3** to re-construct pBF-9. *E. coli* W3110 cells carrying the newly re-constructed and sequenced pBF-9 plasmid were screened for gentamicin resistance and positive clones were assayed for target protein production by induction with IPTG. Unfortunately, no difference in IPTG-dependent protein expression was obtained.

2.3.5 Additional Constructs Tested

Other strategies attempted included i) engineering a His_6-tag to the N-terminus AAC(6')-APH(2") by cloning the gene into plasmid pET15b [149], ii) placing the *aac(6')-aph(2")* under the control of the Lambda promoter/Lambda operator/Cro temperature sensitive repressor combination in two separate plasmids pDOC55 and pSKF301 and finally iii) inserting *aac(6')-aph(2")* into the multi-copy plasmid, pUC19 employing a Lac promoter Lac operator combination [148] (Table 2.1). None of these constructs improved active AAC(6')-APH(2") protein yields compared to *E. coli* W3110/pBF-9.

Through the use of SDS-polyacrylamide gel electrophoresis and especially the aminoglycoside phosphotransferase activity assay, the pBF-9 construct was deemed to be the most successful overexpressing plasmid for active AAC(6')-APH(2") in *E. coli* (Figure 2.8).

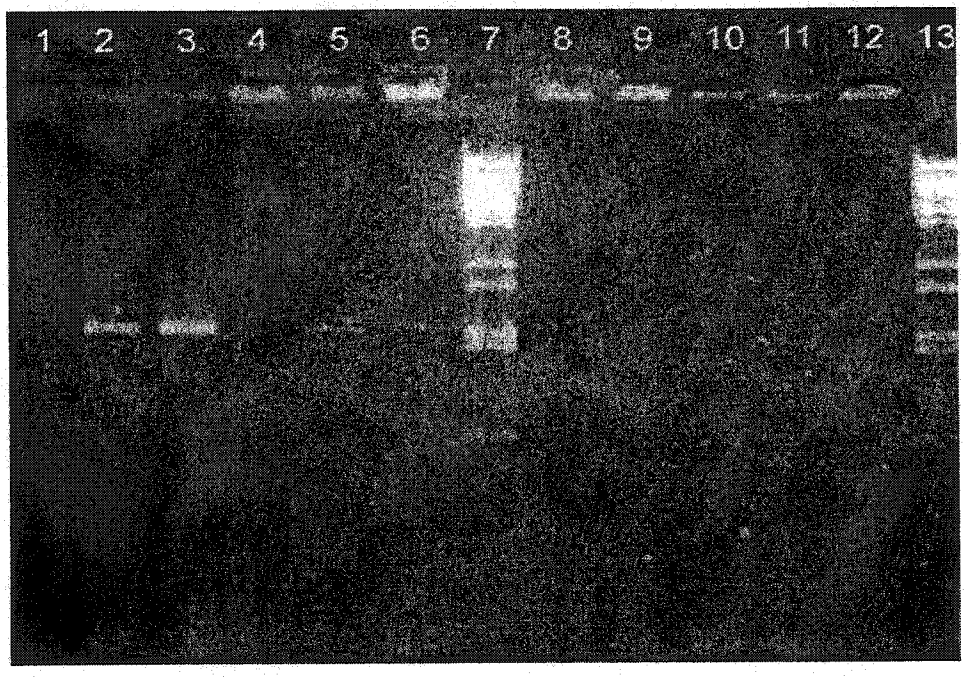


Figure 2.7: PCR screening of gentamicin-resistant clinical isolates for *aac(6')-aph(2")*. Lanes are: 1- no bacteria negative control, 2- *E. coli* W3110/pBF-9 positive control, 3- *E. faecalis* ATCC 49383, 4- MRSA ATCC 49476, 5- MRSA W6137, 6- MRSA 55384, 7 and 13- DNA marker, 8- *E. coli* T6860, 9- *E. coli* W65009, 10- *B. cepacia* M55686, 11- *B. cepacia* M55653, and 12- *P. aeruginosa* X13879.

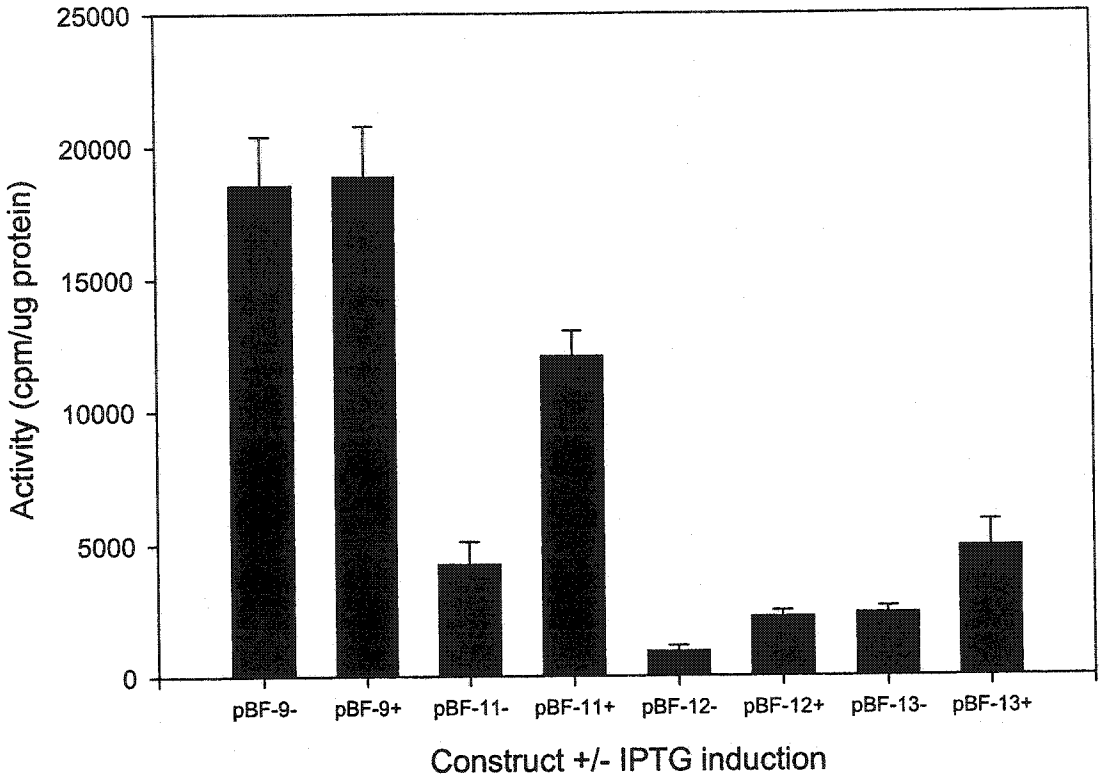


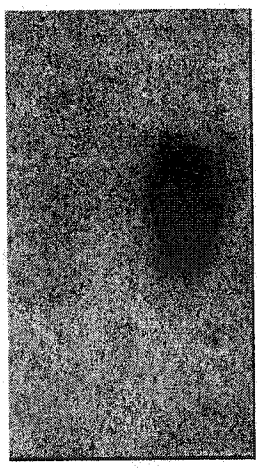
Figure 2.8: Yield of native phosphotransferase active AAC(6')-APH(2") from *E. coli* strains and effect of IPTG induction. Cell cultures were induced and harvested at similar OD_{600} readings. Total protein was quantitated by Bradford protein assay [15]. Equivalent amounts of total protein were added to each reaction. – denotes no induction with IPTG, while + denotes cell cultures were induced by either addition of 1 mM IPTG to the growth media or by temperature shift from 30 °C to 42 °C. APH activity was monitored by the phosphocellulose binding assay with γ^{32}P-ATP (described in Materials and Methods).

2.3.6 Investigation of the Source of the Expression Problem

Having exhaustively investigated overexpression of AAC(6')-APH(2") in *E. coli*, attempts were made to elucidate the reasons at the source of the expression problems. Remaining explanations included: i) potential toxicity of overexpressed AAC(6')-APH(2") in *E. coli*, ii) mRNA instability or iii) codon usage differences between *E. coli* and *E. faecalis*. Northern Blot analysis was carried out to test if IPTG induction yielded any increase in messenger RNA levels. This experiment was helpful in understating the underlying problems observed with AAC(6')-APH(2") expression in *E. coli*. Results showed a dramatic increase in mRNA levels upon IPTG induction (Figure 2.9), suggesting that failure to overexpress the bifunctional protein was likely rooted in translation of the message on the ribosome.

2.3.7 A breakthrough while attempting to Purify AAC(6')-APH(2") from *E. coli*

Even though protein expression was unimpressive, attempts to purify the bifunctional enzyme from *E. coli* W3110/pBF-9 led to an unanticipated result and a partial answer to the previous over-expression troubles. Fortuitously, the specific aminoglycoside phosphotransferase activity assay was used to follow the enzyme along the purification. After three purification steps including a final gentamicin-agarose affinity column, what at first appeared as a low molecular weight contaminant by SDS-polyacrylamide gel electrophoresis became a potential answer to our predicament (Figure 2.10).



1 2

Figure 2.9: Autoradiogram of Northern blot analysis of *aac(6')-aph(2")* mRNA levels upon IPTG induction. Total RNA was isolated from both cultures as described in Materials and Methods. The probe consisted of a ^{32}P end-labeled PCR product of *aac(6')-aph(2")*. Lane 1- total RNA from 100 mL *E. coli* W3110/pBF-9 culture without IPTG induction and lane 2- total RNA from 100 mL *E. coli* W3110/pBF-9 culture with IPTG induction to 1 mM final concentration. The samples (duplicates on either side of the gel) were separated in a 1 % agarose/ 2.2 M formaldehyde gel. One half of the gel was stained with ethidium bromide to visualize rRNA while the other half was used for the transfer and autoradiography.

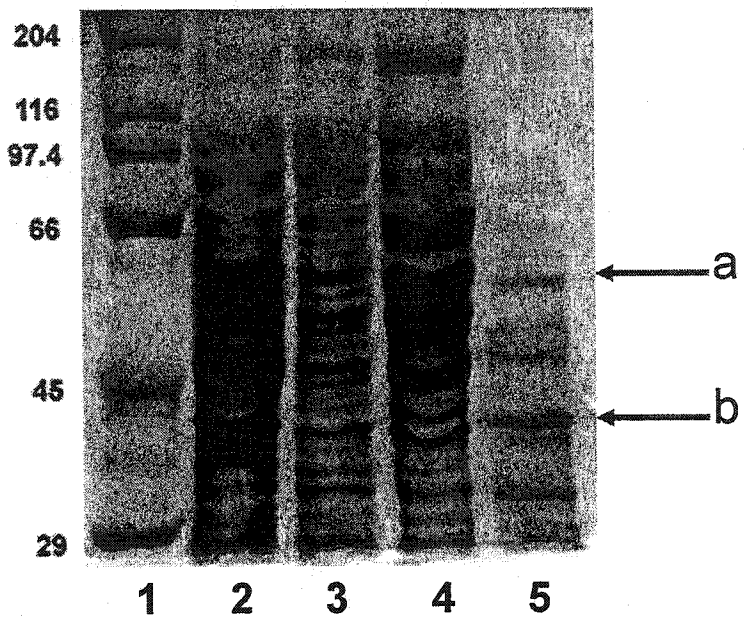


Figure 2.10: 11 % SDS-Polyacrylamide gel of the purification of AAC(6')-APH(2") from *E. coli* W3110/pBF-9. Proteins were stained with Coomassie Brilliant Blue R-250. Lanes are: 1- High molecular weight marker (top to bottom – 204 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 29 kDa), 2- lysate, 3- 25-60 % ammonium sulfate precipitation, 4- Sephadex G100 gel filtration column, 5- Macro prep Q anion exchange column. **a**- denotes partially purified full-length AAC(6')-APH(2"), while **b**- denotes partially purified C-terminal phosphotransferase active APH(2")-Ia.

Luckily AAC(6')-APH(2") and the contaminating 36 kDa protein were separated on the gentamicin-agarose affinity column (Figure 2.11), as APH activity was observed for both AAC(6')-APH(2") and the contaminating protein. A purification table shows that only 1.3 mg of pure AAC(6')-APH(2") can be obtained from 20 L of *E. coli* W3110/pBF-9 with a 430-fold purification (Table 2.2). The ratio of full-length bifunctional protein produced to that of the APH active fragment was approximately 1:4.

2.3.8 Identification of the 36 kDa APH-active co-purified Protein

N-terminal amino acid sequencing identified the first 6 residues of the protein (Met-Glu-Tyr-Arg-Tyr-Asp) and confirmed that the 36 kDa contaminant was a C-terminal fragment of the bifunctional protein possessing aminoglycoside phosphotransferase activity and starting at Met175. Investigation of the DNA sequence upstream of the Met175 codon showed the presence of a sequence strikingly similar to *E. coli* ribosome binding sites ($^{5'}$-GAA GAT -$^{3'}$ compared with the consensus $^{5'}$- GAA GGA -$^{3'}$).

2.3.9 Codon Usage in *aac(6')-aph(2")* as a Source of Expression Problems in *E. coli*

As the level of protein expression of both the full-length and the phosphotransferase active fragment remain low, there must necessarily be other reasons at the source of the low expression. It is potentially a combination of the internal initiation, codon usage differences between *E. coli* and *E. faecalis* (16 of the 18 codons encoding Arg in *aac(6')-aph(2")* (codon AGA 14/16 and codon AGG 2/16) are rare in *E. coli* [202,225]) and mRNA stability

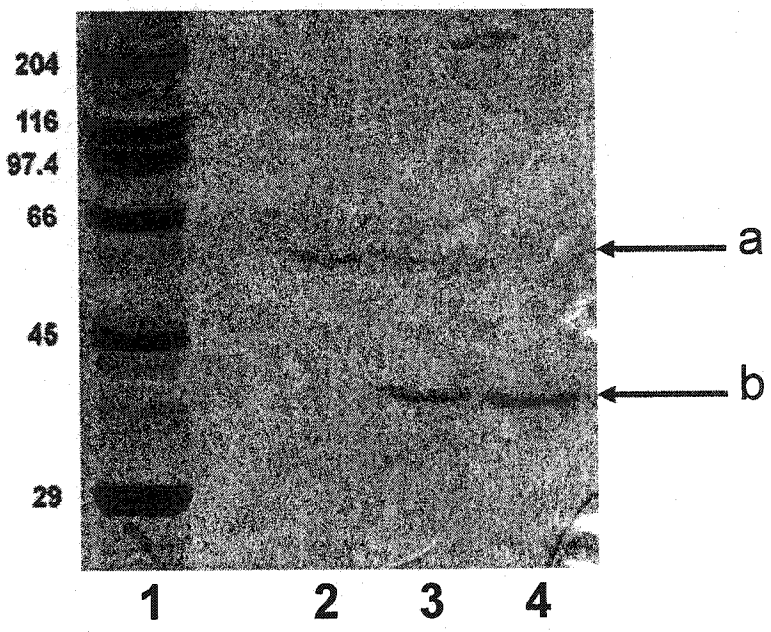


Figure 2.11: 11 % SDS Polyacrylamide gel of the separation of AAC(6')-APH(2") from APH(2")-Ia (175-479) on a gentamicin-agarose affinity column. Proteins were stained with Coomassie Brilliant Blue R-250. Lanes 1- high molecular weight marker (from top to bottom – 204 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 29 kDa), 2- purified AAC(6')-APH(2"), 3- mixture of purified AAC(6')-APH(2") and APH(2")-Ia, and 4- purified phosphotransferase-active C-terminal APH(2")-Ia. The ratio of full-length bifunctional protein produced to that of the APH active fragment was approximately 1:4.

Table 2.2: Purification of AAC(6')-APH(2") from *E. coli* W3110/pBF-9.

Step	Total protein (mg)	APH activity[a] (U)	Specific Activity (U/mg)	Recovery(%)	Purification (n-fold)
Cell lysate	6434	4.2	7	100	---
Sephadex G100	134	3.4	25	81	3.9
Macro prep Q	4.6	3.2	700	76	108
Gentamicin-agarose	3.1	3.5	1100	83	174
Mono Q	1.3	3.6	2800	86	431

1 U = 1 nmole/min. [a]APH activity was monitored by the phosphocellulose binding assay.

in *E. coli* that are responsible. Whatever the case may be, expression of AAC(6')-APH(2") in *E. coli* did not yield the amount of AAC(6')-APH(2") required for our studies.

2.4 Conclusions

A successful purification scheme was developed for AAC(6')-APH(2") that enabled the purification of minute quantities of this protein from *E. coli*. The lack of overexpression observed with *aac(6')-aph(2")* in the numerous constructs tested was at least in part the result of an internal initiation resulting in the production of both the full-length protein and a 36 kDa C-terminal phosphotransferase active fragment. As a result of this internal initiation, the low overall expression levels and our need for copious amounts of full-length AAC(6')-APH(2") for structural and mechanistic characterization, other overexpression systems had to be considered.

2.5 Materials and Methods

2.5.1 Chemicals

Gentamicin and Tris-HCl were obtained from Sigma (St.Louis, MO). γ-^{32}P-labelled ATP was from NEN Life Sciences Products (Boston, MA). All restriction enzymes were purchased from New England Biolabs (Mississauga, ON). IPTG was obtained from Boehringer Mannheim (Laval, PQ). Sephadex G100 and Macro prep Q resins were from Pharmacia (Baie D'Urfe, PQ).

Plasmid pSF815A [55] was the kind gift of Dr. G. Eliopoulos of the New England Deaconess Hospital (Boston, MA).

2.5.2 Cloning and Overexpression

The expression cassette PCR method was used to clone *aac(6')-aph(2")* with complementary primers and plasmid pSF815A as template. The primer to the 5'-end of the gene ($^{5'}$- CCA GGT ACC CAT *ATG* AAT ATA GTT GAA AAT GAA -$^{3'}$) was designed to incorporate a unique *Nde I* restriction site (underlined) ahead of the ATG start codon (Italics), while the primer at the 3'-end incorporated unique *HindIII* and *BamHI* sites (underlined) ($^{5'}$- CCA AAG CTT GGA TCC TCA ATC TTT ATA AGT CCT TTT -$^{3'}$).

Plasmid pBF-7 was constructed by inserting PCR-amplified *aac(6')-aph(2") Nde I* to *Hind III* into plasmid pET22b(+) (Novagen, Madison, WI) [149]. This placed the gene under the control of the bacteriophage T7 promoter which requires the presence of the T7 RNA polymerase for transcription to occur. *E. coli* BL21(DE3) cells which have the T7 polymerase encoded chromosomally were used as the host strain. To avoid having to sequence the entire gene, an internal 1377 bp *Hpa I* to *Hind III* fragment was replaced with that from the sequenced template pSF815A to give plasmid pBF-8. The 80 bp ahead of the *Hpa I* site was sequenced in house by the dideoxy termination sequencing procedure.

Plasmid pBF-9 was constructed by first eliminating an *Nde I* site on the backbone of plasmid pKK223-3 [174] to make plasmid pKK223-3*, then inserting a new Shine Delgarno sequence [150] *Xba I* to *Nde I* (5'- TCT AGA AGG AGA TAT ACA T*AT G* -3', restriction sites are underlined and the start site is in italics) 5' to the *Nde I* and translational initiation start site in the multiple cloning region to make plasmid pKK223-3** (The ribosome binding site was added by sub-cloning the gene encoding *E. coli* D-ala D-ala ligase B with the RBS from plasmid pTB2 *Xba I* to *Hind III* into pKK223-3* [216]), and

finally sub-cloning in *aac(6')-aph(2") Nde I* to *Hind III* from pBF-8 to maintain the RBS and form pBF-9. The cells transformed with this plasmid were *E. coli* W3110.

Plasmid pBF-10 was constructed by PCR amplifying *aac(6')-aph(2")* from pBF-9 with primers which incorporate and a 5'- *Nde I* site (underlined) ($^{5'}$- CCA GGT ACC CAT *ATG* AAT ATA GTT GAA AAT GAA -$^{3'}$) and a 3'- *Xho I* site (underlined) ($^{5'}$- CCA AAG CTC GAG ATC TTT ATA AGT CCT TTT -$^{3'}$) and inserting it into plasmid pET15b (using *E. coli* BL21(DE3) cells) [149]. This placed an N-terminal Histidine tag on AAC(6')-APH(2").

Plasmid pBF-11 was constructed by sub-cloning *aac(6')-aph(2") Nde I* to *Hind III* from pBF-9 into plasmid pDOC55 [213]. This placed the gene under the control of the heat-inducible lambda promoter. This clone was used to transform competent *E. coli* N4830-1 cells which possess the lambda lysogen (lambda repressor protein) encoded chromosomally.

Plasmid pBF-12 was made by cloning *aac(6')-aph(2") EcoRI* to *Hind III* from pSF815A into plasmid pUC19 [148]. This placed the gene under the control of the Lac promoter. *E. coli* JM105 cells were transformed with pBF-12.

Plasmid pBF-13 was constructed by cloning *aac(6')-aph(2") Nde I* to *Hind III* from pBF-9 into plasmid pSKF301. This placed the gene again under the control of the lambda promoter. *E. coli* N4830-1 cells were transformed with this construct.

2.5.3 AAC(6')-APH(2") Purification from *E. coli*

All purification steps were carried out at 4 °C. All Tris buffers were pH adjusted with 4 N HCl after buffers had reached this temperature. Using a fermenter pilot plant, a 20

L culture of Luria Bertani broth (LB) containing 100 μg/mL ampicillin was inoculated with 200 mL of an overnight culture of *E. coli* W3110/pBF-9. Cells were grown at 37 °C to early/mid-log phase (OD_{600} of ~ 1.0) and induced with IPTG to a final concentration of 1 mM. The culture was induced for 4-5 h (reaching OD_{600} of ~ 3). The cells were harvested using a New Brunswick Scientific CEPA model LE continuous flow bench top centrifuge at 50,000 x g. The cells were washed once with ice-cold 0.85 % NaCl and centrifuged at 20,000 x g for 20 min in a Sorval RG-45 floor model centrifuge. The cells were resuspended in 50 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 mM PMSF and 0.1 mM DTT) and lysed by two passes through a French press at 20,000 psi. The cell debris was removed by centrifugation at 15,000 x g for 20 min. The supernatant containing AAC(6')-APH(2") was precipitated using ammonium sulfate precipitations of i) 0-25 % ii) 25-60 % and iii) >60 %. According to results of a specific APH enzyme activity assay, the 25-60 % ammonium sulfate step retained 80 % of enzymatic activity and was dialyzed in buffer A (50 mM Tris-HCl pH 8.0, 1 mM EDTA) and subsequently loaded onto a Sephadex G100 gel filtration column (bed volume = 530 mL) pre-equilibrated with buffer A. Fractions containing AAC(6')-APH(2") were identified by the APH enzyme activity assay and pooled. This sample was loaded onto an Macro prep Q anion-exchange column (Pharmacia) pre-equilibrated with buffer A and chromatographed using a linear gradient of buffer B (Tris-HCl pH 8.0, 1 mM EDTA + 1 M NaCl). Fractions containing AAC(6')-APH(2") activity which eluted at ~50 % B were pooled, dialyzed and stored at 4 °C. A purification table at this point showed that from 7 g of starting material, 36 mg of partially purified enzyme were obtained with a 246-fold purification by the Macro prep Q step. An SDS-

PAGE gel of the purification shows that the bifunctional enzyme is nearly purified to homogeneity, with only 2-3 contaminating proteins observed by silver staining. To eliminate the last few contaminants, a gentamicin-linked agarose column (bed volume of 20 mL) was used as a final chromatographic step. A linear gradient of buffer A (0 to 1 M NaCl) over 20 column volumes was used to elute the protein from the resin. The protein was eluted between 400 and 450 mM NaCl, concentrated to 5 mg/mL using a combination of an Amicon 8200 stirred cell concentrator and Amicon centrifugal filters (10,000 Da cutoff), dialyzed against buffer A with 15 % glycerol and stored at -80 °C.

2.5.4 Preparation of Gentamicin C-agarose Affinity Column

Affigel 10 (*N*-hydroxy succinimide activated agarose) (BioRad, Missassauga, ON) was washed with two volumes of isopropanol and six volumes of water. Gentamicin C complex (250 mg/ml) was added in one volume of 100 mM $NaHCO_3$ pH 8.3 and the slurry was gently rocked at 4 °C for 2 h followed by incubation at room temperature for 2 h. The resin was filtered, placed in 1 M ethanolamine pH 8.3 to block unreacted sites, and shaken at room temperature for 90 min. The suspension was filtered, washed with ten volumes of water and resuspended in 50 mM HEPES pH 7.5, 1 mM EDTA and kept at 4 °C and stored in 5 mM NaN_3 when not in use.

2.5.5 Specific APH Enzyme Activity Assay

Phosphorylation of gentamicin by AAC(6`)-APH(2") was monitored using a phosphocellulose binding assay in which γ-labeled ^{32}P-ATP was supplemented to unlabeled

ATP at a final concentration of 1 mM (1.2×10^5 cpm/nmole). The reaction was composed of an assay buffer (50 mM Tris-HCl pH 8.0, 40 mM KCl and 10 mM $MgCl_2$), gentamicin at 100 μM , γ- ^{32}P-ATP and an aliquot of a fraction containing AAC(6')-APH(2") all in a final volume of 10uL. The reaction was incubated for 30 min at 37 °C and applied onto pre-cut P-81 phosphocellulose paper (~ 1.5cm^2). The paper was washed in a 70 °C water bath for 4 min and washed three consecutive times in a 37 °C water bath for 3 min. The paper was dried and placed in scintillation vials with 5 mL of scintillation fluid and counted with a Beckman liquid scintillation counter. Controls for this assay were: i) a specific activity control (γ-^{32}P-ATP and cold ATP added to reaction in 5 mL scintillation fluid), ii) a no enzyme control and iii) a no aminoglycoside control.

2.5.6 Determination of the Best Overexpression Construct

Using the specific APH enzyme activity assay, all the constructs (*E. coli* i) JM105/pSF815A, ii) BL21(DE3)/pBF-8, iii) W3110/pBF-9, iv) N4830-1/pBF-11, v) JM105/pBF-12 and vi) N4830-1/pBF-13) were tested for differential expression of AAC(6')-APH(2"). All cultures were assayed in triplicate, induced and harvested at similar OD_{600} readings. The cells were lysed by French Press at 20,000 psi and assayed for phosphotransferase activity using the phosphocellulose binding assay. The results were related to the total amount of protein as determined by Bradford assay using Bovine serum albumin as standard protein [15].

2.5.7 Northern Blot Analysis [adapted from 4]

2.5.7.1 Radio-labeled Probe Preparation

The PCR product obtained with the primers AB2716 ($^{5'}$- CCA GGT ACC CAT *ATG* AAT ATA GTT GAA AAT GAA -^{3}) and AB2717 ($^{5'}$- CCA AAG CTT GGA TCC TCA ATC TTT ATA AGT CCT TTT -$^{3'}$) and pSF815A as template was used to make a specific radio-labeled probe. The 5' terminal phosphates were removed using calf intestinal phosphatase (CIP). The reaction mixture contained 12.5 pmol of PCR product, 20 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$ 1 mM $ZnCl_2$ 0.1 U of CIP and water to 50 µL. The reaction was incubated at 37 °C for 30 min followed by inactivation of CIP by heating the reaction at 75 °C for 10 min and precipitation of DNA using 95 % ethanol. The pellet was resuspended in 5 µL of T4 polynucleotide kinase buffer pH 7.5. The reaction mixture to radiolabel the probe was: 12.5 pmol of 5'-dephosphorylated DNA, 1.5 µg BSA, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 pmol (150 µCi) γ-^{32}P-ATP and 20 U of T4 polynucleotide kinase. This reaction was incubated at 37 °C for 60 min and stopped by heating at 75 °C for 10 min. The next step was to separate radio-labeled probe from γ-^{32}P-ATP by loading the sample onto a Sephadex G50 gel filtration/desalting column and assaying 200 µL fractions with a Geiger counter or by scintillation counting. The probe was ~1500 bp in length and end-labeled with ^{32}P-phosphate. The specific activity of the probe was determined to be ~1.2 x 10^7cpm/µg.

2.5.7.2 Isolation of RNA from *E. coli*

All glassware were autoclaved at 120 °C for 30 min. All glassware, tips, solutions, forceps and centrifuge tubes were treated with 0.1 % diethylpyrocarbonate (DEPC), and subsequently autoclaved to sterilize and inactivate DEPC. Two 100 mL cultures of *E. coli* W3110/pBF-9 were grown at 37 °C to an OD_{600} of ~ 0.5, one culture was induced with IPTG to a final concentration of 1 mM while the other was an uninduced control. Induction time was 2 h and growth was stopped by the addition of 1/20 volume of stop buffer [20 mM sodium azide, 20 mM aurintricarboxilic acid (ATA-RNase inhibitor), 20 mM EDTA and 200 mM Tris-HCl pH 8.0] The cells were harvested by centrifugation at 5,500 x g, resuspended in 2 mL of STET lysing solution [8 % (w/v) sucrose, 5 % (v/v) Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 7.0] and 100 µL of 200 mM vanadyl ribonucleoside complex-RNase inhibitor (VRC). This was followed by a 2 mL (1:1) phenol-chloroform extraction with centrifugation at 10,000 X g, 4 °C for 10 min. The top aqueous phase was collected and total nucleic acids were precipitated by adding 1/10 volume 3 M sodium acetate and 2 volumes of ice-cold 100 % ethanol. This solution was centrifuged at 10,000 x g , 4 °C for 10 min. The pellets were resuspended in 2 mL of 10 mM vanadyl-ribonucleoside complex (VRC; Gibco/BRL). This was followed by two other (1:1) phenol:chloroform extractions and re-precipitation of nucleic acids to give a final pellet for both induced and negative control which were resuspended in 6 mL of DEPC-treated water.

2.5.7.3 Purification of RNA on CsCl Gradients

To the re-suspended pellet, 4.5 g of solid CsCl were added and the volume was adjusted to 9 mL with DEPC-treated water. In an ultra clear SW-41Ti ultracentrifugation tube, 3 mL of 5.7 M CsCl was added as a cushion, the sample (9 mL) was layered carefully on top so as to keep the two layers well separated. This was followed by ultracentrifugation using an SW-41Ti rotor for 24 h at 30,000 rpm (150,000 x g) at 20 °C.

After ultracentrifugation, the ultra clear tubes were removed from the SW-41Ti rotor and the DNA at the interface was carefully extracted. The upper CsCl layer was taken off with a sterile Pasteur pipette and the remaining CsCl cushion poured out. The pellet which remained is strictly RNA. The pellet was resuspended in 360 µL of DEPC-treated water and transferred to a microcentrifuge tube. One tenth volume of 3 M sodium acetate and 2.5 volumes of 100 % ethanol were added to precipitate RNA at -70 °C for 20 min. Centrifugation for 5 min at 12,000 x g, at 4 °C was performed to pellet the RNA. The RNA pellet was washed with 70 % ethanol and air dried by lyophilization. The pellet was further dissolved in 200 µL of DEPC-treated water (0.1% treatment followed by autoclaving) and quantified by A_{260}/A_{280} nm readings. The concentration was adjusted to 4 mg/mL. Samples were frozen at -70 °C for long term storage.

2.5.7.4 Agarose/Formaldehyde Gel Electrophoresis

MOPS running buffer (400 mM MOPS pH 7.0, 100 mM sodium acetate and 10 mM EDTA), formaldehyde and formamide were added to the RNA samples as well as a formaldehyde loading buffer (1 mM EDTA, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v)

xylene cyanol, and 50 % (w/v) glycerol). These samples (run in duplicate, that is one set for staining and one set for transfer) were run on an 1 % agarose/ 2.2 M formaldehyde gel in an RNase-free gel boat at 25 V for 3.5 h. One half of the gel was stained with ethidium bromide, visualized and photographed and the other half was used for the transfer.

2.5.7.5 Transfer of RNA from Gel to Nitrocellulose

The unstained portion of the gel was placed in an RNase-free glass dish and rinsed several times with ddH_2O and then 10 gel volumes of 20 X SSC (sodium chloride/sodium citrate –20X stock: 3 M NaCl, 0.3 M Na_3citrate, pH 7) to remove formaldehyde. The transfer chamber consisted of a sponge soaked in 20 X SSC, three pieces of Whatman 3 MM paper, the agarose/formaldehyde gel, the nitrocellulose membrane, five sheets of Whatman 3 MM paper cut to the same size as gel and paper towels cut to size and enough to put pressure on the sandwich when the chamber lid was in place. The transfer proceeded overnight at room temperature.

The membrane was rinsed and the orientation of the gel and position of the wells were marked. The nitrocellulose paper was baked under vacuum for 2 h at 80 °C to fix RNA to the membrane. The transfer efficiency was determined by staining the gel used for the transfer with ethidium bromide.

2.5.7.6 Hybridization Analysis

The membrane was soaked in 6 X SSC, placed RNA side up in a sealed bag and 1 mL of formamide prehybridization/hybridization solution (5 X SSC (from 20 X stock), 5 X

Denhardt solution, 50 % (w/v) formamide, 1 % (w/v) SDS and 100 µg/mL salmon sperm DNA) (Denhardt solution – 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin in 500 mL water and filter sterilized) was added per 10 cm^2 of membrane. The bag was placed in a hybridization oven with rotation for 3 h at 42 °C. The double stranded probe was denatured for 10 min at 100 °C, transferred to ice, and then a desired volume of probe was pipetted into the hybridization tube and the incubation continued overnight. The hybridizing solution was then poured off and an equal volume of 2 X SSC/0.1 % SDS was added and this was incubated at room temperature for 5 min. This step was repeated a second time with a low stringency wash by replacing the wash solution with 0.2 X SSC/0.1 % SDS and again incubated for 5min at room temperature. The 0.2 X step was repeated again and this was followed by autoradiography.

Chapter 3

AAC(6')-APH(2") purification and characterization from *Bacillus subtilis*

Adapted from:

Daigle, D.M., Hughes, D.W., and Wright, G.D., *Chem. & Biol.* 1999, vol. 6, pp. 99-110.

Chapter 3

3.1 Abstract

The bifunctional AAC(6')-APH(2") resistance enzyme has been expressed in *Bacillus subtilis*. The purified enzyme is found as a monomer of 56,883 kDa and possesses the predicted activities, an N-terminal *N*-acetyltransferase and a C-terminal *O*-phosphoryltransferase. Through the construction of an N-terminal truncation, the APH activity has been determined to be located between residues 175 to 479. AAC(6')-APH(2") possesses a broad substrate specificity exhibited by both activities, which accounts for the extensive resistance profiles encountered in the clinics. Surprisingly, two aminoglycosides having a 6'-hydroxyl group instead of a 6'-amino group, lividomycin A and paromomycin, were acetylated by the bifunctional acetyltransferase. Base hydrolysis and infrared spectroscopy indicated the first occurrence of *O*-acetyltransfer to an aminoglycoside catalyzed by an AAC enzyme. Large-scale inactivations together with mass spectrometry and nuclear magnetic resonance spectroscopy elucidated a unique regiospecificity of phosphoryltransfer for the 4,5-disubstituted aminoglycosides. Unexpectedly, phosphoryltransfer occurred primarily on the 3'-OH of the 6-aminohexose ring A. Diphosphorylated species were also found with an additional phosphate at the 3'''-OH of ring D of neomycin C. The only exception to this pattern was the 3'-deoxy aminoglycoside Lividomycin A which was phophorylated on the 5"-OH of pentose ring C. Therefore, the bifunctional AAC(6')-APH(2") is capable of inactivating virtually all known

aminoglycoside substrates by a combination of *N*- and *O*-acetyltransfer of the functionality at the 6'-position and phosphorylation of multiple hydroxyl groups (3', 2", 5" and 3"'). These findings present a daunting challenge for future of aminoglycoside drug design towards Gram positive pathogens like enterococci and staphylococci.

3.2 Introduction

Conventional attempts to clone and overexpress AAC(6')-APH(2") in *E. coli* were complicated by the presence of an internal protein translational start site in *aac(6')-aph(2")* which lead to expression of not only the full-length AAC(6')-APH(2"), but also a 36 kDa C-terminal APH(2") active protein. Aside from the presence of the truncated APH(2")-Ia, the levels of expression of the full-length protein were not sufficient for proper structural and mechanistic characterization of the enzyme, therefore, other protein expression systems had to be investigated.

There are alternatives to expressing bacterial proteins in *E. coli*, however most of these systems are eukaryotic in nature. Protein expression in yeast (*Saccharomyces cerevisiae* or *Pichia pastoris*) [47,142,161,212] and baculovirus vector infection of *Spodoptera frugiperda* (Sf9) insect cells [144,171,182,226] are both frequently used to overproduce heterologous proteins, but are most often employed for expression of eukaryotic proteins. A third option was protein expression in *Bacillus subtilis*, a Gram positive sporulating bacteria [18,19,83,95,159,177].

Although development of protein expression systems in *B. subtilis* was limited, there were a series of *E. coli/B. subtilis* shuttle vectors which simplified cloning efforts

[19]. One obvious advantage of the use of *B. subtilis*, is that it is a prokaryotic system consisting of a Gram positive bacterium, similar to the original hosts of AAC(6')-APH(2"), *E. faecalis* and *S. aureus* [88,154,170]. A second frequently exploited strategy available with *B. subtilis* protein expression is the ability of bacillus to secrete proteins into the growth medium [96]. In fact, several species of bacilli are utilized industrially for the production of various proteins such as α-amylases and alkaline proteases which are native to this organism [53,221]. For these reasons, further attempts to overexpress and characterize AAC(6')-APH(2") were carried out in *B. subtilis*.

Although some work had been done on protein characterization and the regiospecificity of phophoryl and acetyl transfer to the 4,6-disubstituted aminoglycosides [6,130], the regiospecificity of the 4,5-disubstituted aminoglycosides such as neomycin and lividomycin A had not been elucidated. This chapter describes the overexpression and purification of AAC(6')-APH(2") from *B. subtilis* along with the characterization of substrate specificity for both the *N*-acetyltransferase and the *O*-phophoryltransferase as well as the regiospecificity of phosphoryltransfer to the 4,5-disubstituted aminoglycosides. Furthermore, we find that AAC(6')-APH(2") is capable of *O*-acetyl transfer.

3.3 Results and Discussion

3.3.1 Expression of AAC(6')-APH(2") in *B. subtilis*

The *aac(6')-aph(2")* gene was amplified from the source plasmid pSF815A [55] and cloned into the shuttle vector pRB374 (kind gift of Dr. Reinhold Brückner, Heidelberg, Germany) to generate the expression plasmid pBF-14 (Figure 3.1) [19]. This clone placed

the gene under the control of the *vegII* promoter giving rise to constitutive expression of the target gene on a self-replicating plasmid in *B. subtilis* [19]. This construct expressed the bifunctional protein to high levels and did not give rise to any measurable levels of truncated protein. A three-step purification yielded 70 mg of functional 57 kDa AAC(6')-APH(2") from 10 L of culture (Figure 3.2 ,Table 3.1).

3.3.2 Substrate Specificity and Unique *O*-acetyltransfer Activity of AAC(6')-Ie

Using a specific acetyltransferase activity assay [76,208] (Figure 3.3), all 6'-amino containing aminoglycosides were found to be substrates with practically invariant efficiencies ranging from 10^4-10^5 $M^{-1}s^{-1}$ for the 15 6'-amino aminoglycosides tested (maximum difference $\Delta k_{cat}/K_m = 12.6$) (Table 3.2). Unlike other AAC(6')'s, AAC(6')-APH(2") confers resistance to fortimicin through *N*-acetylation (Figure 3.4). The K_m values varied from 1-26 μM and the k_{cat} values ranged from 0.2 to 3 s^{-1}. Unexpectedly, 6'-hydroxyl containing aminoglycosides which are inhibitors of other AAC(6')-class enzymes such as AAC(6')-Ii from *E. faecium* [215], were substrates of the AAC(6')-Ie. The drugs lividomycin A and paromomycin were found to possess similar K_m values to other aminoglycosides but with k_{cat} levels reduced approximately 10-fold (Table 3.2). This suggested the possibility of acetyl transfer to amino groups other than at the 6'-position or *O*-acetyltransfer to the 6'-hydroxyl, both of which were unprecedented for this class of enzyme. Susceptibility to mild saponification conditions was used to distinguish the type of modification obtained with lividomycin A and paromomycin.

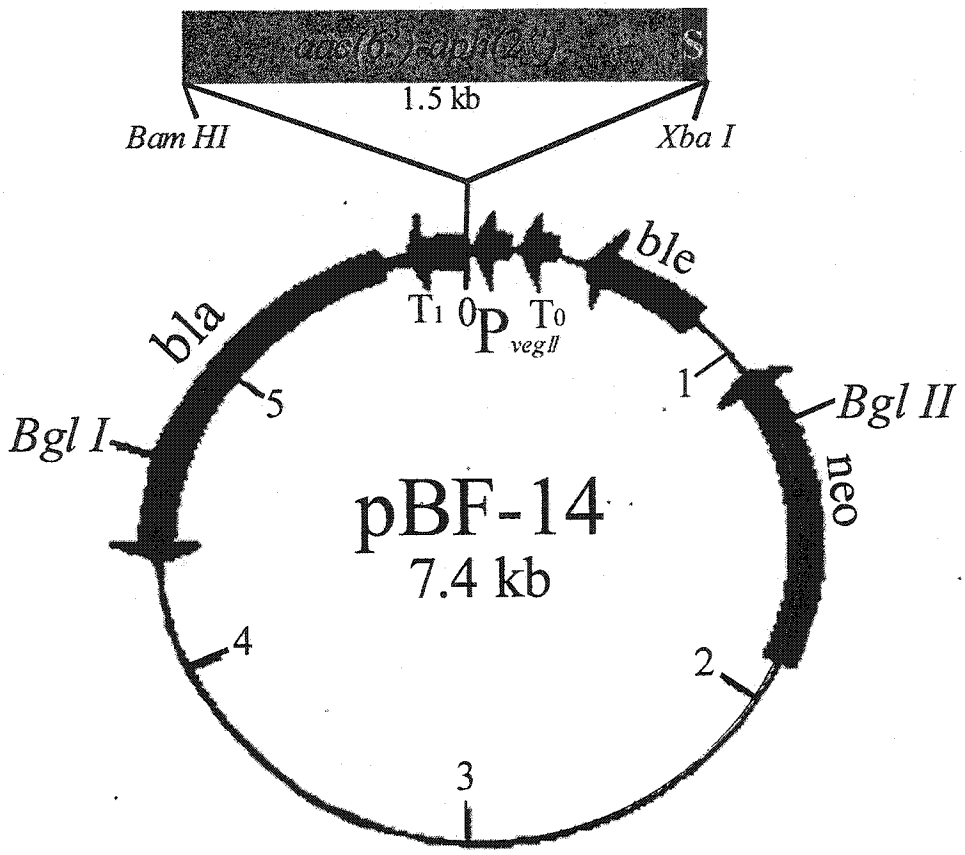


Figure 3.1: Map of *B. subtilis/E. coli* shuttle vector pBF-14 [19]. Location of resistance genes (*ble, bla* and neo), terminators (T_0 and T_1) and the *vegII* promoter (P*vegII*) are marked. A 1.5 kb *XbaI/BamHI* insert containing *aac(6')-aph(2")* and a ribosome binding site (S) from the bacteriophage T7 gene are represented as a green and orange rectangle. The plasmid size is 7.4 kb which consists of the parent plasmid pRB374 (5.9 kb) and the insert (1.5 kb).

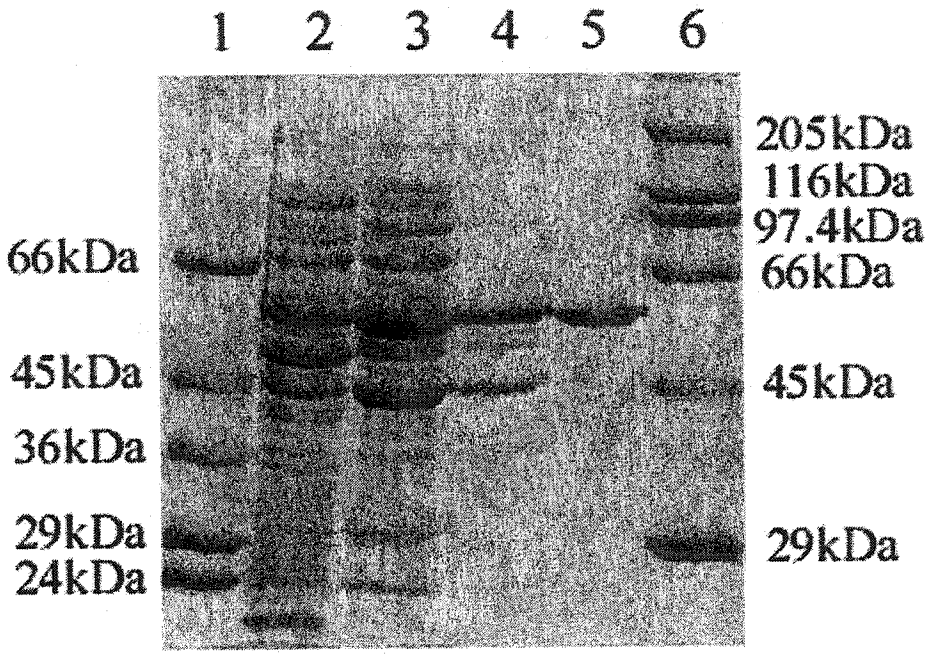


Figure 3.2: 11 % SDS-polyacrylamide gel stained with Coomassie Blue R-250 showing the purification of AAC(6')-APH(2") from *B. subtilis* 1A752/pBF-14. Lane 1 and 6, low and high molecular weight markers from BioRad; lane 2, cell lysate - generated by French Press (S1); lane 3, Q Sepharose anion exchange; lane 4, Sephadex G100 gel filtration; and lane 5, Gentamicin C-agarose affinity chromatography.

Table 3.1: Purification of AAC(6')-APH(2") from *B. subtilis* 1A752/pBF-14.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (n-fold)
Cell Lysate	1950	APH[1] ---- AAC 47.3	APH ------ AAC 0.024	APH ----- AAC 100	APH ----- AAC 0
Q Sepharose	629	APH 22.4 AAC 27.8	APH 0.036 AAC 0.044	APH 100 AAC 59	APH ----- AAC 1.9
Sephadex G100	255	APH 26.3 AAC 24.8	APH 0.103 AAC 0.098	APH 117 AAC 53	APH 4.3 AAC 4.0
Gentamicin-agarose	71.8	APH 15.8 AAC 13.0	APH 0.22 AAC 0.181	APH 71 AAC 28	APH 9.2 AAC 7.5

1 U = 1 μmole/min. [1] High levels of background ATPase activity prevented the determination of APH activity. APH activity was monitored by a coupled assay consisting of pyruvate kinase and lactate dehydrogenase (PK/LDH, see Materials and Methods).

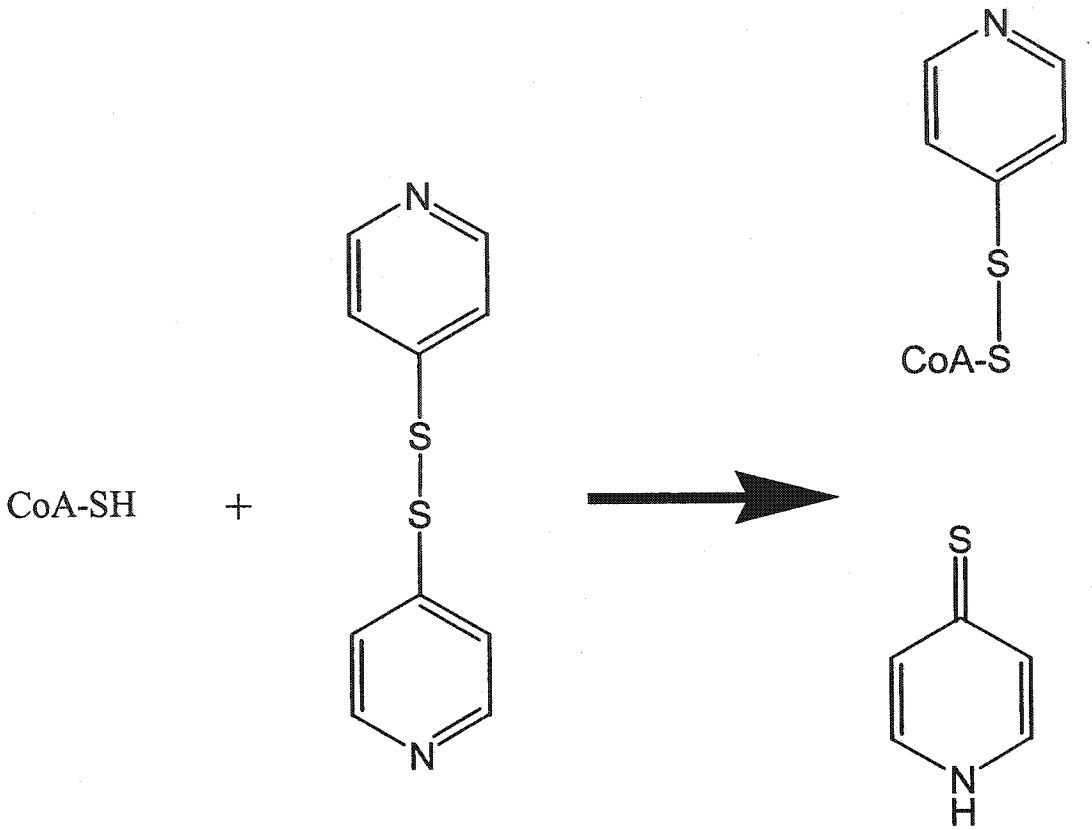


Figure 3.3: Acetyltransferase assay using 4,4'-dithiodipyridine. The assay monitors the production of the thiol of CoASH by reacting to form 4-thiopyridone which has a λ_{max} of 324 nm and a molar absorptivity coefficient of 19,800 M^{-1} cm^{-1} [208].

Table 3.2: Kinetic Parameters for AAC(6')-Ie Activity.

Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	K_i (μM)[d]	k_{cat}/K_m ($M^{-1}s^{-1}$)
kanamycin A	1.4 ± 0.5	0.31 ± 0.05	50.2 ± 22.3	2.21×10^5
kanamycin B	10.6 ± 2.9	1.7 ± 0.3	10.5 ± 3.4	1.61×10^5
tobramycin	2.3 ± 0.9	0.68 ± 0.1	50.0 ± 20.1	3.0×10^5
dibekacin	11.1 ± 6.4	4.9 ± 2.1	6.2 ± 4.5	4.41×10^5
amikacin	20.9 ± 5.3	2.11 ± 0.35	23.7 ± 7.8	1.01×10^5
gentamicin C[b]	4.6 ± 1.5	0.85 ± 0.15	76.6 ± 39.7	1.85×10^5
gentamicin B	1.1 ± 0.2	0.3 ± 0.01		2.73×10^5
isepamicin	18.7 ± 9.0	1.66 ± 0.50	88.3 ± 52.7	8.87×10^4
netilmicin	3.2 ± 0.5	0.87 ± 0.04		2.72×10^5
sisomicin	6.5 ± 2.1	2.92 ± 0.31		4.5×10^5
neomycin B	4.2 ± 0.8	1.53 ± 0.09		3.64×10^5
ribostamycin	6.1 ± 2.5	2.06 ± 0.47	54.7 ± 31.4	3.38×10^5
butirosin A	25.8 ± 2.7	1.27 ± 0.06		4.92×10^4
neamine	15.0 ± 7.0	5.26 ± 1.95	6.7 ± 2.9	3.51×10^5
fortimicin	2.2 ± 0.4	0.19 ± 0.01		8.64×10^4
lividomycin A	10.1 ± 2.8	0.020 ± 0.001		2.0×10^3
paromomycin	17.5 ± 5.0	0.032 ± 0.02		1.8×10^3
acetyl CoA[c]	5.9 ± 1.4	0.73 ± 0.04		1.24×10^5

[a] Acetyl CoA concentration was held at 100 μM for aminoglycoside assays.
[b] Gentamicin C is a complex of gentamicin C1, C1a and C2.
[c] fixed substrate was kanamycin A at 100 μM.
[d] Substrate inhibition observed with aminoglycoside tested.

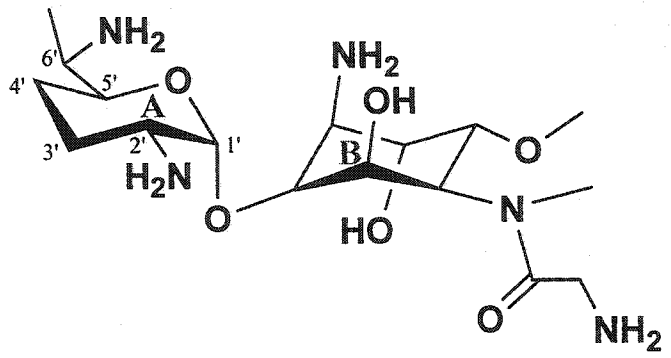


Figure 3.4: Structure of fortimicin, a substrate of the AAC(6')-Ie.

The purpose of this experiment was to distinguish between the ester linkage that would be found in the *O*-acetylated product versus the amide linkage that would be expected from *N*-acetylation. The ester linkage is more susceptible to hydrolysis than the amide bond that would be found in 6'-*N*-acetylated kanamycin A, which was used as a control. This was indeed the case as the ^{14}C-acetylated lividomycin A and paromomycin selectively loss their acetyl group more rapidly than did ^{14}C-acetylated kanamycin A under mild base hydrolysis conditions (Figure 3.5). Because of the added lability of the ester bond, we were unable to purify significant amounts of acetylated paromomycin and lividomycin A for mass spectrometry and NMR analysis, however we were able to purify sufficient quantities to perform infrared spectroscopy. This analysis identified a prominent signal at 1719 cm^{-1}, consistent with the formation of a 6'-acetyl ester with both aminoglycosides (Figure 3.6 and 3.7).

While unprecedented with aminoglycoside acetyltransferases, *O*-acetyltransfer is not uncommon as enzymes involved in acetylation of toxic and mutagenic hydroxy-substituted arylamines in *E. coli* and *Salmonella typhimurium* perform *O*-acetyltransfer [173,219]. O-acetyltransfer is also observed with chloramphenicol acetyltransferase and an enzyme involved in L-Cysteine biosynthesis in *E. coli,* Serine acetyltransferase (SAT) [48,123]. This unique activity exhibited by AAC(6')-Ie complements its' already prodigious substrate profile. This behavior will therefore have to be taken into consideration when designing new aminoglycosides which require hydroxyls and amino groups for antibacterial activity.

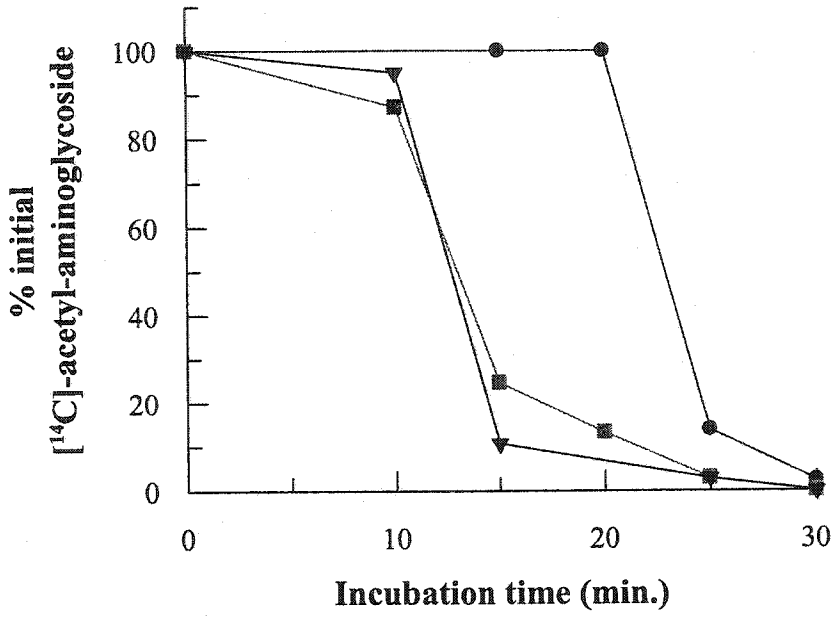


Figure 3.5: Sensitivity of [^{14}C]-acetylated aminoglycosides to mild alkaline treatment. The amount of label remaining on the AAC(6')-APH(2") acetylated aminoglycosides was determined using a phosphocellulose-binding assay as described in Materials and Methods section. ● [^{14}C] acetyl-kanamycin A; ■ [^{14}C] acetyl-lividomycin A; ▼ [^{14}C] acetyl-paromomycin.

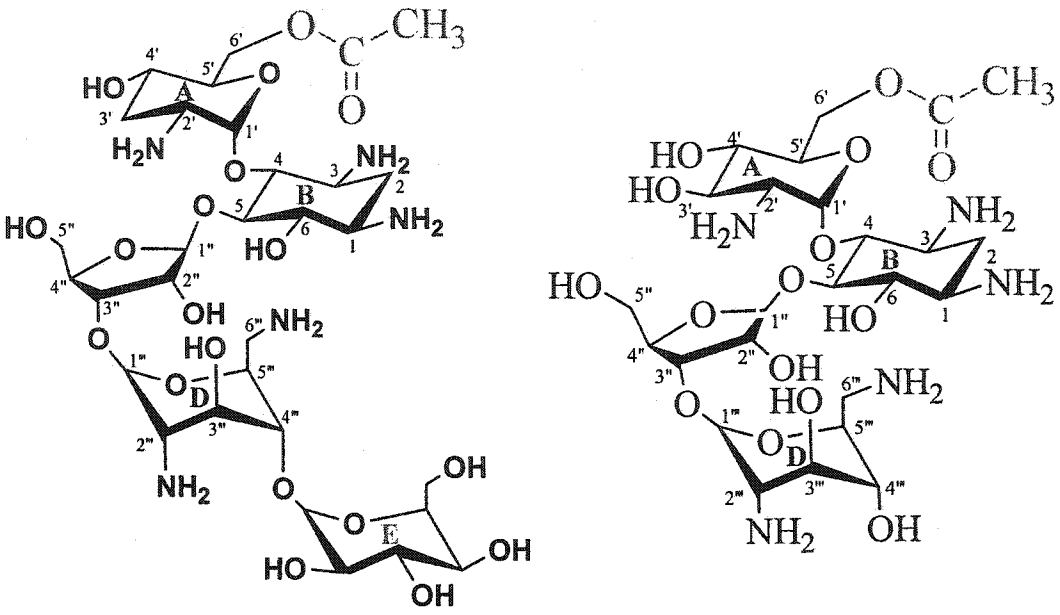


Figure 3.6: Structures of *O*-acetylated lividomycin A and paromomycin.

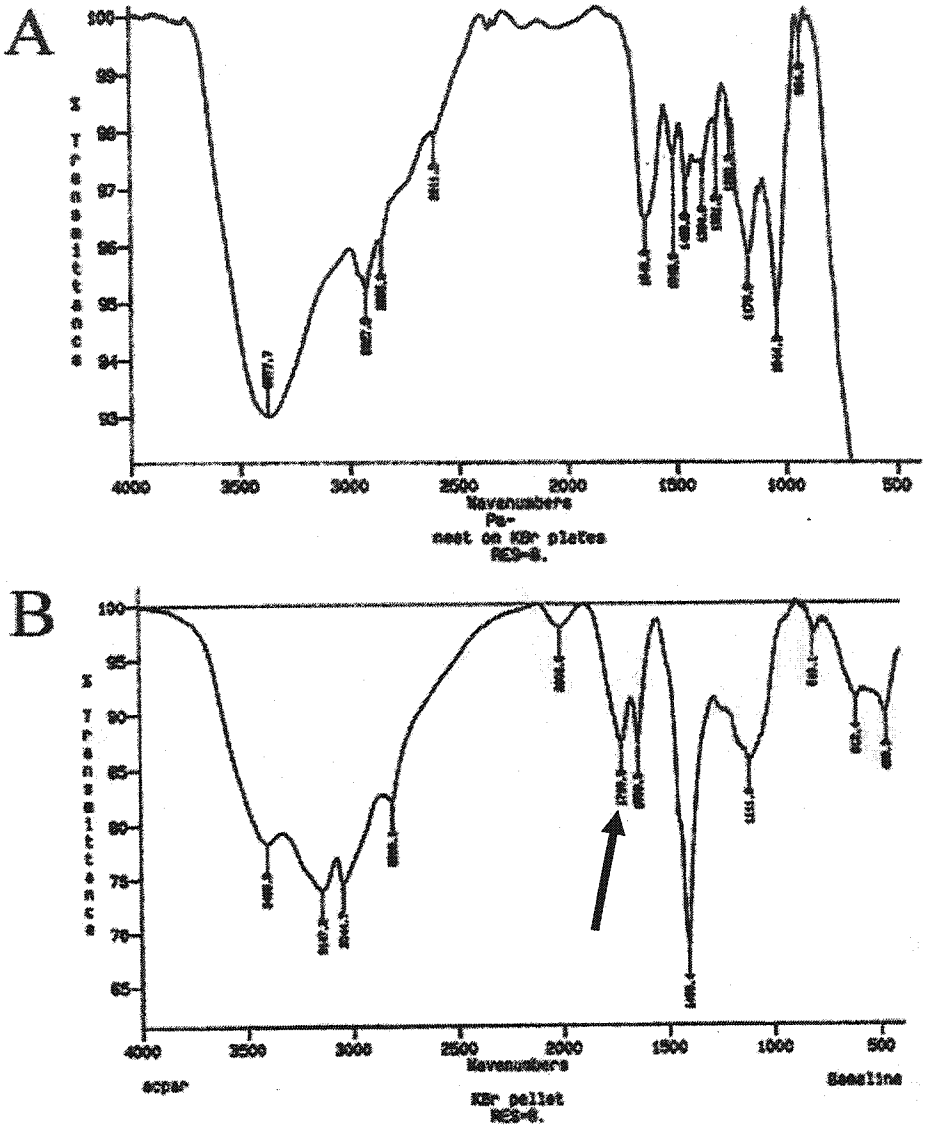


Figure 3.7: Infrared spectroscopy spectrum of purified acetyl-paromomycin: evidence for *O*-acetyltransfer. (A) IR spectrum of paromomycin. (B) IR spectrum of acetyl-paromomycin. A prominent signal denoted by a black arrow at 1719 cm^{-1} is consistent with the formation of a 6'-acetyl ester.

3.3.3 Substrate Specificity of APH(2")-Ia

Using a pyruvate kinase/lactate dehydrogenase coupled assay (figure 3.8), the K_m and k_{cat} values for various aminoglycoside substrates of APH(2")-Ia were determined. The substrate specificity of the phosphotransferase did not show similar efficiencies between drugs as did the acetyltransferase. The kinetic constant K_m varied from 2-200 μM while k_{cat}/K_m values varied from 10^3-10^5 $M^{-1}s^{-1}$ (Table 3.3). These values are similar to those found for other phosphotransferases such as APH(3')-IIIa [135], however, they are lower than those found for other APH(3') enzymes for which k_{cat}/K_m values approach the diffusion limit [183].

APH(2")-Ia is capable of tolerating multiple substitutions on the 6-aminopyranose ring (Ring A – figure 3.9). Similarly, substitutions on the 2-deoxystreptamine ring (Ring B) with a pentose at position C6 or a hexose at positions C5 also have minimal effects on specificity. Aminoglycosides containing two rings (neamine), three rings (ribostamycin and butirosin) and four rings (neomycin and paromomycin) are equivalently phosphorylated by AAC(6')-APH(2"), however the five ring aminoglycoside lividomycin, shows a 15-fold increase in the observed K_m. The most critical substitutions which alter substrate specificity are located at the *N*1 position of the central 2-deoxystreptamine ring (Ring B). Comparison between different aminoglycosides which differ only in *N*1-substituents allows the quantitative assessment of the impact of such a modification on substrate specificity. For example, amikacin, which is *N*1-4-amino-2-hydroxy butyryl kanamycin A, shows a 22-fold increase in K_m and a 2.5-fold decrease in k_{cat} over that of kanamycin A (Table 3.3).

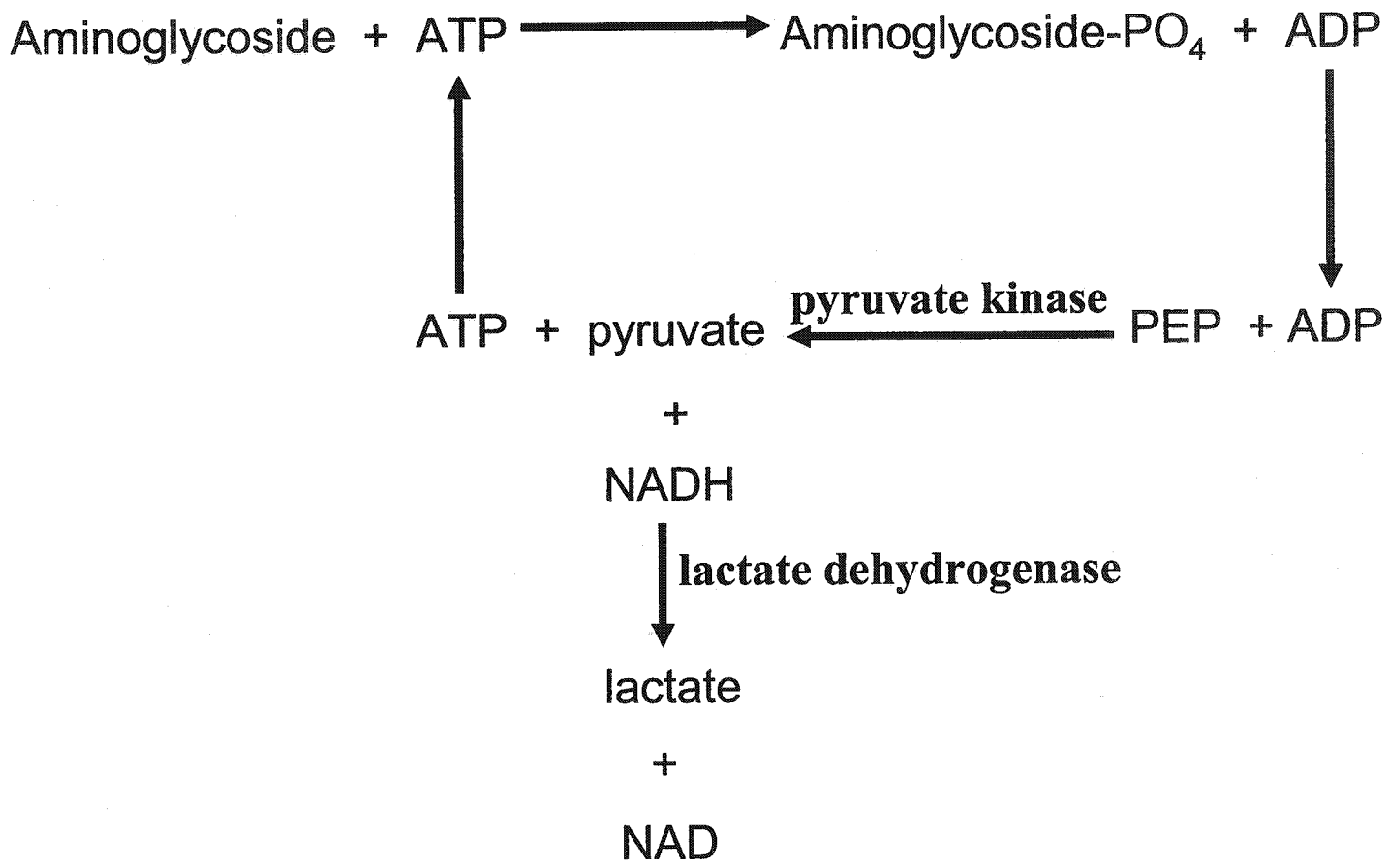


Figure 3.8: Coupled enzyme assay system to monitor APH(2")-Ia activity. The assay regenerates ATP from ADP using pyruvate kinase and lactate dehydrogenase and phosphoenolpyruvate (PEP). Enzymes are denoted in bold red.

Table 3.3: Kinetic Parameters for APH(2")-Ia Activity.

Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	K_i (μM)[d]	k_{cat}/K_m ($M^{-1}s^{-1}$)
kanamycin A	7.0 ± 0.9	0.41 ± 0.02		5.84×10^4
kanamycin B	9.3 ± 2.2	1.07 ± 0.16	65.4 ± 19.6	1.15×10^5
tobramycin	4.8 ± 1.1	0.33 ± 0.02		6.9×10^4
dibekacin	3.6 ± 0.8	0.34 ± 0.02		9.4×10^4
amikacin	158 ± 33	0.16 ± 0.01		1.0×10^3
gentamicin C[b]	3.4 ± 0.5	0.23 ± 0.01		6.8×10^4
gentamicin B	38.9 ± 8.5	0.65 ± 0.09	173 ± 49	1.67×10^4
isepamicin	197 ± 44	0.59 ± 0.04		3.0×10^3
netilmicin	36.4 ± 5.7	0.21 ± 0.01		5.77×10^3
sisomicin	12.2 ± 1.1	0.4 ± 0.01		3.28×10^4
neomycin B	1.9 ± 0.2	0.19 ± 0.01		1.0×10^5
paromomycin	3.4 ± 1.5	0.49 ± 0.1	77.5 ± 36.1	1.44×10^5
lividomycin A	32.9 ± 8.1	0.22 ± 0.02		6.7×10^3
ribostamycin	2.0 ± 0.8	0.25 ± 0.04	176 ± 108	3.71×10^5
butirosin A	11.6 ± 1.4	0.43 ± 0.09		3.71×10^4
neamine	9.6 ± 3.1	0.17 ± 0.01		1.8×10^4
ATP[c]	24.1 ± 6.1	0.17 ± 0.02		7.1×10^3

(a) ATP concentration was held at 1 mM for aminoglycoside assays.
(b) Gentamicin C is a complex of gentamicin C1, C1a and C2.
(c) The fixed substrate was kanamycin A held at 100 μM.
(d) Substrate inhibition observed with aminoglycoside tested.

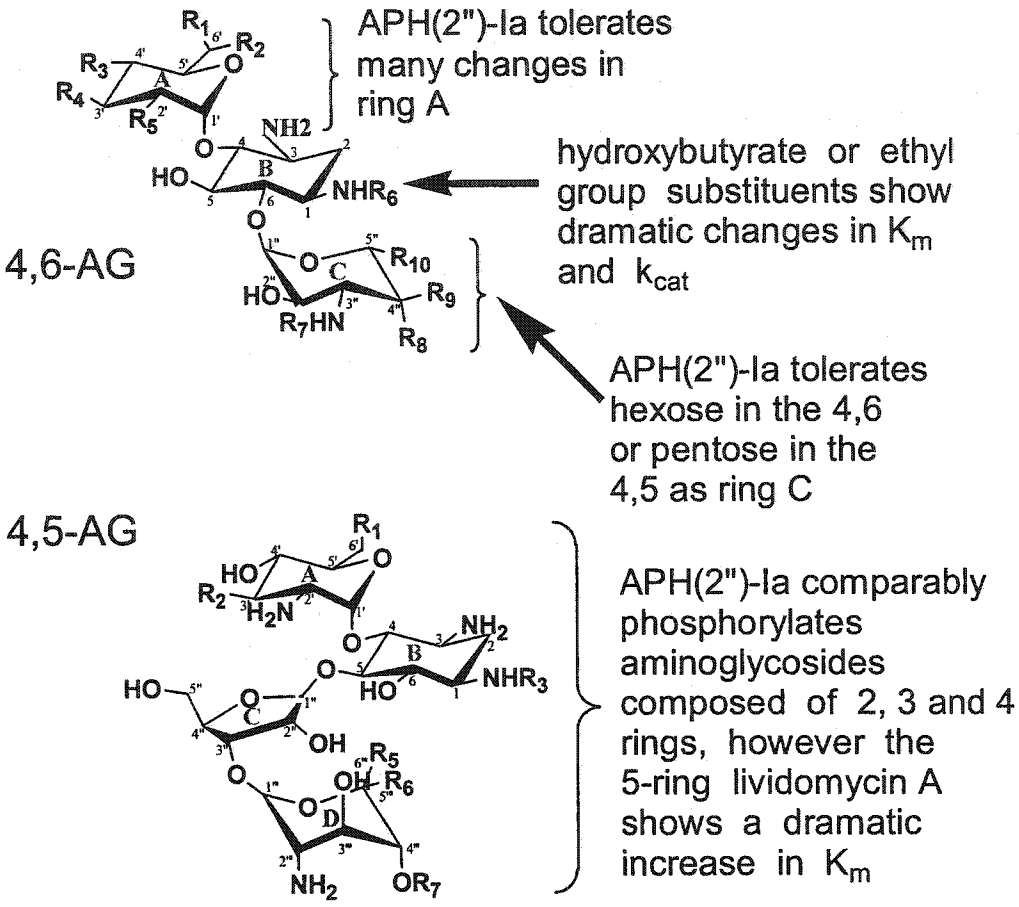


Figure 3.9: Summary of APH substrate specificity towards 4,6- and 4,5-disubstituted aminoglycosides.

Butirosin, which consists of *N*1-4-amino-2-hydroxybutyryl ribostamycin, has a 5.8-fold higher K_m than its parent drug ribostamycin (Table 3.3). The clinically important aminoglycoside netilmicin, *N*1-ethylsisomicin, displayed a 3-fold increase in K_m over that of sisomicin and finally, isepamicin (*N*1-4-amino-2-hydroxybutyryl-N3"-acetylgentamicin B) had a 5-fold increase in observed K_m as compared to gentamicin B (Table 3.3). Taken together, these results report that any substitution to the *N*1 of the 2-deoxystreptamine ring is unfavorable to the APH activity (Figure 3.10). Finally, APH(2")-Ia has been shown to possess the broadest substrate spectrum of all known aminoglycoside kinases.

3.3.4 Regiospecificity of APH(2")-Ia Catalyzed Phosphotransfer to 4,5-disubstituted Aminoglycosides

The regiospecificity of acetyl transfer and phosphoryl transfer to kanamycin A has already been determined using nuclear magnetic resonance spectroscopy to be the 6'-amino group and the 2"-hydroxyl group respectively [6]. Substrate specificities for the acetyltransferase, as well as those for the phophoryltransferase with the 4,6-disubstituted aminoglycosides are consistent with these observations. The regiospecificity of phosphoryl transfer to the 4,5-disubstituted aminoglycosides had not yet been determined. Therefore, a large-scale inactivation of neomycin C followed by purification and ESI-mass spectrometry analysis was performed. This purification revealed two products that were identified as mono-phosphorylated neomycin C (m/z 695.4) and diphosphorylated neomycin C (m/z 775.3) (Table 3.4).

To determine the sites of phosphorylation on neomycin C, a combination of one and

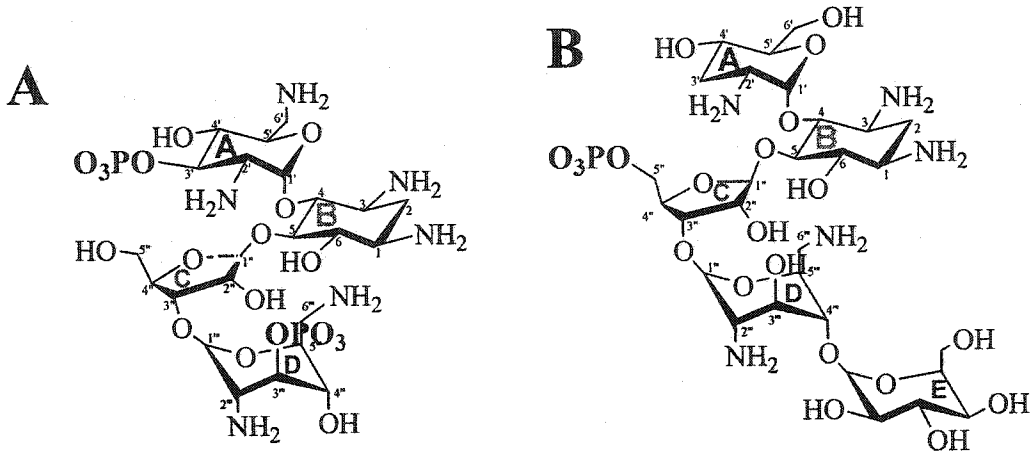


Figure 3.10: Summary of the determined regiospecificity of phosphorylation to the 4,5-disubstituted aminoglycosides by AAC(6')-APH(2"). Represented are: **(A)**-diphosphorylated neomycin C for which single phosphorylation occurs on the 3'-hydroxyl, and **(B)**- phosphorylated lividomycin A on the 5"-hydroxyl of ring C.

two-dimensional NMR techniques were employed. NMR analysis was performed by Dr. Don Hughes (department of Chemistry, McMaster University). ^{1}H, ^{13}C and ^{31}P-NMR spectra for mono-phosphorylated neomycin C were consistent with phosphorylation occurring on the 3'-OH of pyranose ring A as had been previously been demonstrated with APH(3')-IIIa [193]. The second site of phosphorylation on diphosphorylated neomycin C was determined to be at the 3'''-hydroxyl of Ring D (Figure 3.10). The unexpected identification of these sites of phosphorylation on neomycin as being the 3' and 3'''-hydroxyls prompted us to examine the phosphorylation of the 3'-deoxy aminoglycoside lividomycin A. NMR analysis revealed that phosphorylation occurred on the 5"-OH on pentose ring C of lividomycin A (Figure 3.10). Consequently, the bifunctional phosphotransferase (APH(2")-Ia) has the ability to phosphorylate 4,5-disubstituted aminoglycosides on the 3', 5" and 3'''-hydroxyl groups, making this APH a highly potent aminoglycoside resistance determinant.

3.3.5 Delineation of the APH Functionality

Previous experiments to delineate the functionalities had been performed and showed that the AAC was located between residues 1 and 230, while the APH was located between residues 138 and 479 [76] (Figure 3.11).

Fortuitously, through protein expression and purification of AAC(6')-APH(2") in *E. coli*, an internal translational initiation site was found. This resulted in the production of both a full length AAC(6')-APH(2") and a C-terminal APH(2")-Ia, which after N-terminal amino acid sequencing was determined to start at Met 175. The DNA sequence

Table 3.4: Electrospray mass spectrometry of phosphorylated 4,5-disubstituted aminoglycosides.

Phosphorylated aminoglycoside	MW of parent compound (g/mol)	m/z of mono-phosphorylated species	m/z of diphosphorylated species
Neomycin C	614.7	695.4	775.4
Lividomycin A	762.3	842.3	-----[a]

[(a)] No diphosphorylated species observed.

upstream of the Met 175 codon displayed significant similarity to *E. coli* ribosome binding sites. Subsequently, we have cloned, expressed and purified both proteins separately (AAC – amino acids 1-174 and APH – amino acids 175-479) (Figure 3.12) from *E. coli* and have found that only the APH(2")-Ia (175-479) is active exhibiting only a minor impact on enzyme efficiency compared to the full-length AAC(6')-APH(2") (Figure 3.12, Table 3.5). The two overexpression plasmids constructed to express both enzymes consisted of the pBF-9 construct where *aac(6')-aph(2")* was replaced by *aac(6')*-Ie (bases 1-522 with the addition of a new stop codon) or *aph(2")*-Ia (bases 175-1440 including the original stop codon).

Finally, polyclonal antibodies raised against full-length AAC(6')-APH(2") were able to detect the two separate resistance proteins determined by Western blotting (Figure 3.13a). Additionally, when these antibodies were used to detect AAC(6')-APH(2") in lysates of *E. faecalis* ATCC 49476, only the full-length protein was detected suggesting that the APH active fragment observed in *E. coli* does not occur in its natural host.

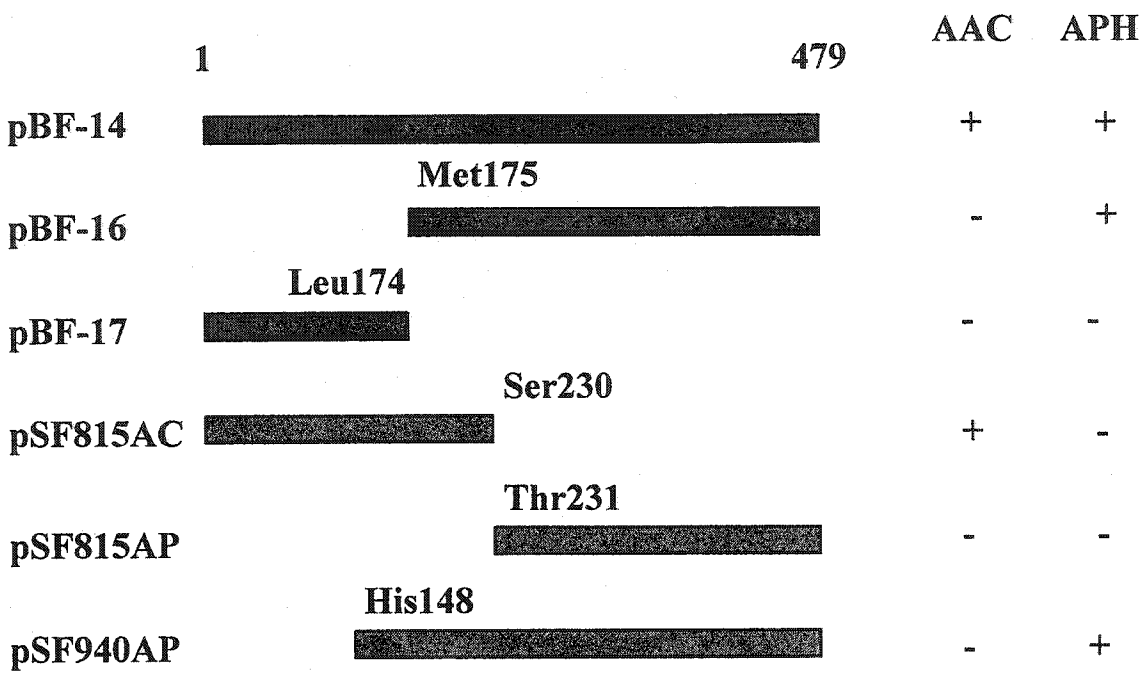


Figure 3.11: Delineation of the topology of activities in AAC(6')-APH(2"). Constructs in blue were made in this work, while constructs in green were previously reported [55]. The two columns denoted AAC and APH designate the presence or absence of the observed activity by assignment of a + or -.

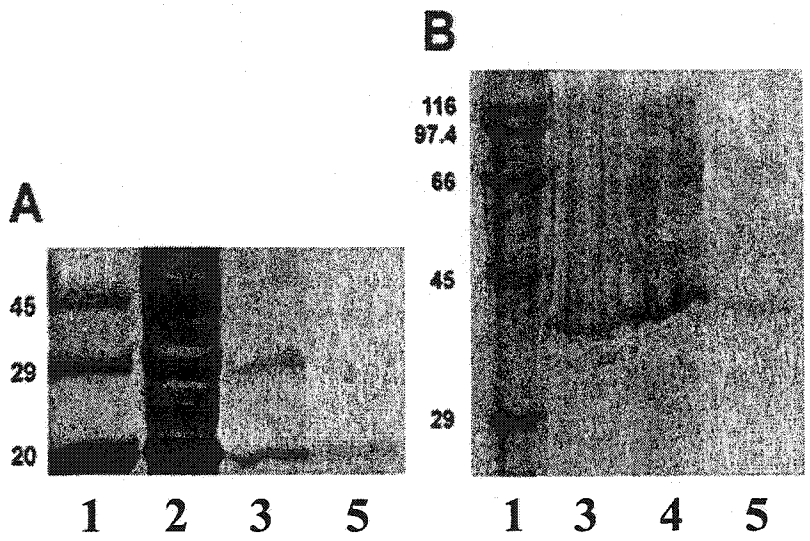


Figure 3.12: SDS-polyacrylamide electrophoresis gels of the purifications of **(A)** 20 kDa AAC(6')-Ie (1-174) and **(B)** 36 kDa APH(2")-Ia (175-479). Lanes 1: molecular mass markers; lanes 2: cell lysate; lanes 3: Q Sepharose anion exchange; Lane 4: Sephadex G100 gel filtration and lanes 5: gentamicin-agarose affinity chromatography.

Table 3.5: Kinetic Characterization of APH(2")-Ia (175-479).

Enzyme	Aminoglycoside	n-fold reduction of k_{cat}/K_m [a]
APH(2")-Ia (175-479)	Kanamycin A Gentamicin C Adenosine triphosphate	1.8 2.1 2.2

[a] k_{cat}/K_m values are in comparison to those presented in Tables 3.2 and 3.3.

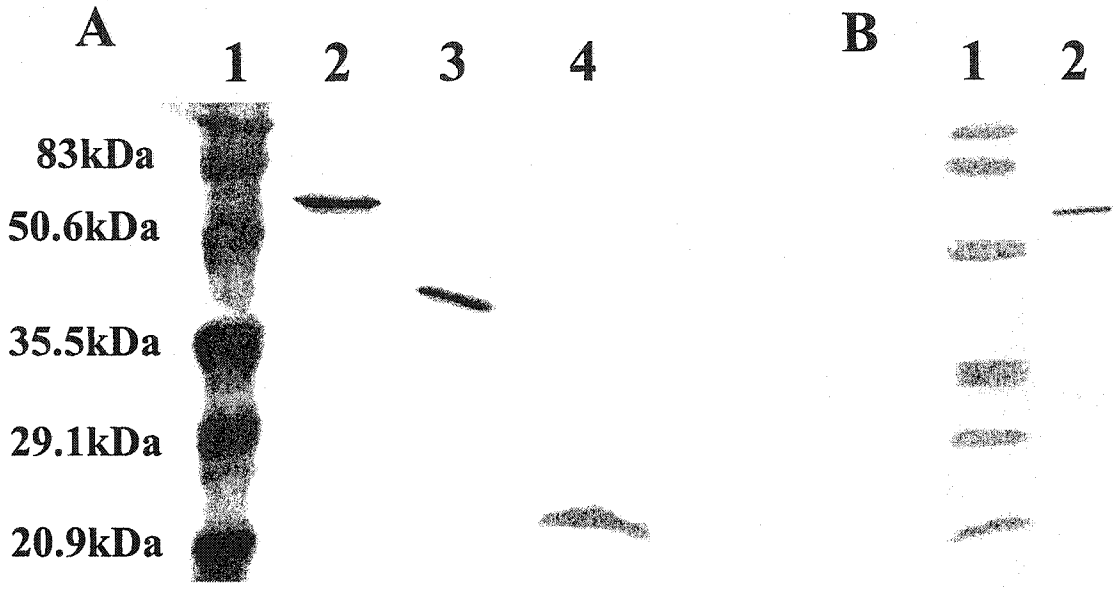


Figure 3.13: Western blotting of AAC(6')-APH(2") from *B. subtilis* 1A752 and *E. faecalis* ATCC 49476. **(A)** Lane 1: pre-stained molecular mass marker; lane 2: purified AAC(6')-APH(2") from *B. subtilis* 1A752/pBF-14; lane 3: purified APH(2")-Ia (175-479) from *E. coli* W3110/pBF-16; and lane 4: purified AAC(6')-Ie (1-175) from *E. coli* W3110/pBF-15. **(B)** lane 1: pre-stained molecular mass marker; and lane 2: clarified lysate from *E. faecalis* ATCC 49476.

3.4 Conclusions

The ability of the bifunctional kinase to phosphorylate rings A, C and D of aminoglycosides coupled to the extraordinary ability of the acetyltransferase to catalyze *N*- and *O*-acetyltransfer to the 6' position were unexpected and have dramatic implications to the future production of synthetic aminoglycosides and/or inhibitor design. Based on the mechanism of both the AAC and the APH, and the strict requirement for the co-factors ATP and acetylCoA, finding inhibitors directed towards AAC(6')-APH(2") will be a daunting task. One advantage is that the AAC(6')-APH(2") aminoglycoside binding pockets have the ability to accommodate diverse structural substitutions, based on the extensive substrate profile. As a consequence, the identification of inhibitory compounds directed towards these drug binding pockets may represent a novel route to clinical reversal of aminoglycoside resistance by inhibiting both activities of AAC(6')-APH(2").

3.5 Materials and Materials

3.5.1 Chemicals

Isepamicin, sisomicin and netilmicin were gifts of G. Miller, Schering Plough Research Institute and gentamicin B and fortimicin were gifts of J. Davies, University of British Columbia. Neamine was the gift of Shahriar Mobashery, Wayne State University. All other aminoglycosides, 4,4'-dithiodipyridine and pyruvate kinase/lactate dehydrogenase mix were from Sigma. [γ-^{32}P]-ATP was from Dupont-NEN. All

restriction endonucleases were from New England Biolabs. ATP and acetyl CoA were from Boehringer Manheim.

3.5.2 Overexpression of AAC(6')-APH(2") in *B. subtilis*

The *aac(6')-aph(2")* gene was amplified by PCR using Vent DNA polymerase from plasmid pSF815A [55] (gift of G. Eliopoulos, New England Deaconess Hospital, Boston, MA) using the DNA primers P1 (5'-CCA GGT ACC *CAT ATG* AAT ATA GTT GAA AAT GAA-3') and P2 (5'-CCA *AAG CTT* GGA TCC TCA ATC TTT ATA AGT CCT TTT) that incorporate unique *Nde*I and *Hind*III restriction sites (underlined), respectively. Plasmid pBF-14 was constructed by ligation of the 1.5 kb *Xba*I to *Bam*HI fragment from pBF-9 with plasmid pRB374 [19] (gift from Reinhold Brückner of Mikrobielle Genetik, Universitat Tubingen, Tubingen, Germany). This placed the gene under the control of the constitutive vegetative promoter, *veg II,* and the translational control of the ribosome binding site from gene 10 of bacteriophage T7. This construct was used to transform the protease-deficient strain *B. subtilis* 1A752 (apr, bglT/bglS(∇)EV, eglS(∇)102, his, npr) (obtained from D.R. Zeigler, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH) by electroporation using a BioRad Gene Pulser apparatus equipped with a pulse controller by minor modification of the method of Kusaoke et al. [116]. Plasmid pBF-15 and pBF-16 consisted of the replacement of *aac(6')-aph(2")* in pBF-9 by the genes *aac(6')-Ie* (nucleotides 1-522 plus a TAA stop codon) to form pBF-15 and *aph(2")-Ia* (nucleotides 523-1440 including original stop codon) to form pBF-16 respectively. Primers used to PCR amplify an

NdeI/HindIII insert for pBF-15 were P1 (5'-CCA GGT ACC *CAT ATG* AAT ATA GTT GAA AAT GAA-3') and P2 (5'-GAA TTC *AAG CTT* ATA AAT AAC AAT CTT CT-3'), restriction sites are underlined. Primers used to PCR amplify an *NdeI/HindIII* insert for pBF-16 were P1 (5'-GGG GAT C*CA TAT G*GA ATA TAG ATA TGA TG-3') and P2 (5'-CCA *AAG CTT* GGA TCC TCA ATC TTT ATA AGT CCT TTT-3'), restriction sites are underlined.

3.5.3 Purification of AAC(6')-APH(2") from *B. subtilis* 1A752/pBF-14:

A 25 ml overnight culture of *B. subtilis* 1A752/pBF-14 in Pennassay broth (Difco antibiotic medium #3) served as an innoculum for 10 L of Pennassay broth supplemented with 200 µg/ml of gentamicin C. AAC(6')-APH(2") was purified from *B. subtilis* 1A752/pBF-14. The culture was grown at 37 °C until the end of logarithmic phase (OD_{600} of 1.6). The cells were collected by centrifugation at 5,000 x g for 10 min and resuspended in 40 ml of lysis buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride and 0.1 mM DTT). Cell lysis was achieved by two consecutive passes through a French pressure cell at 20,000 psi. The cell debris was removed by centrifugation at 10,000 x g for 20 min and the lysate was applied to a Q Sepharose FF anion exchange column (bed vol. 200 ml). The matrix was washed with several column volumes of buffer A (50 mM HEPES pH 7.5, 1 mM EDTA) and AAC(6')-APH(2") was eluted with a linear gradient of buffer B (A + 1 M NaCl). Fractions were assayed for both APH and AAC enzymatic activities (described below). Active fractions eluted between 350 mM and 500 mM NaCl. These were pooled and

concentrated to a final volume of 5 ml over an Amicon PM30 ultrafiltration membrane. The concentrated sample was then applied to a Sephadex G100 gel filtration column (bed volume = 560 ml) and eluted with buffer A + 200 mM NaCl and 0.1 mM DTT. Active fractions were pooled, separated into two aliquots and dialyzed against buffer A.

Affigel 15 (*N*-hydroxy succinimide activated agarose, BioRad) was used to prepare a gentamicin-linked agarose affinity column. Both aliquots were separately applied onto the gentamicin C-agarose affinity column (bed volume = 50 ml) and washed with three column volumes of buffer A. The bifunctional enzyme was eluted from the column with a linear gradient of buffer B at a flow rate of 0.5 ml/min. The fractions containing AAC(6')-APH(2") were pooled, concentrated over an Amicon PM30 membrane, and dialyzed against 25 mM HEPES pH 7.5. Pure bifunctional enzyme retained activity for at least 2 months at 4 °C. Protein concentrations were determined using the Bradford method [15].

3.5.4 Enzyme Assays

Routine assays for aminoglycoside phosphorylation during enzyme purification were performed using a phosphocellulose-binding assay as previously described [77] with kanamycin A and [γ-^{32}P]-ATP. For more detailed analyses using purified enzymes, phosphorylation of aminoglycoside substrates by AAC(6')-APH(2") was monitored using a pyruvate kinase/lactate dehydrogenase coupled assay system, which links the release of ADP to the oxidation of NADH to NAD+, and the resulting decrease in absorbance at 340 nm was monitored spectrophotometrically using a Cary 3E UV-visible

spectrophotometer [135]. Reactions contained 950 µl of assay buffer (50 mM HEPES pH 8.0, 10 mM $MgCl_2$, 40 mM KCl, 0.5 mg/ml NADH, 2.5 mM phosphoenolpyruvate and 1 mM ATP). A volume of 25 µl of aminoglycoside solution was added and the mixtures were pre-incubated at 37 °C for 5 min. The reactions were initiated by the addition of 25 µl of enzyme solution (typically 0.8 mg/ml stock solution). Fractions throughout the enzyme purification were assayed with 100 µM kanamycin A and were corrected for the presence of contaminating ATPase activity when required by a control reaction lacking the aminoglycoside substrate.

All substrates for the kinetic analysis were titrated using the coupled assay. Initial rates were obtained directly from the progress curves and then analyzed without external weighting by non-linear least squares fit to equation 1 or equation 2 for reactions where substrate inhibition was observed, using the Grafit 3.0 software [119]. Kinetic constants are reported +/- the standard error obtained from the fit of the data.

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

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

The acetylation of aminoglycoside substrates was monitored by coupling the acetylation of the aminoglycoside to the cleavage of 4,4'-dithiodipyridine which gives an increase in absorbance monitored at 324 nm (ε = 19,800 M^{-1} cm^{-1}) [76,208] . Reactions contained 800 µl of assay buffer (50 mM HEPES pH 7.0, 1 mM EDTA, 2 mM 4,4'-

dithiodipyridine, 80 μM acetyl CoA and 25 μl of aminoglycoside solution). The solutions were pre-incubated at 37 °C for 5 min and then the reactions were initiated by the addition of 25 μl of enzyme solution (0.8 mg/ml). Initial rates were analyzed as described above.

3.5.5 Large-scale Inactivations of Neomycin, Lividomycin and Butirosin

Inactivations were based on an *in vitro* kinase reaction using purified AAC(6')-APH(2"). The reactions consisted of 100 mg of aminoglycoside, 250 mg of ATP in 500 ml of reaction buffer (50 mM HEPES pH 7.5, 40 mM KCl, 10 mM $MgCl_2$). Reactions were initiated with 1 mg of purified AAC(6')-APH(2"), and an additional 1 mg of purified enzyme was added every 12 h until completion. Completion of substrate phosphorylation was monitored by thin layer chromatography using a 5:2 methanol: ammonium hydroxide mobile phase or an ethanol:methanol:acetic acid:water (5:5:4.5:4.5) mobile phase. *B. subtilis* 1A752 streaked plates containing filter disks impregnated with the reaction product, were measured for zones of clearance were also used to determine the completion of the reaction. The reactions were then mixed with pre-equilibrated AG50WX8 cation exchange resin (bed volume = 100 ml). The slurry was mixed for 1 h, filtered over a scintered glass funnel, washed with 5 volumes of water, and the phosphorylated aminoglycoside products were eluted with a 1 % NH_4OH solution. Fractions (50 ml) containing phosphorylated aminoglycosides as determined by TLC were pooled, lyophilized, resuspended in 500 μl of water and loaded onto a pre-equilibrated Sephadex G10 or G50 gel filtration column for desalting purposes. Fractions

(0.5 ml) off the seizing column which contained phosphorylated aminoglycosides were pooled, lyophilized, resuspended in 1 ml of water. 100 µl volumes were loaded onto a Mono S cation exchange column pre-equilibrated in water. The phosphorylated products were monitored at 211 nm and eluted using a 1 % NH_4OH elution buffer. Positive fractions assayed by TLC were pooled and lyophilized. Samples were subjected to electrospray mass spectrometry to confirm the presence of the phosphate and the level of purity prior to NMR experiments. Finally, the samples were exchanged twice in D_2O.

3.5.6 Base Hydrolysis of Acetylated Aminoglycosides

Reactions contained 100 µM aminoglycoside, 36.4 µM [^{14}C]-acetyl CoA (1.2 x 10^5 cpm/nmol), AAC(6')-APH(2") (2.6 pmol) and 50 mM HEPES buffer pH 7.5 in a final volume of 10 µl. Following completion of the acetyl transfer reaction, 10 µl of 80 mM NaOH was added and the reaction mixtures were incubated at 80 °C for various time points (final pH 11-12). Samples were neutralized with the addition of 10 µl of 1 M HEPES pH 7.5 and analyzed by application of the solution on Whatman P-81 phosphocellulose paper. The papers were washed three times in water, dried, and the amount of remaining radioactivity bound was determined by scintillation counting.

3.5.7 Thin-Layer Chromatography, Purification and Infrared Spectroscopy

O-acetylated paromomycin was produced by incubating 5 mg of the aminoglycoside (150 µM), 150 µM acetyl Coenzyme A, 300 nM AAC(6')-APH(2") in 50 ml HEPES pH 7.5 for 4 h. The modified aminoglycoside product was partially

purified by batch anion exchange chromatography on AG50W-X8 resin with a 1% NH_4OH step gradient. The eluted product was concentrated by lyophilization and resuspended in 1 ml water. Samples were then separated on Whatman PLK-5 glass-backed silica plates using a 5:2 methanol:NH_4OH mobile phase. The R_f of the product was 0.23 whereas that of paromomycin was 0.35. Silica was scraped off the plate and the acetylated aminoglycoside was extracted with 15 ml of methanol under mildly acidic conditions. Samples were neutralized by the dropwise addition of NaOH followed by lyophilization. The dried product was ground into a KBr pellet and analyzed by infrared spectroscopy using a BioRad FTS-40 Fourier Transform Infrared Spectrometer. The collection of the IR spectra was performed by George Timmins (department of Chemistry, McMaster University, Hamilton, ON, Canada).

Chapter 4

Inhibition studies on the AAC(6')-APH(2") kinase

Adapted from:

Daigle, D.M., McKay, G.A. and Wright, G.D. *J. Biol. Chem.* 1997, vol. 272, pp. 25755-24758, 1997.

Chapter 4

4.1 Abstract

The crystal structure of aminoglycoside phosphotransferase APH(3')-IIIa [Hon, W., *et al.* (1997) *Cell* **89**, 887-895] revealed that this family of aminoglycoside kinases possess a similar fold to those of eukaryotic protein kinases such as the cAMP dependent protein kinase (PKA) and also suggested that they may be equally susceptible to protein kinase inhibitors. We therefore examined the effect of well known protein kinase inhibitors, all of which were ATP analogs or ATP binding pocket inhibitors, on the bifunctional AAC(6')-APH(2") aminoglycoside kinase. We have found that members of the isoquinolinesulfonamide class of protein kinase inhibitors are successful inhibitors of the bifunctional kinase. The most potent isoquinolinesulfonamide, (H-9) resulted in competitive inhibition versus ATP and non-competitive inhibition versus kanamycin A with mid-micromolar K_i values.

Bisubstrate analogues, composed of adenosine or an isoquinolinesulfonamide (H-9) linked to neamine, were also tested for their ability to inhibit the bifunctional APH. These compounds were expected to potentiate the inhibition observed with the isoquinolinesulfonamides by employing binding energy from both an aminoglycoside component and a nucleotide component. However, the compounds exhibited mixed-type inhibition with respect to both substrates, suggesting the formation of multiple dead-end complexes with different enzyme forms. The strongest inhibition observed was that of

adenosine linked to neamine with a seven carbon atom spacer, bisubstrate compound (C-7). This compound (C-7) gave only a slight improvement in observed K_i values over those of the isoquinolinesulfonamides. Further modifications and co-crystallization followed by structure activity relationships will be required to design successful bisubstrate analog inhibitors towards the AAC(6')-APH(2") kinase. Nonetheless, the bisubstrate compounds and the isoquinolinesulfonamides are successful inhibitors which represent a foundation for future AAC(6')-APH(2") inhibitor design.

4.2 Introduction

4.2.1 Characteristics of Aminoglycoside Phosphotransferases

Antibiotic resistance is a growing problem that seriously threatens human health. This problem has been propagated by years of misuse of antibiotics and the subsequent rapid dissemination of bacterial resistance genes throughout the bacterial population. One mechanism to stay a step ahead of bacterial evolution and keep antibiotic resistance at bay is by rationally designing inhibitors which could be co-administered along with the antibiotics.

The class of aminoglycoside phosphoryltransferases (APH) is composed of many different enzymes which differ in their regiospecificity of inactivation, substrate spectrum and uniqueness of their resistance profiles [reviewed in 179]. These enzymes generally show a low overall amino acid similarity, but all have a signature sequence in common with eukaryotic protein kinases [89]. This sequence H(G/N)DX_{3-4}N, is homologous to the eukaryotic Ser/Thr and Tyr protein kinase sequence (H/Y)RDX_4N [89]. The significance

of this short peptide sequence lies in the fact that the conserved aspartate (Asp190 – in the structure of APH(3')-IIIa, and equivalent to Asp374 of AAC(6')-APH(2")) is required for catalysis in protein kinases [33,128]. Site-directed mutagenesis of this conserved residue, Asp190 of APH(3')-IIIa, resulted in a dramatic reduction in aminoglycoside phosphorylating activity [89], results which agree with similar observations obtained with EPKs [33,128]. The three dimensional structure of one such aminoglycoside phosphotransferase, mainly APH(3')-IIIa, has been solved by X-ray crystallography to 2.2 Å [89]. The structure revealed that APHs have a strikingly similar kinase fold to those found in eukaryotic protein kinases (Figure 4.1). Thus APHs and EPKs share similar overall structure and mechanism of phosphoryl transfer. The close mechanistic and structural relationship between APH(3')-IIIa and protein kinases has led us to explore the sensitivity of a second aminoglycoside kinase, AAC(6')-APH(2"), to known inhibitors of protein kinases. Three classes of kinase inhibitors were examined: i) the indole carbazoles, ii) the flavanoids and iii) the isoquinolinesulfonamides (Figure 4.2).

Protein kinase inhibitors have been useful tools since the early 80's as a means of dissecting eukaryotic signal transduction pathways or identifying cognate enzyme substrate interactions [32,54,72,78,84,86,94,117,218]. Among all protein kinase inhibitors, there are three well-known classes which have seen widespread experimental use.

4.2.2 Isoquinolinesulfonamide Protein Kinase Inhibitors

A large family of isoquinolinesulfonamide compounds inhibit protein kinases by competing with ATP, yet have little effect on other ATP utilizing enzymes such as ATPases or adenylyl cyclases. These compounds are derivatives of naphthalene-sulfonamides in which the naphthalene ring has been substituted by an isoquinoline ring [86]. Selective derivatives exhibit specific inhibition of certain protein kinases based on the side chain used. Cyclic AMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C are inhibited by low micromolar levels of *N*-[2(methylamino)ethyl]-5-isoquinoline-sulfonamide (H-8), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) and *N*-(2-aminoethyl)-5-isoquinolinesulfonamide (H-9) [86,94]. Kinase selectivity between these compounds was observed by the specificity of the sulfonylpiperazine compound (H-7) for protein kinase C while (H-8) and (H-9) displayed specificity for cyclic nucleotide-dependent protein kinases [86,94]. Others such as *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7) and 1-(5-chloroisoquinoline-8-sulfonylpiperazine) (CKI-8) which differ only in the positioning of the ring nitrogen and the addition of a chlorine atom linked to C-5 (Figure 4.2), are selective potent inhibitors of casein kinases [218]. Inhibition by these compounds was shown to be reversible, competitive against ATP and non-competitive with the phosphate acceptor [86,94]. X-ray crystallography structures of CKI-7 bound to casein kinase [218] and (H-7), (H-8) and (H-89) bound to PKA [54] have demonstrated the structural requirements governing inhibition of protein kinases by this family of protein kinase inhibitors [54].

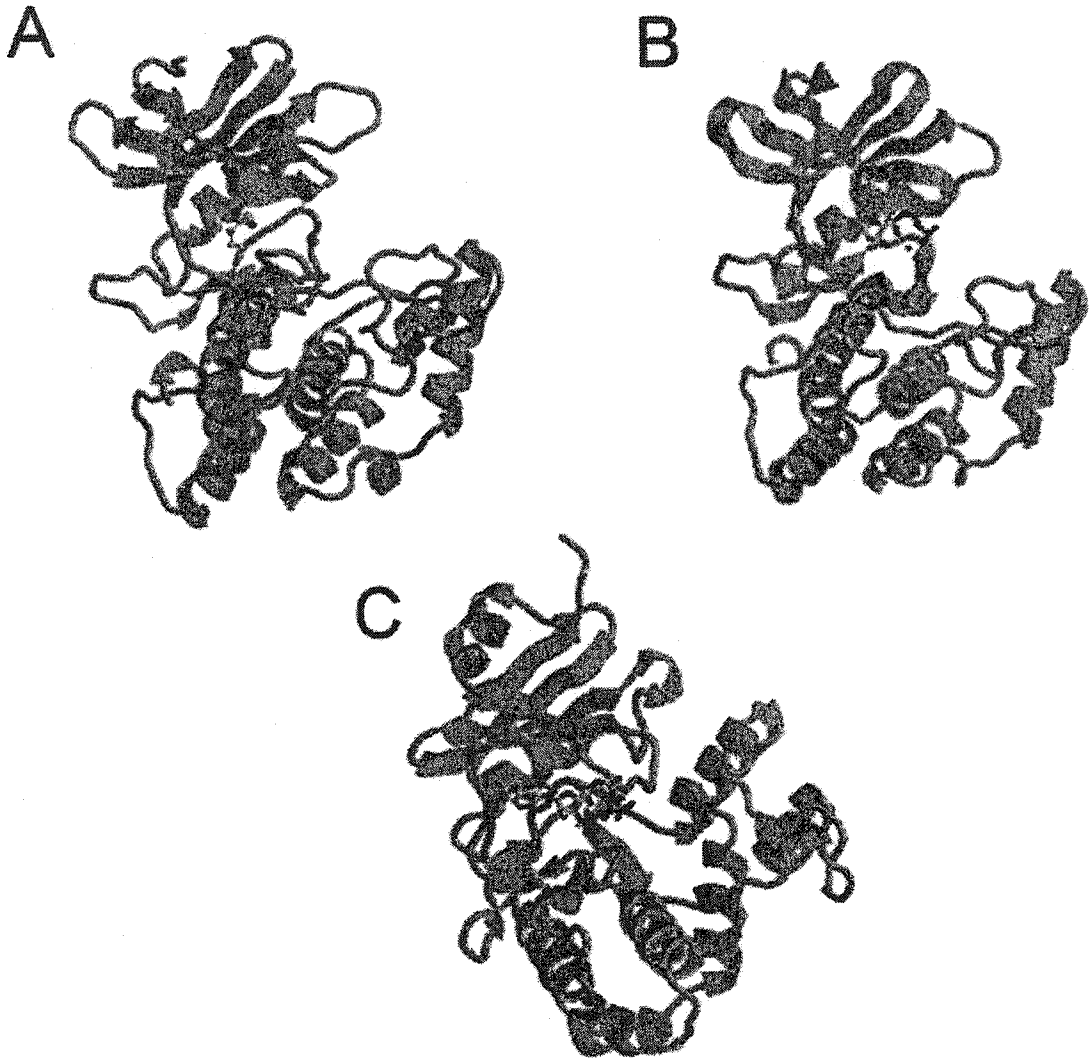
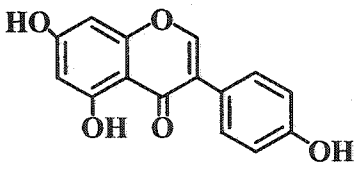
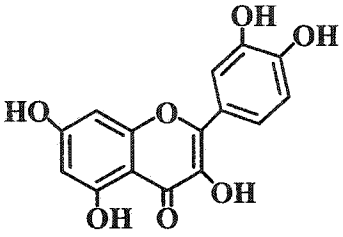


Figure 4.1: Ribbon diagrams of APH(3')-IIIa and casein kinase-1 **(A)** The structure of casein kinase-1 from *S. pombe* bound by the isoquinolinesulfonamide inhibitor CKI-7. Protein data bank (PDB code 2CSN) **(B)** The structure of casein kinase-1 in complex with Mg^{2+}-ATP. The magnesium atom is colored in green. Protein data bank (PDB code 1CSN) **(C)** The structure of APH(3')-IIIa shown is in complex with Mg^{2+}-ADP. The magnesium atom is in red. Protein data bank (PDB code 1J7L). Images created using RasMol version 2.6 [175].

Flavanoids

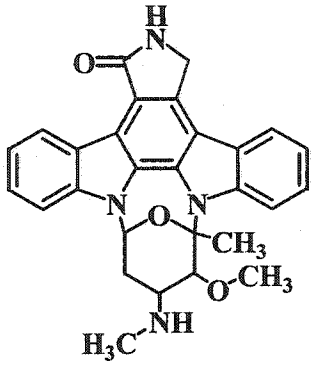


Genistein



Quercetin

Indole carbazole containing alkaloid



Staurosporin

Isoquinoline sulfonamides

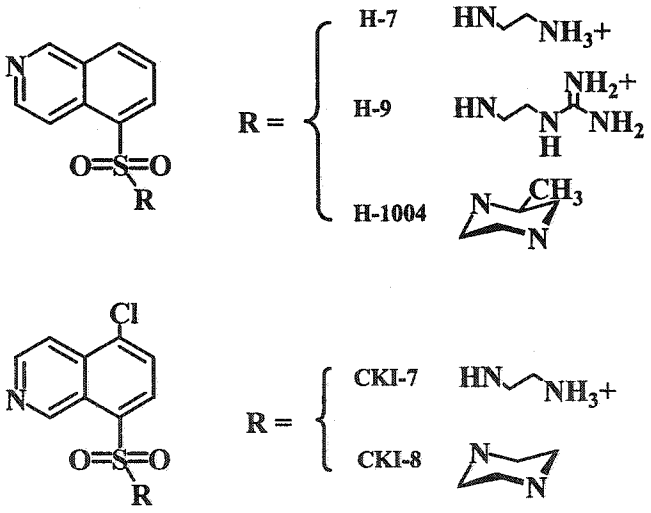


Figure 4.2: Structures of the protein kinase inhibitors used in this study.

A common feature observed with both CKI-7 and the H-series inhibitors is a hydrogen bond between the isoquinoline ring nitrogen and a main chain amide hydrogen in the structures [54]. This bond mimics the interaction between N-1 of ATP in the enzyme-substrate complex, consequently the isoquinolinesulfonamides occupy the same binding pocket and employ identical positioning elements in the active site of protein kinases as ATP.

4.2.3 Staurosporin

The indole carbazole containing alkaloid compound staurosporin is a potent inhibitor of protein kinase C and many other eukaryotic protein kinases with low nanomolar K_i values (Figure 4.2) [84]. Staurosporin also has micromolar potency against tyrosine kinases such as mitogen-activated protein kinase (MAPK), casein kinase-1 (CK1) and C-terminal Src kinase (Csk) [138]. The 2.4 Å structure of Csk bound by staurosporin shows the inhibitor is bound in the ATP binding pocket and as in the case of other protein kinases appears to bind by induced fit [117].

4.2.4 Flavanoids

Flavanoids or bioflavanoids are yet another class of protein kinase inhibitors (Figure 4.2). These are naturally occurring compounds which are potent cyclic nucleotide-independent protein Tyr kinase inhibitors, however it has been observed that modifications to the level of hydroxylation of these structures can yield some compounds which are effective inhibitors of Ser/Thr protein kinases [78]. One of these compounds,

quercetin (3,3',4',5,7-pentahydroxyflavone) inhibits pp60src [72], cyclic nucleotide-independent casein kinase G (CKG) [32], phosphorylase kinase from rat lung [185] and the insulin receptor kinase [78]. A second flavanoid, Genistein (3,4',5,7-trihydroxyflavone), is a potent inhibitor of the epidermal growth factor receptor tyrosine kinase, pp60v-src while showing no inhibition towards PKA, phosphorylase kinase or PKC [1]. In the case of both flavanoids discussed, the inhibition behavior observed is competitive against ATP and non-competitive against the phosphate acceptor [1,32,72,78,185]. The structure of Hsk complexed with quercetin has been solved and shows that this compound specifically binds to the ATP binding pocket [181].

4.2.5 Bisubstrate Analogues Inhibitors

Bisubstrate analogue inhibitors have been successfully designed and employed against several targets [67,118,125,132,141,164,209]. These compounds generally have very low inhibitory constants in view of the fact that the compounds take advantage of the binding energies of two individual substrates. One example of this is DPPG (*N*-dibenzylphospho-*N'*-3-(2,6-dichlorophenyl)-propylguanidine), which is a potent bisubstrate analogue inhibitor of creatine kinase having a K_i in the mid-nanomolar range versus the nucleotide substrate [141] (Figure 4.3). In addition, N-myristoylated peptides make effective bisubstrate analogue inhibitors of Src tyrosine kinase [164]. Non-acylated peptides gave rise to mixed inhibition while myristoylated peptides resulted in competitive inhibition of Src [164].

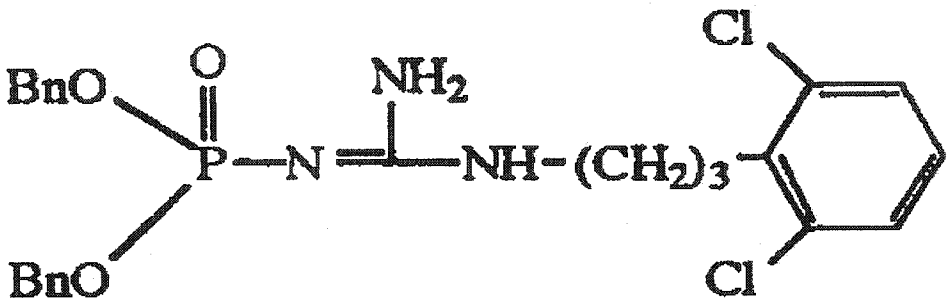


Figure 4.3: Structure of DPPG, a bisubstrate inhibitor of creatine kinase.

Adenosine 5'-carboxylic acid peptide derivatives have also been shown to be good broad application bisubstrate analogue inhibitors of protein kinases [125].

Bisubstrate inhibitor design against the bifunctional AAC(6')-APH(2") would be regarded as advantageous as this strategy would engineer more specificity against this enzyme. Bisubstrate analogues, in theory, impart an entropic advantage as these compounds present both individual substrates and should exploit binding energies from both compounds to make tight binding inhibitors. Successful bisubstrate analogue inhibitors have already been designed and tested against a variety of clinically relevant kinases such as Src and other protein kinases [125,164], yeast thymidylate kinase [118], and Creatine kinase [141]. The use and development of bisubstrate analogue inhibitors has not been restricted to the study of kinases however, as successful compounds have been tested against dopamine beta-monooxygenase [209], catechol *O*-methyltransferase [132] and GlcNAc:beta-1,4 glycosyltransferases [67].

In this study we show that the well-known isoquinolinesulfonamide class of protein kinase inhibitors are also effective inhibitors of the bifunctional APH. Additionally, our initial attempts to test bisubstrate analogs [124] have been met with limited success as the compounds appear to bind to multiple forms of the enzyme yielding mixed-type inhibition and only minimal improvements in potency as compared to the isoquinolinesulfonamide H9.

4.3 Results and Discussion

4.3.1 The Flavanoids and Staurosporin

AAC(6')-APH(2") confers resistance to all known aminoglycosides with the exception of streptomycin and spectinomycin, which makes this protein the most prolific aminoglycoside resistance enzyme and therefore a valid target for inhibitor design [38]. The kinase portion of AAC(6')-APH(2") resides in the C-terminal half of the protein and shows only 16 % overall primary sequence similarity to APH(3')-IIIa. APH(2")-Ia possesses a catalytically important signature motif found in APH(3')-IIIa, mainly $H^{188}XDX_3N$ which in AAC(6')-APH(2") is H^{372}-NDFSCN [89].

All protein kinase inhibitors tested in this study are ATP analogs or compounds which have been shown or are suggestive of ATP-binding pocket inhibitors. One of these, staurosporin, a potent low nanomolar indole carbazole containing alkaloid inhibitor of protein kinases, displayed no inhibition of the bifunctional kinase at 1 μM (the limit of solubility) [84].

The flavanoids genistein and quercetin, specific inhibitors of the EGF receptor kinase and other tyrosine kinases [1,185], also did not display any inhibitory activity against the bifunctional APH. Evidence of some similarity within the ATP binding pocket of tyrosine kinases and aminoglycoside kinases is shown by the mild inhibitory action of quercetin on another aminoglycoside kinase, mainly APH(3')-IIIa [39].

4.3.2 The Isoquinolinesulfonamides

The isoquinolinesulfonamide inhibitors are well known competitive inhibitors of ATP for many protein kinases [29,54,86,94,218]. These compounds have been shown to possess remarkable specificity by varying side chain constituents and especially positioning of the isoquinoline ring nitrogen [reviewed in Ref. 87]. Structural studies of protein kinases with isoquinolinesulfonamides have shown that these compounds use similar positioning elements in the active site as ATP [54]. By analogy, the X-ray crystallographic structure of ADP bound to APH(3')-IIIa also shows a hydrogen bond between N-1 of ADP and the main chain amide hydrogen of Ala-93 (Figure 4.4) [89]. APH(3')-IIIa is also successfully inhibited by five members of the isoquinolinesulfonamide family, mainly CKI-7, CKI-8, H-7, H-9 and H-1004 [39]. Therefore, on the basis of the similar kinase fold between APH(3')-IIIa and protein kinases and by extension of this to other aminoglycoside kinases despite low overall amino acid sequence similarity, we suspected that the isoquinoline ring would bind in a similar fashion in the bifunctional kinase.

CKI-7, H-7, H-9 and H-1004 were found to be competitive inhibitors of ATP with the bifunctional APH (Table 4.1, appendix 4A –section 4.1). In addition, as expected H-9 displayed non-competitive behavior towards the aminoglycoside substrate kanamycin A. Significant differences in the potency of inhibition of these compounds observed against APH(3')-IIIa and the bifunctional APH indicate that AAC(6')-APH(2) is more sensitive to the methylpiperazine compound H-7 than APH(3')-IIIa, which is surprising since no inhibition was observed with the homologue CKI-8 [39].

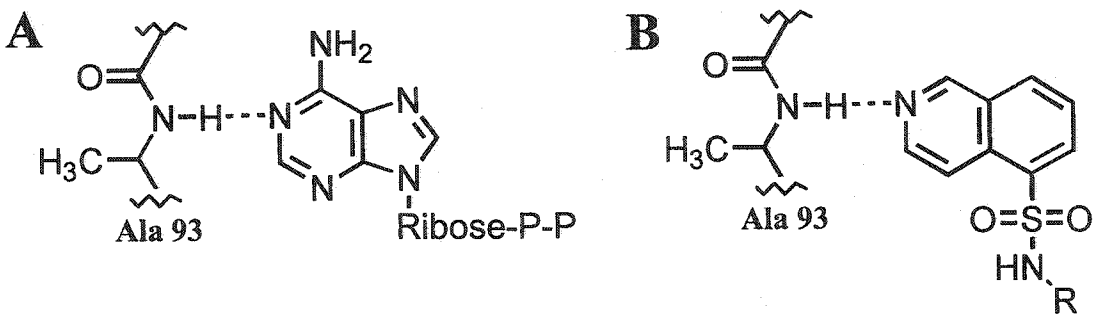


Figure 4.4: Proposed interaction of isoquinolinesulfonamides with a member of the aminoglycoside phosphotransferases APH(3')-IIIa. **(A)** Interaction of ADP bound to APH(3')-IIIa as determined by the three dimensional structure [89]. **(B)** proposed interaction of APH(3')-IIIa and other aminoglycoside kinases with isoquinolinesulfonamides.

Table 4.1: AAC(6')-APH(2") phosphotransferase inhibition by protein kinase inhibitors[f].

Inhibitor	Variable substrate	Pattern[a]	K_{is} (μM)	K_{ii} (μM)	Equation[b]
CKI-7	ATP	C	87.1 ± 7.5		1
CKI-8	ATP	-[c]			
H-7	ATP	C	137 ± 29		1
H-9	ATP	C	63 ± 19		1
H-9	Kanamycin A	NC	998 ± 307	125 ± 49	2
HA-1004	ATP	C	151 ± 42		1
Genistein	ATP	-[d]			
Quercetin	ATP	-[d]			
Staurosporin	ATP	-[e]			

[a] C, competitive; NC, non-competitive.
[b] Data fit to equation under "Experimental Procedures."
[c] No inhibition at 500 μM
[d] No inhibition at 500 μM, dimethyl sulfoxide required to solubilize these compounds dramatically reduced enzyme activity (approximately 50 % at 5 % dimethyl sulfoxide); thus these compounds may bind to the enzyme, but nonetheless with poor affinity.
[e] No inhibition at 1 μM
[f] Structures of the protein kinase inhibitors can be seen in figure 4.2 (page 124).

None of the inhibitors tested in this study affected activity of the AAC(6')-Ie portion of the bifunctional enzyme. Based on the inhibition behavior observed, and the identical behavior observed with these compounds against APH(3')-IIIa [39], some conclusions can be drawn: i) these compounds are similar ATP binding pocket inhibitors of the bifunctional APH and ii) the bifunctional APH likely adopts a similar kinase fold to that of APH(3')-IIIa and protein kinases. In contrast to the effective inhibition of the bifunctional APH *in vitro*, antibiotic resistance in liquid cultures or on solid media of *E. faecalis* harboring *aac(6')-aph(2")* was unaltered by the presence of 100 μM H-9 (corresponding to 20-30 μg/mL).

4.3.3 Bisubstrate analogues tested against APH(2")-Ia

The kinetic mechanism of the AAC(6')-APH(2") kinase is random BiBi [130], which implies that both substrates must be present in the active site for phosphoryl transfer to occur. Information regarding the mechanism and the studies with the isoquinolinesulfonamides were then applied to the design of bisubstrate analogues. Through a collaboration with Dr. Shahriar Mobashery (Wayne State University, Detroit, MI), we received five newly synthesized compounds composed of adenosine or the isoquinolinesulfonamide (H9) linked covalently to the 3'-hydroxyl of neamine (an aminoglycoside). The linkage consisted of an all-methylene hydrocarbon tether of 5-8 carbon atoms. We tested the hypothesis that compounds exhibiting at once the characteristics of the nucleotide ATP and components of the aminoglycoside would potentiate the inhibition observed, as binding of each component would be expected to

entropically favor the binding of the second tethered component. Neamine was chosen to ensure that there was significant occupancy of the aminoglycoside binding region in the AAC(6')-APH(2") kinase active site, as previous studies have shown that neamine is the minimal substrate that will be phosphorylated by either the AAC(6')-APH(2") bifunctional kinase or APH(3')-IIIa [135].

The five compounds differed mainly in the linkage unit between both molecules with the exception of the compound ISO, which was coupled to a derivative of the isoquinolinesulfonamide H-9 instead of adenosine (Figure 4.5). The linking unit between the two compounds was varied to accommodate different binding pocket geometries while the effect on the AAC(6')-APH(2") aminoglycoside kinase was tested. Our results show that these bisubstrate compounds are in fact inhibitors of the bifunctional aminoglycoside kinase with inhibitory constants in the low to mid-micromolar range (Table 4.2). Unfortunately, adding an aminoglycoside moiety and a linker to an isoquinolinesulfonamide, resulted in only a two-fold increase in inhibition observed from that of H-9 alone (Table 4.2). One possible explanation for the minimal improvement in inhibition observed with these compounds is that there is a significant loss of binding energy by replacing the triphosphate of ATP with the hydrocarbon tether found in these compounds. A second is that these compounds did not display the expected inhibition behavior of true bisubstrate analogues. Instead of competitive-type behavior towards both substrates, the inhibition patterns were mixed non-competitive for both aminoglycoside and ATP with all bisubstrate compounds tested (Table 4.2,

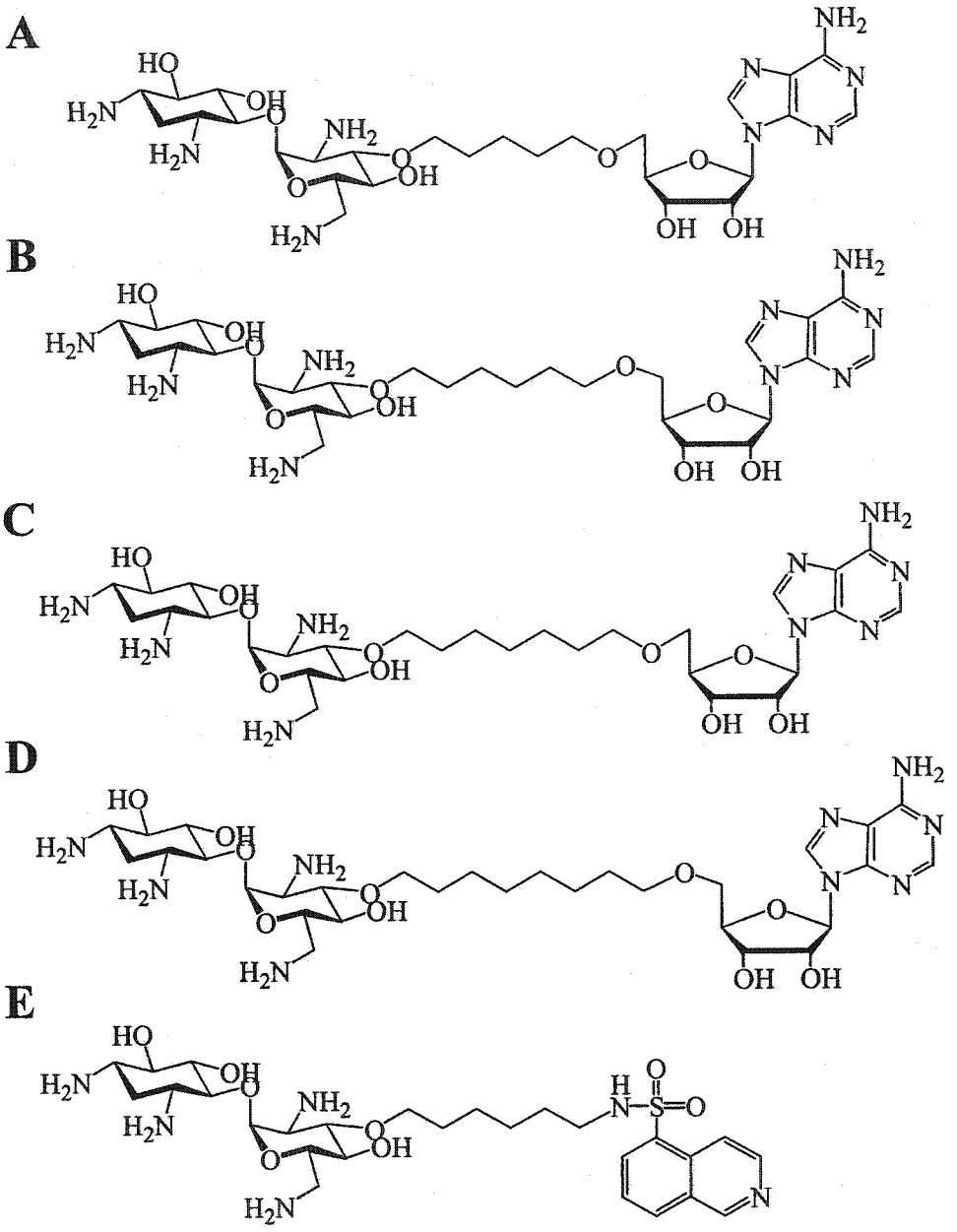


Figure 4.5: Structures of the bisubstrate analogues tested in this study. **A**- C5, **B**- C6, **C**- C7, **D**- C8, and **E**- ISO. Compounds were synthesized by Liu et al.[124].

Table 4.2: AAC(6')-APH(2") phosphotransferase inhibition data obtained with bisubstrate analogues.

Compound	Type of inhibition	Substrate	K_{ei} (μM)	K_{esi} (μM)
C5	Mixed NC	kanamycin	3.3 ± 1.2	14.1 ± 3.4
C6	Mixed NC	kanamycin	1.6 ± 1.7	40.0 ± 16.3
C7	Mixed NC	kanamycin	3.6 ± 1.2	8.7 ± 1.3
C8	Mixed NC	kanamycin	11.1 ± 4.2	108.0 ± 48.1
ISO	Mixed NC	kanamycin	149.9±99.6	50.3 ± 8.6
C5	Mixed NC	ATP	14.6 ± 5.4	102.2 ± 70.9
C6	Mixed NC	ATP	18.6 ± 14.2	30.0 ± 16.9
C7	Mixed NC	ATP	30.6 ± 24.1	11.6 ± 3.5
C8	Mixed NC	ATP	30.9 ± 20.5	29.4 ± 8.3
ISO	Mixed NC	ATP	39.1 ± 17.9	47.2 ± 8.3

appendix 4A –section 4.2). Inhibitory constants varied from 1.6-150 μM when kanamycin A was the variable substrate and from 11.6-102 μM when ATP was varied. No slow-binding kinetics was observed with any of the compounds tested. The data suggested that these analogs were probably not binding equally to both sites and/or forming various dead-end complexes with multiple enzyme forms resulting in mixed non-competitive inhibition patterns. Conversely, these compounds have been reported as successful competitive bisubstrate inhibitors of APH(3')-IIa [124].

It is possible that these molecules bind in the active site and do not interfere with binding of the variable substrate. If the potency of the bisubstrate compounds was more significant, a detailed analysis of the binding constants (K_d) for these compounds to multiple enzyme forms by a method such as isothermal calorimetry would have been informative [129]. Site-directed mutagenesis and computer modeling of APH(3')-IIIa has previously shown, that aminoglycosides have multiple binding modes [195]. This former phenomenon is believed to contribute to the broad aminoglycoside spectrum exhibited by these enzymes. The recent structures of APH(3')-IIIa bound by ADP and an aminoglycoside, either kanamycin A or neomycin B, have determined that there are sub-sites within the aminoglycoside binding pocket which are employed to accommodate the various substrate structures [57]. The exact mode of binding of these bisubstrate analogues will only be confirmed once they have been co-crystallized with APH(3')-IIIa or the APH(2")-Ia.

4.4 Conclusions

Several isoquinolinesulfonamide protein kinase inhibitors, mainly CKI-7, H7, H9 and H1004, were found to also be successful inhibitors of the APH(2")-Ia. Bisubstrate analogues were also tested as potential inhibitors of APH(2")-Ia. These compounds exhibited unusual inhibition behavior towards APH(2")-Ia which suggested that the compounds were binding to multiple enzyme forms yielding mixed inhibition patterns towards both the aminoglycoside and nucleotide substrates. Both APH(3')-IIIa and AAC(6')-APH(2") appeared to favor a certain tether length (i.e. carbon atom spacer) as would be expected depending on the dimensions of their respective enzyme active sites. Both the APH(2")-Ia and the APH(3')-IIIa preferred the compounds C-6 and C-7 [39]. A close look at the APH(3')-IIIa structure complexed with ADP and kanamycin A shows a pocket lined with negatively charged residues that places the 3'-OH of the aminoglycoside approximately 3.4 Å away from the γ-phosphate of ATP [57] (Figure 4.6). The distance between the 5'-OH of adenosine and the 3'-OH of ring A of an aminoglycoside is 8.3 Å - 12.1 Å, depending on whether there is a straight path or if the tether mimics the triphosphate backbone respectively. These distances do correlate with the findings that the best bisubstrate compounds are C6 and C7 as they would possess tethers of 10.5 Å and 12.0 Å respectively.

Finally, this study demonstrates the potential effectiveness of protein kinase inhibitors as inhibitors of aminoglycoside phosphotransferases while providing a basis for future inhibitor design against AAC(6')-APH(2") and reversal of aminoglycoside antibiotic resistance conferred by this prominent resistance determinant.

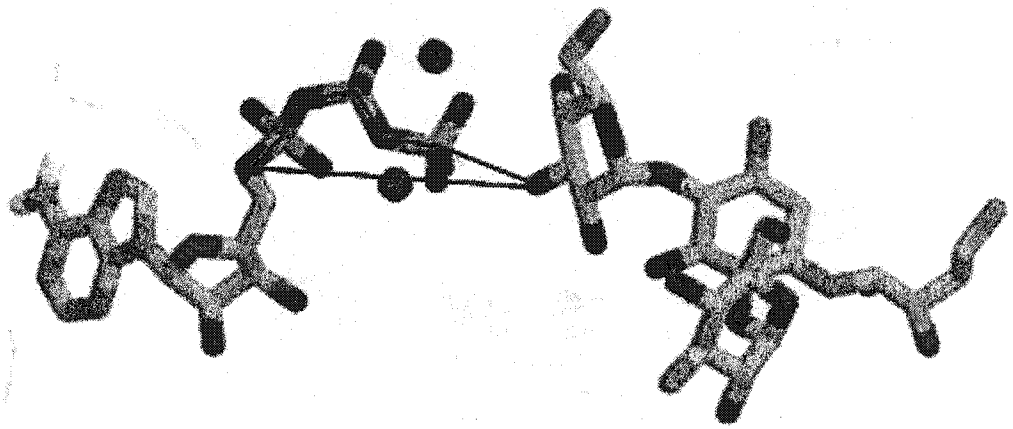


Figure 4.6: Close-up of the structure of APH(3')-IIIa bound by ADP and amikacin. The distance of a direct path from the 5'-hydroxyl of adenosine to the 3'-hydroxyl of the aminoglycoside amikacin is 8.3 Å. The distance observed if the methylene tether were to conform to the triphosphate binding pocket is 11.4 Å. Magnesium atoms are in magenta (PDB code 1L8T).

4.5 Materials and Methods

4.5.1 Chemicals

Genistein, quercetin and staurosporin were from Sigma. N-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7) and 1-(5-chloroisoquinoline-8-sulfonyl) piperazine (CKI-8) were from Seikagaku America (Rockville, MD). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), N-(2-aminoethyl)-5-isoquinoline-sulfonamide (H-9), and N-(2-guanidinoethyl)-5-isoquinolinesulfonamide (H-1004) were from Research Biochemicals International (Nattick, MA). AAC(6')-APH(2") was purified as described in [38]. Bisubstrate compounds C5, C6, C7, C8 and ISO were obtained as kind gifts from Dr. Shahriar Mobashery (Wayne State University, Detroit, MI).

4.5.2 Enzyme Assays and Data Analysis:

Aminoglycoside kinase activity was monitored by coupling of ADP release to NADH oxidation by pyruvate kinase and lactate dehydrogenase in the presence of excess phosphoenolpyruvate [135]. Assays were performed at 37 °C and contained kanamycin A (0.12 mM) when ATP was the variable substrate, 0.28 mM NADH, 2.5 mM phosphoenolpyruvate, 5 units of lactate dehydrogenase, 3.5 units of pyruvate kinase, 50 mM HEPES, pH 7.5, and inhibitor dissolved in water or dimethyl sulfoxide in the case of the isoflavanoid compounds. The amount of DMSO never exceeded 5% (v/v) and did slightly diminish the activity of AAC(6')-APH(2"). Reactions were initiated by the addition of 0.21 nmol of pure AAC(6')-APH(2"). For experiments with varying

kanamycin A, ATP was held at 1 mM. All substrates for the kinetic analysis were titrated using the coupled assay. The inhibition data, obtained in duplicate for at least four different inhibitor concentrations, was analyzed without external weighting by non-linear least squares fit Equation 1 for competitive inhibition or Equations 2 for non-competitive inhibition using Grafit 3.0 software [119]. Kinetic constants are reported +/- the standard error obtained from the fit of the data.

$$v = V_m[S]/(K_m(1 + [I]/K_{is}) + [S]) \quad (1)$$

$$v = V_m[S]/(K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})) \quad (2)$$

where K_m is the Michaelis-Menton constant, V_m is the maximal velocity, K_{is} is the slope inhibition constant, and K_{ii} is the intercept inhibition constant, and K_i is the substrate inhibition constant.

4.6 Appendix 4A:

4.6.1 Section A – Inhibition plots of H-9 with respect to ATP and kanamycin A:

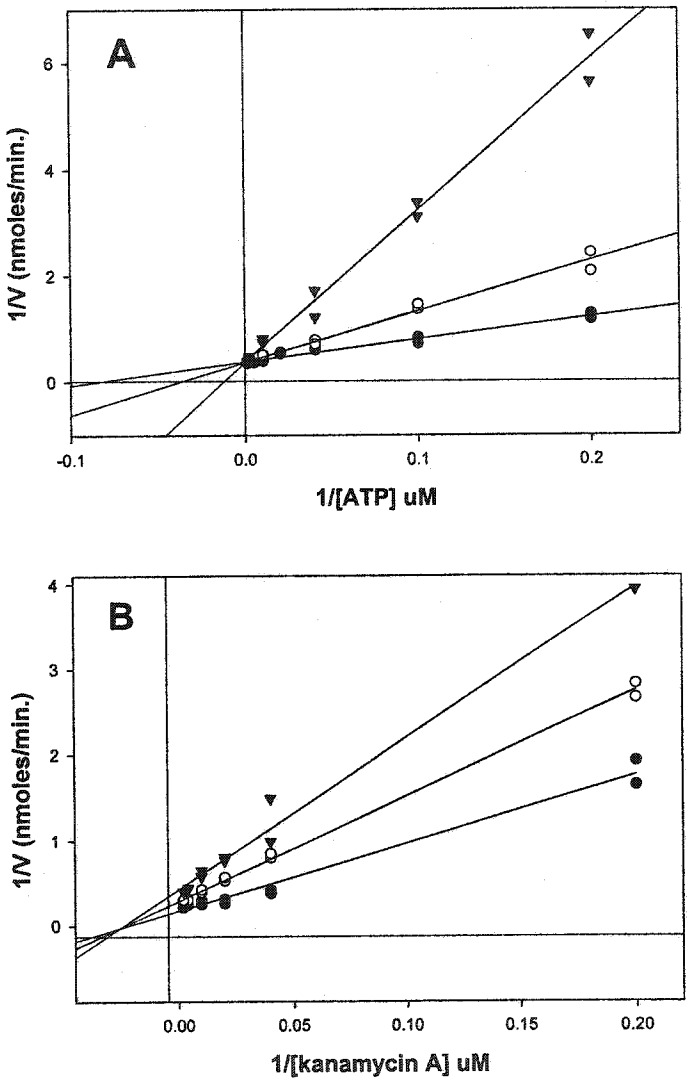


Figure 4.7: Inhibition of the AAC(6')-APH(2") kinase by the isoquinolinesulfonamide (H9). **A**- (H9) versus ATP: ▼ 300 μM (H9), ○ 100 μM (H9), and ● 0 μM (H9). ATP was varied from 5-500 μM at saturating 120 μM kanamycin A. **B**- (H9) versus kanamycin A: ▼ 400 μM (H9), ○ 200 μM (H9), and ● 0 μM (H9). Kanamycin A was varied from 5-500 μM at saturating ATP of 1 mM.

4.6.2 Section B – Inhibition plots of (C7) with respect to ATP and kanamycin A:

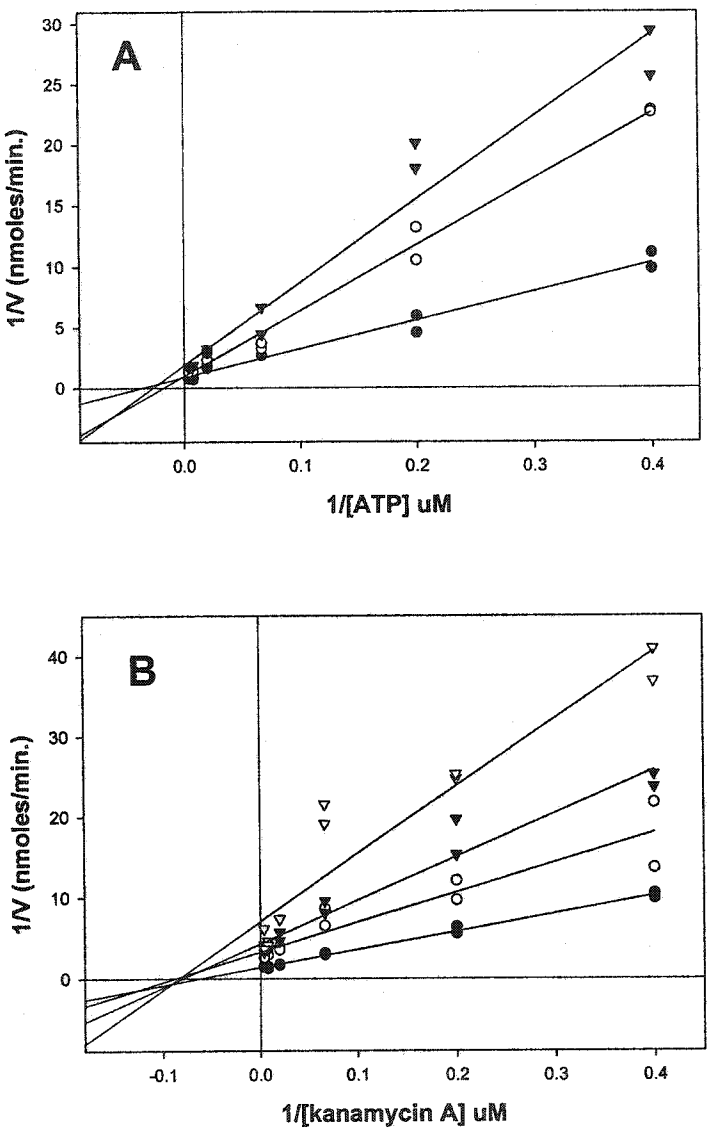


Figure 4.8: Inhibition of the AAC(6')-APH(2") kinase by the bisubstrate compound C7. **A**- C7 versus ATP: ▼ 30 μM C7, ○ 5 μM C7, and ● 0 μM C7. ATP was varied from 5-500 μM at saturating 120 μM kanamycin A. B- C7 versus kanamycin A: ▽ 30 μM C7, ▼ 12.5 μM C7, ○ 5 μM C7, and ● 0 μM C7. Kanamycin A was varied from 5-500 μM at saturating ATP of 1 mM.

Chapter 5

AAC(6')-APH(2") protein kinase activity

Adapted from:

Daigle, D.M., McKay, G.A., Thompson, P.R., and Wright, G.D. *Chem.& Biol.* 1999, vol. 6, pp. 11-18.

Chapter 5

5.1 Abstract

The structure of APH(3')-IIIa obtained by X-ray crystallography demonstrated striking similarities in the overall kinase fold to that of eukaryotic protein kinases (EPK). As a result, it is predicted that other aminoglycoside kinases such as the bifunctional AAC(6')-APH(2") phosphotransferase would also adopt a similarly conserved core tertiary structure. This predicted structural similarity coupled with the fact that both the APH(3')-IIIa and AAC(6')-APH(2") kinases are effectively inhibited by eukaryotic protein kinase inhibitors suggested the possibility that the AAC(6')-APH(2") phosphotransferase may phosphorylate EPK substrates. Using a variety of peptides and proteins to assess the specificity of phosphorylation, it has been determined unequivocally that AAC(6')-APH(2") can phosphorylate several EPK substrates including Myelin basic protein (MBP), protamine and small peptide derivatives of myristoylated alanine-rich C-kinase substrate (MARCKS K and MARCKS R). Phosphoamino acid analysis of protein and peptide phosphorylation by AAC(6')-APH(2") showed that phosphorylation occurred exclusively on Ser residues. Phosphorylation of Ser/Thr protein kinase substrates follows first order kinetics, and the observed rates with peptide substrates were found to be 50-100-fold lower than those with aminoglycosides under identical assay conditions, which is consistent with the primary biological role of the enzyme in bacteria. These results contribute to our

understanding of aminoglycoside kinases and demonstrate a functional relationship between aminoglycoside and protein kinases suggesting an evolutionary link between the two.

5.2 Introduction

Insight into the tertiary structure of aminoglycoside kinases was attained by the three dimensional structure of the APH(3')-IIIa [89]. Significant structural similarity especially at the core of the kinase fold to that of eukaryotic protein kinases (EPK) was observed [89]. Consequently, all APH's are predicted to adopt a kinase fold similar to those of Ser/Thr and Tyr protein kinases despite having a lack of significant amino acid sequence homology (<10 % similarity) [89]. Apart from the expected overall structural similarity, the APHs also share a signature sequence with eukaryotic protein kinases. The sequence, H(G/N)DX_{3-4}N, from the APHs is homologous in both structure and function to the sequence (H/Y)RDX_4N from EPK [89]. The aspartate in this sequence has been proposed as the catalytic base involved in deprotonating the substrate hydroxyl for both cyclic AMP-dependent protein kinase (PKA) and the tyrosine kinase Csk [23,33,128]. The first crystallographic evidence for this was obtained by the solved binary and tertiary structures of cyclic AMP-dependent protein kinase (PKA) bound by a 20 residue phosphorylated peptide or ADP and the 20 residue peptide respectively [128]. Mutagenesis of the conserved aspartate (190-APH(3')-IIIa) found in this signature sequence, shows dramatic reduction in enzymatic activity when mutated to Ala [89], which also parallels results obtained for EPKs [23,33,69,128], however, this residue in

APH(3')-IIIa has the role of properly positioning the aminoglycoside hydroxyl during phosphate transfer, and is not a general base [14]. Mutagenesis results and the appropriate positioning of the aspartate 190 in the active site of APH(3')-IIIa [89,14], implicated the equivalent residue in AAC(6')-APH(2"), Asp374, as the active site amino acid essential for optimal phosphoryl transfer.

The similarities in both the structure and mechanism of APH(3')-IIIa with that of EPKs and the structural predictions for the kinase domain of AAC(6')-APH(2"), led to further investigation through the use of specific EPK inhibitors including flavanoids and isoquinolinesulfonamides [39]. The isoquinolinesulfonamides were determined to be effective inhibitors of both the APH(3')-IIIa and the AAC(6')-APH(2") kinase [39]. These compounds are competitive inhibitors of ATP and non-competitive inhibitors of aminoglycoside substrates for both APH(3')-IIIa and AAC(6')-APH(2") displaying K_i values in the low-micromolar range (<100 μM) for the best derivatives. Consequently, it is assumed that APHs and EPKs share a similar overall three dimensional fold, chemical mechanism of phosphoryl transfer, and sensitivity to inhibitors.

To further investigate and establish the functional and mechanistic relationship between APHs and EPKs, the ability of AAC(6')-APH(2") to phosphorylate several known substrates of EPKs was investigated. The results demonstrate that APHs phosphorylate the substrates on Serine residues and thus act as Ser/Thr kinases.

5.3 Results and Discussion:

5.3.1 Similarities between APHs and EPKs

Previous studies have demonstrated that APH(3')-IIIa, and likely all aminoglycoside phosphotransferases, share overall structure, chemical mechanism and sensitivity to inhibitors with EPKs. The bifunctional AAC(6')-APH(2") kinase, which has modest sequence similarity with APH(3')-IIIa (10 % identity and 21 % similarity), is also sensitive to EPK inhibitors [39] which supports the theory that these enzymes form a larger structurally related kinase family which also likely includes the type IIβ phosphoinositide phosphate kinase for which a structure has been determined [165]. The structural and functional similarities between APHs and EPKs suggested that aminoglycoside kinases, including the AAC(6')-APH(2"), may be able to catalyze phosphoryl transfer to an EPK substrate.

5.3.2 Peptide Substrates Tested

We examined various protein and peptide substrates to establish if protein kinase activity could be observed with AAC(6')-APH(2"). Our choices of peptide and protein substrates were made to investigate overall substrate diversity as well as Tyrosine or Serine/Threonine phosphorylation specificity (Table 5.1). Ser/Thr kinase specific substrates included: i) MARCKS K, MARCKS R (derivatives of myristoylated alanine-rich C-kinase substrate (MARCKS) protein) [188], and protamine sulfate which are PKC substrates [71,160], casein, a substrate of casein kinase I and II [80], histone H1 a substrate of protein kinases A , C and G [115], Kemptide, a PKA substrate [75] and

Table 5.1: Peptide and protein substrates tested.

Substrate	Sequence	Associated kinase
MARCKS K	Ac-FKKSFKL-NH_2	PKC
MARCKS R	Ac-FRRSFRL-NH_2	PKC
Kemptide	LRRASLG	PKA
Casein		Casein kinases
Histone H1		PKA, PKC, PKG
Protamine		PKC
MBP		PKA, PKC and MAP kinases
Poly(Glu,Tyr) 4:1		Csk
$p60^{c\text{-}src}$ Substrate II	Ac-IYGEF-NH_2	c-Src

Myelin basic protein (MBP) a substrate of various protein kinases including calmodulin-dependent protein kinases, cAMP-dependent protein kinases [31], protein kinase C [122] and mitogen-activated protein kinases (MAP kinases) [114,122]. Two protein tyrosine kinase substrates were also assayed, poly Glu-Tyr, a synthetic peptide substrate for various tyrosine kinases (e.g. csk [33]) and $p60^{c\text{-}src}$ substrate II a substrate of c-src kinase [146]. These peptides and proteins represented a broad spectrum of protein kinase activities which subsequently gave a specific profile to the bifunctional APH protein kinase activity.

5.3.3 APH(2")-Ia Phosphorylates Ser/Thr Kinase Substrates

Several known substrates of Ser/Thr kinases examined were found to be substrates of AAC(6')-APH(2") (Table 5.1 & Figure 5.1). These were MARCKS peptides, protamine sulfate and MBP while Kemptide, histone H1 and casein were not determined to be substrates. The tyrosine kinase specific substrates, poly (Glu,Tyr) (4:1) and p60c- src substrate II were not substrates of AAC(6')-APH(2") at concentrations of up to 500 μM.

Phosphorylation of MBP was investigated further by the use of site-directed variants of AAC(6')-APH(2") and APH(3')-IIIa in the signature sequence to verify that the activity observed was in fact due to the aminoglycoside kinases and not a contaminating kinase in either the enzyme or substrate preparations. Mutagenesis of equivalent signature sequence residues Asp374Asn of AAC(6')-APH(2") and Asp190Ala of APH(3')-IIIa, which highly impact on aminoglycoside kinase activity are equivalent

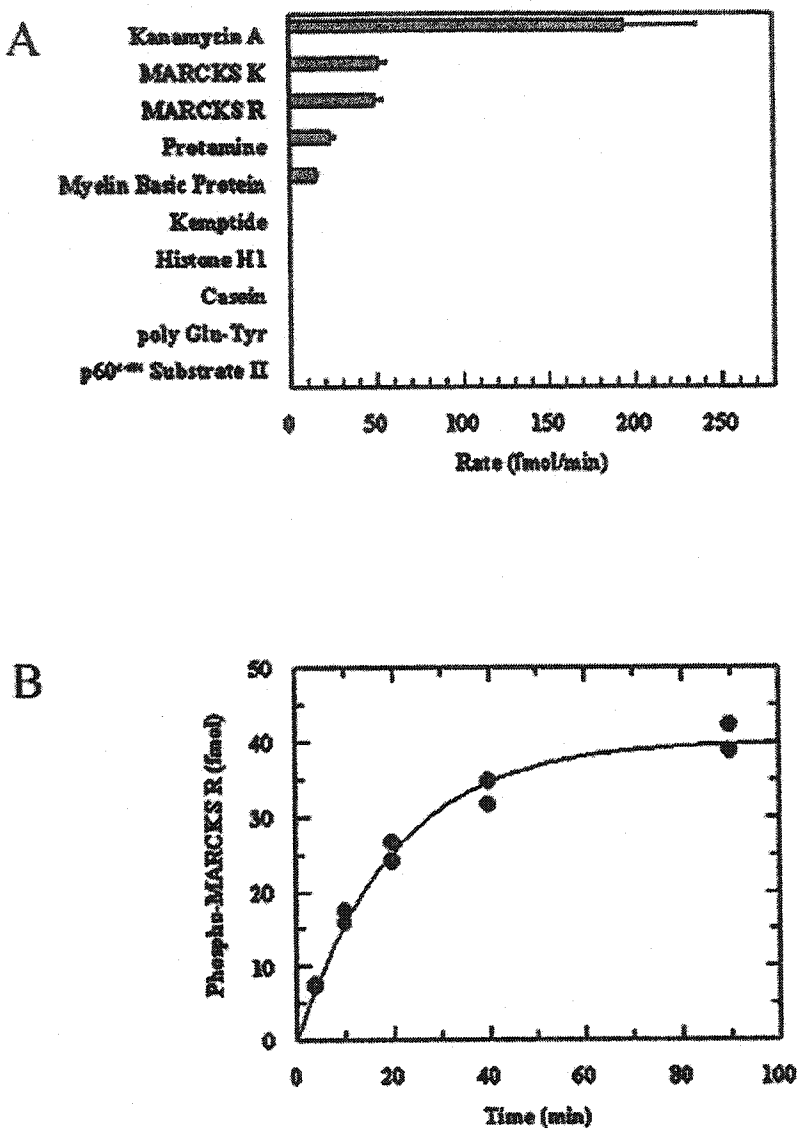


Figure 5.1: Protein phosphorylation by AAC(6')-APH(2"). Potential substrates (50 μM) were incubated with purified AAC(6')-APH(2") as described in Materials and Methods. The rate of reaction was determined by fitting to a first-order rate equation. **(A)** Specificity of substrate phosphorylation. **(B)** Example of a rate determination for the AAC(6')-APH(2")-catalyzed phosphorylation of the MARCKS R peptide. Figure taken from [40] with copyright approval.

residues to Asp210 of PKA (Figure 5.2a and 5.2b) [69]. A second variant of APH(3')-IIIa, Asp208Ala, was also tested for its' ability to phosphorylate MBP as it possessed no detectable aminoglycoside kinase activity. Both the Asp190Ala and the Asp208Ala variants of APH(3')-IIIa were kind gifts from Dr. Paul Thompson (dept. Biochemistry, McMaster University, Hamilton, Ontario, Canada). The bifunctional APH site-directed variant had a greater than 250-fold reduction in k_{cat} towards aminoglycoside substrates (from 0.42 to 0.0019 s^{-1}) and also showed as expected, a marked decrease in the ability to phosphorylate MBP (6.7 % wild-type activity) (Figure 5.3). The Asp190Ala variant of APH(3')-IIIa is dramatically impacted in k_{cat} (>500-fold) for aminoglycoside substrates [89]. As anticipated, this APH(3')-IIIa variant displayed a reduced ability to phosphorylate MBP (52 % wild-type activity) while the greatest impact on catalysis was obtained with the Asp208Ala mutation that exhibited no detectable phosphorylation of MBP as determined by autoradiography of a 15 % SDS-PAGE gel (Figure 5.3). Phosphorylation of MBP by the variants was determined following a four hour incubation and quantified by scintillation counting of the excised band from the dried gel. As an additional control for background kinase activity, the MBP preparation was boiled prior to assays with no detectable effect on MBP phosphorylation by either the bifunctional kinase or APH(3')-IIIa. It is therefore concluded that both the APH(3')-IIIa and the bifunctional APH are phosphorylating MBP and that there is no contaminating kinase.

(a)

APH(3′)-IIIa	H_{188}	GDLGDSNIFVKDGKVSG-----FIDLG
AAC(6′)-APH(2″)	H_{372}	NDFSC-NHLLLDGNNRLTG---IIDFG
PKA(yeast TPK1)	Y_{208}	RDLKPENILLDKNGHIK-----ITDFG
PKC-a	Y_{461}	RDLKLDNVMLDSEGHIK-----IADFG
CK-I	Y_{129}	RDIKPDNFLIGRPSSRNANMVYMVDRG
c-Src	H_{387}	RDLRAANILVGENLVCK-----VADFG
CDK2	H_{125}	RDLKPQNLLINTEGAIK-----LADFG

(b)

Figure 5.2: Active site region of protein in aminoglycoside kinases. **(a)** Sequence alignment of aminoglycoside and protein kinases highlighting conserved residues including APH(3')-IIIa Asp190 (green, APH(3')-IIIa numbering), the putative active site base, and the Mg^{2+} ligands, Asp195 (pink) and Asp208 (red). Genbank accession numbers: APH(3')-IIIa, VO1547; AAC(6')-APH(2"), M13771; TPK1, M17072; Human PKCα, S09496; *S. pombe* CK-1, U06930; Human $pp60^{c\text{-}src}$, K03218; Human CDK2, X61622. **(b)** Close up of the APH(3')-IIIa active site (1J7L) displaying ADP (orange) Mg2+ (cyan), the ADP coordinating residue Lys44 (light green), Asp190 (dark green), Asn195 (magenta), Asp208 (red). Gly189 is white (PDB code 1J7L). The structure was drawn using the programs MOLSCRIPT [100] and RASTER 3D [139].

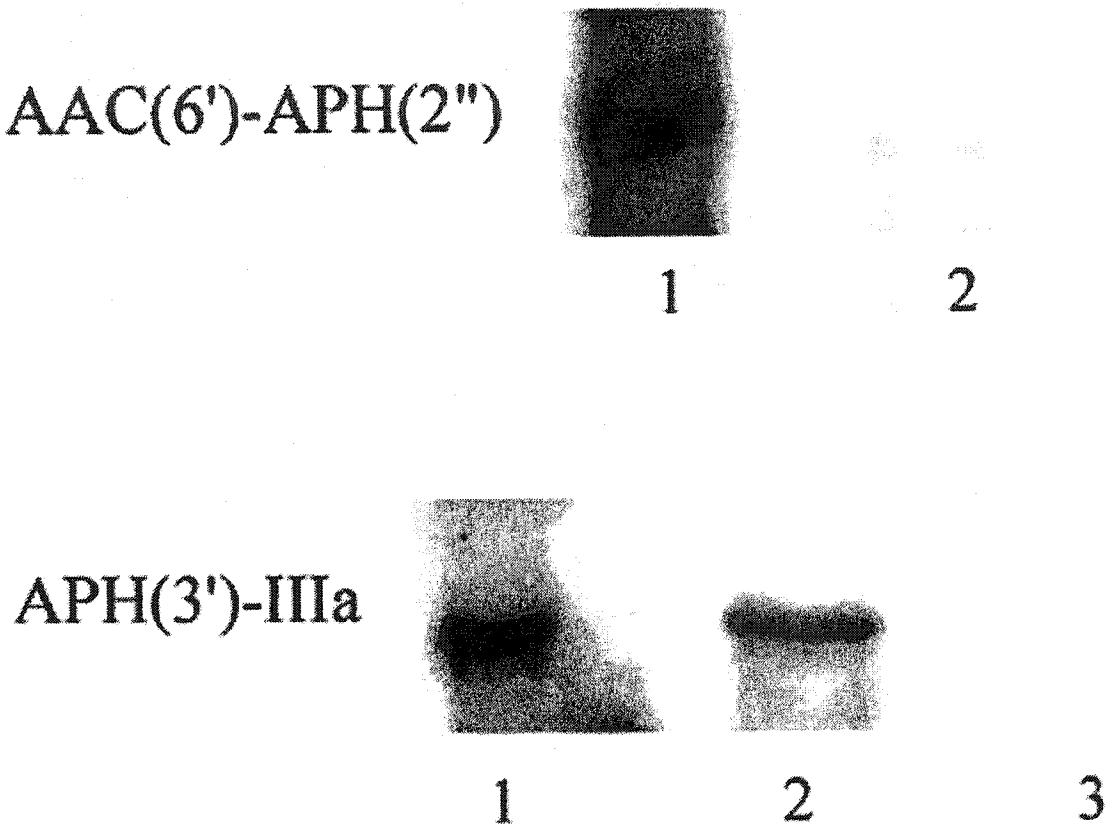


Figure 5.3: Phosphorylation of MBP by APH(3')-IIIa and AAC(6')-APH(2"). MBP was phosphorylated with [γ^{32}P]-ATP by (a) AAC(6')-APH(2") or (b) APH(3')-IIIa, separated on a 15 % SDS-polyacrylamide gel and analyzed by autoradiography. Bovine MBP has a molecular weight of 18.3 kDa, but when phosphorylated, migrates at/or around 23 kDa. Top panel lane 1, wild-type AAC(6')-APH(2"); Lane 2, AAC(6')-APH(2") Asp374Asn. Lower panel lane 1, wild-type APH(3')-IIIa; lane 2, APH(3')-IIIa Asp190Ala; lane 3, APH(3')-IIIa Asp208Ala. Identical results were obtained with native MBP and with MBP which was first boiled to inactivate any potential contaminating kinases.

5.3.4 Rates of APH(2")-Ia Peptide and Protein Phosphorylation

Attempts to determine V_{max} and K_m for these substrates were unsuccessful as the quantities of peptide required were incompatible with the assay. We therefore turned to a measure of k_{cat}/K_m using a sub-K_m concentration of MBP with equimolar enzyme. Under these conditions, the Michaelis-Menten equation simplifies to:

$$v = [S][E_{tot}]k_{cat}/K_m \quad (1)$$

The value for k_{cat}/K_m for MBP was determined to be 8.0×10^2 $M^{-1}s^{-1}$ for AAC(6')-APH(2"). This catalytic efficiency is 75-fold lower than that obtained for kanamycin A using the phosphocellulose binding assay under similar reaction conditions. In comparison, the k_{cat}/K_m for PKC-catalyzed MBP phosphorylation is 10^6 $M^{-1}s^{-1}$ [92]. Even though the efficiency of the bifunctional kinase catalyzed reaction is not comparable to those of typical EPKs, they still exhibit a noticeable activity. Measurements of rates of incorporation of radio-labeled $\gamma[^{32}P]$-PO_4 into the products using a phosphocellulose binding assay demonstrated that the natural substrate kanamycin is phosphorylated at a much higher rate whereas the peptide substrate values were generally 10 to a 100-fold lower under identical assay conditions.

5.3.5 Characteristics of APH(2")-Ia Phosphorylated Peptides and Proteins

The peptides and proteins which were phosphorylated by AAC(6')-APH(2") have an overall positive charge in common (pI = 10 for MARCKS peptides, protamine and MBP). Acidic peptides and proteins were not phosphorylated. The criteria for binding and phosphorylation of these compounds is not exclusively based on charge however, as

Histone H1 and Kemptide are both positively charged at pH 7.5, and are not substrates. PKC, a kind gift of Dr. Richard Epand (dept. Biochemistry, McMaster University, Hamilton, Ontario, Canada) and commercially purchased casein kinase I were able to phosphorylate these substrates under identical assay conditions [40]. The fact that there is a charge selectivity towards the positively charged EPK substrates is consistent with the fact that the binding pocket for aminoglycoside substrates in the APH(3')-IIIa structure is lined with negatively charged residues [89], an observation that is consistent with the aminoglycoside substrates being polycationic molecules. The AAC(6')-APH(2") has an overall negative charge at pH 7.5 and presumably also has aminoglycoside binding pockets lined with negatively charged residues. These results are however striking when the structures of both eukaryotic protein kinases (Figure 5.4a) and APH(3')-IIIa (Figure 5.4b) are compared. Although they share a similar three dimensional fold, the accessibility of the active sites is very different.

In the case of the protein kinases, the active sites are open and freely accessible while in the case of the APH(3')-IIIa structure, the active site is found in a cleft near the center of the protein and is blocked by a series of two alpha helices from the C-terminus and a loop from the N-terminus (Figure 5.4b and 5.4c). Differences in the accessibility aside, the fact the peptides and especially MBP are able to access the catalytic residues shows that APH active sites are quite accommodating.

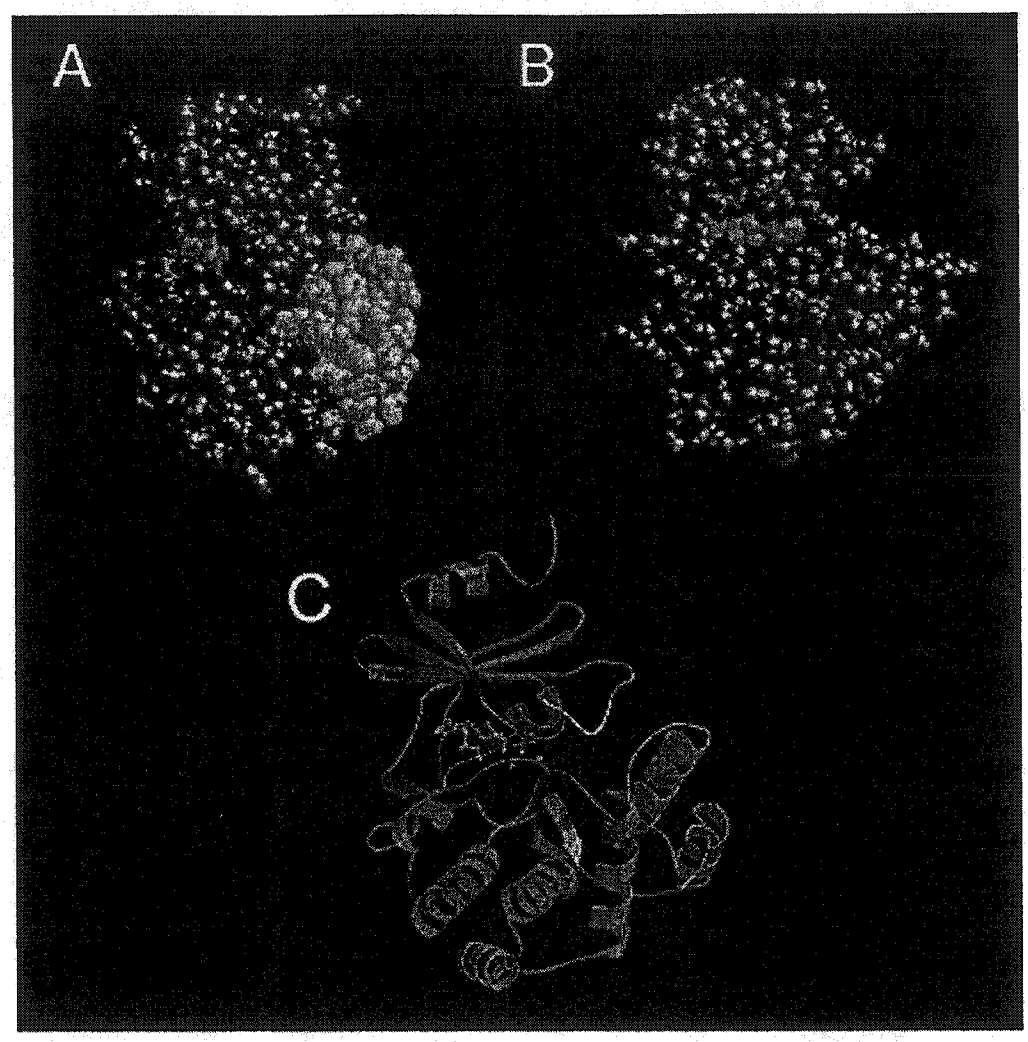


Figure 5.4: Three dimensional structures of APH(3')-IIIa and casein kinase-1. Comparison of the three dimensional structures of **(a)** APH(3')-IIIa bound by Mg^{2+}-ADP (PDB ID code 1J7L) and **(b)** casein kinase-1 bound by Mg^{2+}-ATP (PDB ID code 1CSN). The nucleotide co-substrates are indicated in green and the Mg^{2+} ions are in magenta. The large cleft in casein kinase-1, which provides access to the active site for the protein and peptide substrates, is blocked in part in APH(3')-IIIa by two helices between residues 135 and 178 shown in orange in the APH(3')-IIIa structure. **(c)** Ribbon diagram of APH(3')-IIIa. A loop between residues 49-56 that lines the second substrate binding pocket of the active site is in white. Structures **(a)** and **(b)** were drawn with the program RasMol v.2.6 [175], while **(c)** was drawn with MOLSCRIPT [100] and visualized by RASTER 3D [139].

5.3.6 Phosphoamino Acid Analysis

To further define and characterize the site of phosphorylation, a phosphoamino acid analysis was performed on MARCKS R, MARCKS K and protamine sulfate phosphorylated by AAC(6')-APH(2") or PKC (negative control). Results demonstrated that in all cases, with all three substrates, phosphorylation occurred exclusively on Ser residues (Figure 5.5), thereby leading to the conclusion that APHs are protein Ser kinases.

The reciprocal reaction of phosphorylation of aminoglycosides (kanamycin A or neomycin at 1 mM) by EPKs was assayed using casein kinase I and PKC in a 60 min reaction, however no detectable phosphorylation of these aminoglycosides occurred (detection limit 0.7 fmol).

5.4 Conclusions

The results presented here demonstrate that both the bifunctional kinase and APH(3')-IIIa have intrinsic protein kinase activity and suggests that other APHs will also possess this property. APHs and EPKs therefore share similar three dimensional structural features, mechanism of phosphoryl transfer, sensitivity to inhibitors and the ability to phosphorylate peptides and proteins. This data also suggests a direct evolutionary relationship between APHs and EPK which perhaps originated from Ser/Thr kinases found in many bacterial species (reviewed in [99,224]). It is perhaps not coincidental that several Ser/Thr kinases have been implicated in secondary metabolism

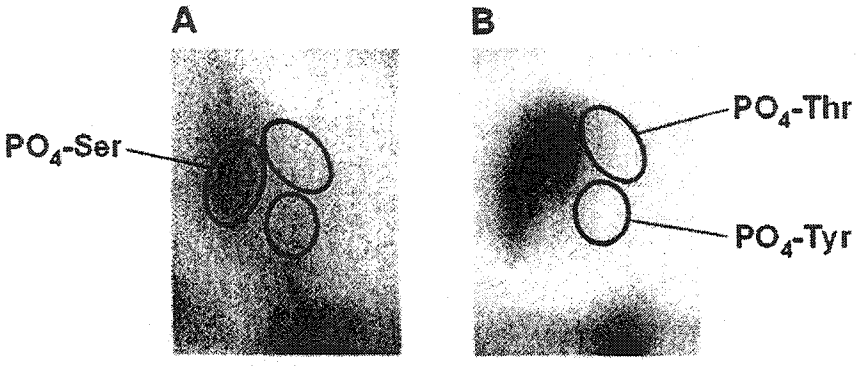


Figure 5.5: Phosphoamino acid analysis of MARCKS K phosphorylated by AAC(6')-APH(2") and PKC. MARCKS K was phosphorylated by **(a)** AAC(6')-APH(2") and by **(b)** PKC, hydrolyzed under acidic conditions and separated by two-dimensional thin layer electrophoresis as described in the Materials and Methods section. The relative positions of standard phosphoserine, phosphotyrosine and phosphothreonine are indicated. Phosphorylation of MARCKS K, MARCKS R and protamine by AAC(6')-APH(2") occurred exclusively on serine residues.

in actinomycetes [90,133,186,199,205], of which many are important aminoglycoside producing bacteria, harboring APH resistance genes [44]. These actinomycetes may represent the source of origin for antibiotic resistance enzymes evolved from intrinsic protein kinases.

The biological relevance if any of these findings is presently unknown. They do suggest the possibility of some potential impact on *in vivo* mammalian gene expression/signal transduction experiments where G418 or hygromycin B are used as selection. The *neo* (APH(3')-II) and *hyg* (APH(4)-I) genes confer resistance to G418 and hygromycin B, respectively. Our findings that two such aminoglycoside kinases possess low but significant protein kinase activity should therefore be taken as a cautionary note to the use of such selection with *in vivo* mammalian systems and be considered in experimental design.

5.5 Materials and Methods

5.5.1 Chemicals:

Myelin-basic protein (MBP) from bovine brain, kemptide, casein, poly (Glu-Tyr)(4:1) and kanamycin were from Sigma. Histone H1 from calf thymus and $p60^{c\text{-}src}$ substrate II were from Calbiochem. Protamine sulfate, MARCKS R were kind gifts of Dr. Richard Epand, Department of Biochemistry, McMaster University. MARCKS K peptide was prepared by R.E. Williams, Institute for Biological Sciences, National Research Council, Ottawa, Ont. APH(3')-IIIa and AAC(6')-APH(2") were purified as previously described [38,135]. Site-directed mutants were generated using Quick-change

mutagenesis kit (Strategene) using primer ($^{5'}$-GTT TAT GCC ATA ATA ATT TTA GTT GTA ATC A-$^{3'}$), and the reverse complement sequence for AAC(6')-APH(2") Asp374Asn. APH(3')-IIIa, Asp190Ala APH(3')-IIIa and Asp208Ala APH(3')-IIIa were kind gifts of Dr. Paul Thompson (McMaster University, department of Biochemistry). Rat brain PKC preparation (a mixture of α, β and γ-isoforms) was the kind gift of Dr. Richard Epand (McMaster University, department of Biochemistry). Casein kinase-I from *Schizosaccharomyces pombe* was purified by Dr. Geoff McKay (McMaster University, department of Biochemistry) from *E. coli* BL21/pT7II-ckiΔ298 (kind gift of Jeff Kuret, Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL) as previously described [27].

5.5.2 Aminoglycoside Kinase Assays

Phosphorylation of aminoglycosides was monitored by coupling the release of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase or by phosphocellulose binding assays described previously [135].

5.5.3 Protein Kinase Assays

5.5.3.1 Phosphocellulose Binding Assay

Phosphorylation of peptide and aminoglycoside substrates was monitored by three separate assays. A phosphocellulose binding assay was employed for substrates that are positively charged at neutral pH and generally amenable to this protocol [77]. The assay consisted of peptide substrate (50-100 μM final concentration for rate determinations and

600 μM for determination of linearity with enzyme), or aminoglycoside substrate (50 μM final), purified AAC(6')-APH(2") (15-60 μg) or purified APH(3')-IIIa (7.5-30 μg), 10 mM γ^{32}P-ATP (1.2 x 10^5 cpm/nmol), 50 mM Tris pH 8.0, 40 mM KCl, 10 mM $MgCl_2$ to a final volume of 10 μL. The reactions proceeded at room temperature and were terminated after 5 min to 6.5 h by application onto Whatman P-81 phosphocellulose paper, washed three consecutive times with water, air dried and placed in scintillation vials with 5 ml of scintillation fluid. The prepared samples were analyzed by liquid scintillation counting.

5.5.3.2 Glass Microfibre Filter Binding Assay

Substrates with a negative or neutral charge at neutral pH and/or those that weren't amenable to trapping on phosphocellulose paper, were assayed using filtration on glass microfibre filters. The reactions were prepared as in the above procedure. At appropriate time points, samples were applied onto Whatman GF/C glass microfibre filters and suction-filtered through a Millipore vacuum filtration apparatus. The filters were washed several times with the reaction buffer, dried and placed in scintillation vials with 5 mL of scintillation fluid.

5.5.3.3 Phosphorylation of Histone H1 and MBP

Incubations consisted of 0.4 mg/ml histone H1 or MBP and 10 mM [γ^{32}P]-ATP (1.2 x 10^5 cpm/nmol) in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 40 mM KCl. The assays were initiated by the addition of 10-20 μg of AAC(6')-APH (2") and allowed to

progress for 4 h at ambient temperature. The reactions were quenched by the addition of EDTA to a final concentration of 35 mM followed by the addition of an equal volume of 2 X SDS loading buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 10 % glycerol, 4 % SDS) and separated on a 15 % SDS-polyacrylamide gel. Phosphorylation of the substrates was monitored by autoradiography of the dried gels.

5.5.4 Protein Kinase Phosphorylation of Kanamycin

Two protein kinases, casein kinase I and PKC, were assayed by phosphocellulose filter binding assays for their ability to modify the aminoglycoside kanamycin. Assay conditions were identical to those described above with the exception that 25 μg of either partially purified casein kinase I or PKC were used instead of an APH. For PKC activity assays, large unilamellar vesicles composed of phosphotidylserine and phosphotidylcholine were added to a final concentration of 100 μM. The reactions were allowed to proceed for 1-4 h and then applied onto Whatman p81 phosphocellulose paper and analyzed as above.

5.5.5 Analysis or Rate Data

Plots of the amount of phosphate incorporated into substrate proteins or peptides as a function of time were fit by non-linear least squares method to a first order rate equation (1):

$$v = k\,[S] \qquad (1)$$

5.5.6 Phosphoamino Acid Analysis of Phosphorylated Peptides

Peptides (MARCKS R, MARCKS K and protamine) were first subjected to an *in vitro* kinase reaction that consisted of 15 μg of AAC(6')-APH(2"), 32 μg of peptide, [γ^{32}P]ATP to 10 mM final (1.20 x 10^5 cpm/nmol) in 20 μL of 50 mM Tris pH 7.5, 40 mM KCl and 10 mM $MgCl_2$. Reactions proceeded for 2 h. Separation of the phosphorylated peptide from ATP and enzyme is accomplished by binding to the Whatman P-81 phosphocellulose paper followed by three consecutive washes and drying.

The dried 1 cm^2 piece of phosphocellulose was placed in a screw cap eppendorf tube with 1 mL of 6 N HCl. The tube was sealed and incubated in a 110 °C oven for 90 min. The hydrolysate was dried under vacuum and the sample was then dissolved in 10 μL of water.

Separation and identification of phosphoamino acids was performed by two-dimensional thin-layer electrophoresis [5]. A 5 μL volume of the sample was applied in 0.5 μL aliquots followed by drying on an origin of a 20 cm x 20 cm x 100 μM glass-backed cellulose thin layer chromatography plate. Non-radioactive standard phosphoamino acids (Phosphoserine, threonine and tyrosine) (1 μl) was also applied at the origin in 0.5 μL aliquots. Blotter paper was used to apply the electrophoresis buffer for the first dimension of electrophoresis at pH 1.9. This buffer consisted of 50 mL of 88 % formic acid (0.58 M final concentration), 156 mL of glacial acetic acid (1.36 M final concentration) and 1794 mL water all at pH 1.9. The samples were separarted for 20 min at 1.5 kV in a Hunter Thin Layer Peptide Mapping System Model# HTLE-7000 powered

by a Bethesda Research Laboratories Model 4000 power supply. Following electrophoresis the plate was removed and air dried.

The second dimension consisted of 100 mL of glacial acetic acid (0.87 M final concentration), 10 mL of pyridine (0.5 % (v/v) final concentration, 10 mL of 100 mM EDTA (0.5 mM final concentration) and 1880 mL of water, at pH 3.5. The plate was rotated 90° to the first dimension electrophoresis and separated for 16 min. at 1.3 kV. The plate was dried, sprayed with 0.25 % ninhydrin in ethanol and heated to visualize the standards. Autoradiography was then used to visualize the samples.

Concluding Remarks

Biochemical characterization of AAC(6')-APH(2") has revealed a highly efficient enzyme possessing a broader substrate range and activity profile than was first anticipated. It is capable of modifying any 2-deoxystreptamine aminoglycoside antibiotic by *N*- or *O*-acetyltransfer at the 6'-position and phosphoryl transfer to multiple hydroxyl positions. These include not only the 2"-hydroxyl of 4,6-deoxystreptamine aminoglycosides, but also phosphorylation on the 3' and 3'''-hydroxyl groups. The only exception to this rule is the 3'-deoxy aminoglycoside lividomycin A which is phosphorylated on the 5"-hydroxyl. The combination of these broadened activities and the apparent variability of aminoglycoside binding and modification characteristics exhibited by this enzyme will make it very challenging to design successful inhibitors. The lack of a crystal structure of AAC(6')-APH(2") or any part thereof has been disappointing and has limited investigation on this clinically important modifying enzyme. The future of this project will lie in obtaining such a structure and subsequently in the structure driven design and modeling of small molecule inhibitors. It is hoped that the inhibitory studies detailed in this work will lay a foundation for future inhibitor design towards AAC(6')-APH(2") and the reversal of the high-level aminoglycoside resistance resulting from the presence of this protein in bacteria.

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