Structure and function of the bifunctional aminoglycoside-modifying enzyme AAC(6')-Ie/APH(2'')-Ia

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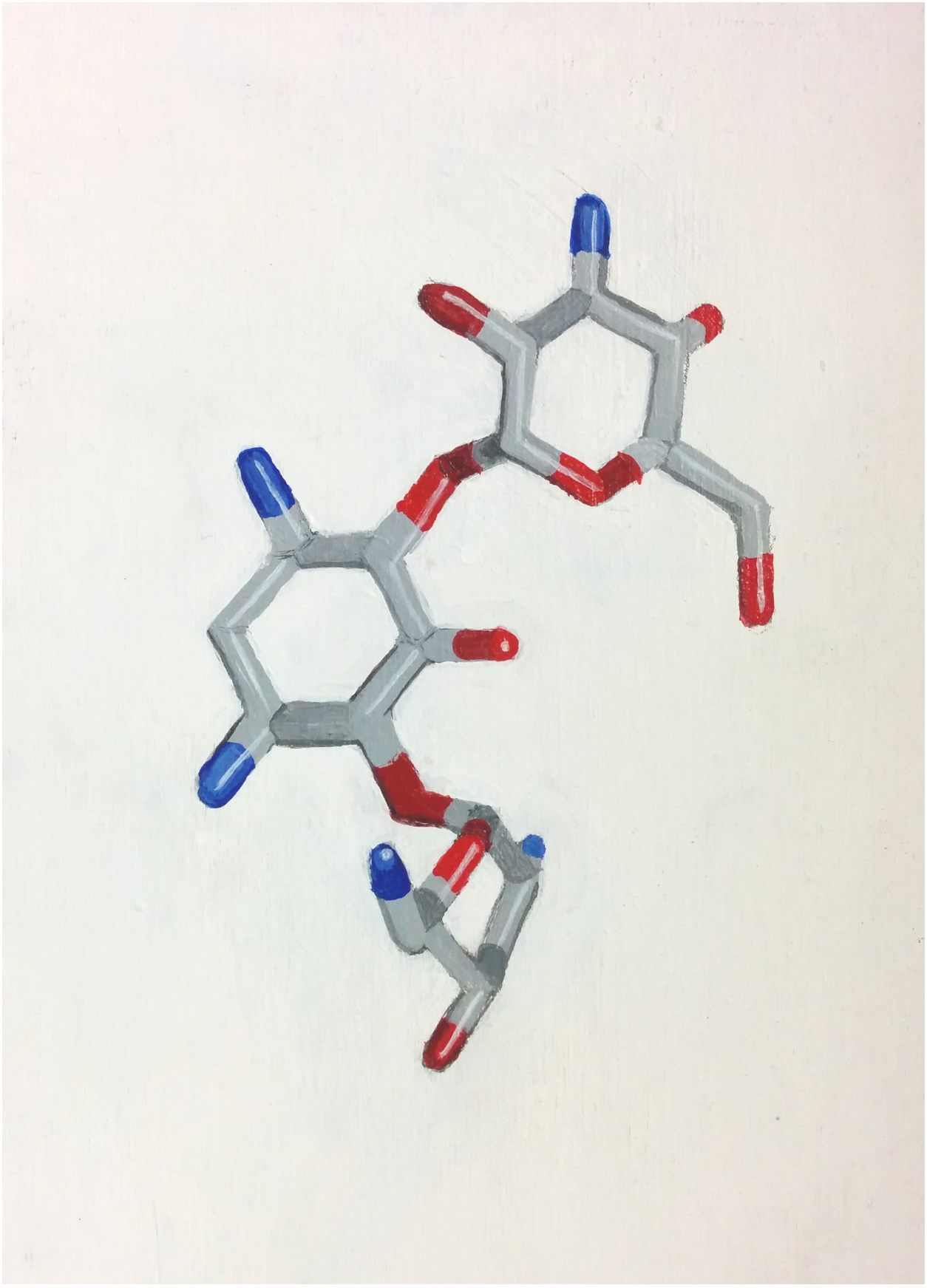

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Abstract

The bifunctional aminoglycoside-modifying enzyme AAC(6')-Ie/APH(2")-Ia is one of the most prolific resistance factors for aminoglycoside antibiotics. An understanding of the structure of this protein is necessary for the development of inhibitor compounds, new aminoglycosides, and to gain insight about the evolution of this antibiotic resistance enzyme.

I studied this enzyme using small-angle X-ray scattering as well as X-ray crystallography to probe the enzyme's structural architecture, interaction with substrates, and conformational changes upon binding of substrates to drive catalysis.

Small-angle X-ray scattering analysis indicated that the protein is not flexible and packs as a rigid bi-domain particle in solution. Addition of donor substrates GTP and acetyl-coenzyme A showed little appreciable change in the enzyme's scattering profile, indicating modest structural changes and the maintenance of a rigid conformation between domains. Rigid structure, but absence of large conformational changes suggest a possible model of bifunctionality in AAC(6')-Ie/APH(2")-Ia where the two enzymatic domains gain function by acting as reciprocal binding modules to aid the enzyme in capturing and sequestering aminoglycoside from solution.

Crystallization of the C-terminal APH(2")-Ia domain of the protein in complex with guanosine nucleosides allows study of the aminoglycoside-binding profile of the enzyme, and demonstrated that 4,5- and 4,6-disubstituted aminoglycosides are bound to the phosphotransferase domain in the same orientation. This illustrates tight binding of both types of compound, but precludes catalytic modification of the 4,5-disubstituted family. This shows that this enzyme continues a trend of broad-profile antibiotic-modifying enzymes that bind to conserved regions *via* specificity for the conserved neamine-based rings of the compounds. It also indicates the possibility that the enzyme confers low-level resistance to some aminoglycosides by binding alone and not chemical modification.

The nucleoside triphosphate-bound structure of APH(2")-Ia revealed a novel conformation of the guanosine triphosphate group in the enzyme active site. This stabilized conformation of triphosphate is not compatible with productive catalysis. This conformation is in equilibrium with the activated, catalytically-competent conformation of the triphosphate, and this switch is

responsive to the binding of aminoglycosides to the enzyme. This catalytic switch allows regulation of the enzyme in response to antibiotic binding, to conserve the activity of the enzyme co-substrate for when it productively binds antibiotic. The enzyme conserves features with eukaryotic protein kinases, and the shared Gly-loop of the enzyme plays a lynchpin role in co-ordinating this function. While this enzyme shares the central catalytic machinery with eukaryotic protein kinases, it has developed a novel means of regulating its activity. This finding changes our understanding of antibiotic resistance enzymes, instead of a passive factor that is constitutively active, this enzyme can modulate its activity to optimize its effectiveness and modulate fitness cost.

Finally, the binding of N1-substituted aminoglycosides to APH(2")-Ia was investigated. Modification of the N1 group of aminoglycosides blocks binding to the enzyme, as does a clinical mutation of serine 376 to asparagine. Structures of this mutant enzyme illustrates that this mutant disrupts the means of binding most aminoglycosides and does not undergo structural rearrangement that accommodates N1-substituted aminoglycosides. A co-crystal structure of amikacin bound to the wildtype enzyme indicates a possible weak binding mode that could be responsible for low levels of aminoglycoside phosphorylation. Some of the contacts used for binding the native enzyme substrates are conserved, but the central ring avoids contact with the neamine binding site previously identified. This binding mode tolerates substitution at the S376 position, suggesting that this mutation improves resistance toward amikacin and arbekacin by biasing the enzyme toward this mode of binding.

Together, these studies indicate that the structure and function of AAC(6')-Ie/APH(2")-Ia is more nuanced than previously estimated, and place this resistance factor in the resistance armamentarium as a complex, dynamic, and highly evolved clinical antibiotic resistance factor. It has highly tuned behaviour regulated toward a balanced environmental role.

Résumé

L’enzyme bifonctionnelle AAC(6')-Ie/APH(2")-Ia, agissant sur les aminoglycosides, est l’un des plus importants facteurs de résistance à ces antibiotiques. La connaissance de la structure de cette protéine est essentielle au développement d’inhibiteurs et de nouveaux aminoglycosides ainsi qu’à la compréhension de l’évolution de cette enzyme.

J’ai investigué cette protéine au moyen de la diffusion des rayons X aux petits angles et de la cristallographie aux rayons X afin d’en déterminer la structure, les interactions avec ses substrats et les changements de conformation lors de la liaison aux substrats à des fins catalytiques.

L’analyse par la diffusion des rayons X aux petits angles a démontré que la protéine n’est pas flexible, mais a un arrangement rigide à deux domaines en solution. L’ajout des substrats donneurs GTP et acétyl-coenzyme A a causé peu de changement au profil de diffusion, indiquant l’absence de réarrangements majeurs entre les domaines. Cette structure rigide m’amène à proposer un modèle de la bi-fonctionnalité de AAC(6')-Ie/APH(2")-Ia, selon lequel les deux domaines enzymatiques fonctionnent en tant que modules de liaison réciproques afin de capter et de retenir l’aminoglycoside.

La cristallisation du domaine C-terminal APH(2")-Ia en complexe avec des nucléosides de guanosine m’a permis d’explorer les propriétés de liaison aux aminoglycosides de cette enzyme, démontrant que les aminoglycosides à deux substituants 4,5- ou 4,6- se lient au domaine phosphotransférase dans la même orientation. Bien que les deux classes de composés soient étroitement liées à la protéine, la famille 4,5- ne peut être modifiée par la catalyse. Ces résultats placent cette protéine au sein d’une lignée d’enzymes modificatrices d’antibiotiques à large spectre qui se lient à leur substrat par la reconnaissance d’éléments conservés sur ces derniers. De plus, ils ouvrent la possibilité d’un mécanisme de faible résistance à certains aminoglycosides par la simple liaison sans modification chimique.

La structure de APH(2")-Ia en complexe avec un nucléoside triphosphate a aussi révélé une nouvelle conformation de celui-ci dans le site actif de l'enzyme. Cette conformation stabilisée n'est pas disposée pour la catalyse, et existe en équilibre avec la conformation activée du groupe triphosphate. L'équilibre est affecté par la liaison de l'aminoglycoside à la protéine. Cet interrupteur catalytique permet de restreindre l'activité de l'enzyme seulement au substrat antibiotique correctement lié. La protéine possède des caractéristiques communes aux protéines kinases eucaryotes, incluant la boucle riche en résidus de glycine, qui joue un rôle clef dans la coordination de cette fonction. Bien que les éléments catalytiques majeurs dans cette enzyme soient conservés par rapport aux protéines kinases eucaryotes, ce mécanisme de régulation est unique. Cette découverte ouvre une nouvelle perspective sur les enzymes de résistance aux antibiotiques. Loin d'être un facteur passif constamment activé, AAC(6')-Ie/APH(2")-Ia est capable de moduler son activité afin d'optimiser le coût à la valeur sélective.

Finalement, la liaison des aminoglycosides substitués à la position N1- à l'APH(2")-Ia a été étudiée. La modification du groupe N1 bloque la liaison de l'aminoglycoside à l'enzyme, comme le fait une mutation clinique de la sérine 376 en asparagine. Des structures de cette enzyme mutée montre qu'elle perturbe les moyens de liaison de la plupart des aminosides et ne subit pas de réarrangement structural permettant de lier les aminoglycosides substitués en N1. Une structure co-cristalline de l'amikacine liée à l'enzyme native indique possiblement un mode de liaison faible qui pourrait être responsable du bas niveau de phosphorylation des aminoglycosides. Quelques-uns des contacts utilisés pour lier les substrats de l'enzyme native sont conservés, mais l'anneau central évite tout contact avec le site de liaison de la néamine précédemment identifié. Ce mode de liaison peut tolérer une substitution à la position S376, suggérant que cette mutation améliore la résistance à l'amikacine et à l'arbekacin en sollicitant l'enzyme vers ce mode de liaison.

En résume, ces études ont exposé des nuances auparavant insoupçonnées dans la structure et la fonction de AAC(6')-Ie/APH(2")-Ia, un facteur de résistance aux antibiotiques complexe, dynamique et très évolué. Son action est hautement régulée pour un rôle balancé dans son environnement.

List of Abbreviations

2-DOS 2-deoxystreptamine

Å Ångstrøm (0.1 nanometres)

AAC Aminoglycoside acetyltransferase

AAC(6')-Ie Aminoglycoside-6'-*N*-acetyltransferase type Ie

AAC(6')-Ie/APH(2'')-Ia

Bifunctional aminoglycoside-6'-*N*-acetyltransferase type Ie / aminoglycoside-2''-*O*-phosphotransferase type Ia fusion protein

AcCoA Acetyl-coenzyme A

AHB (*S*)-4-amino-2-hydroxybutyric acid

AME Aminoglycoside-modifying enzyme

ANT Aminoglycoside nucleotidyltransferase

APH Aminoglycoside phosphotransferase

APH(2'')-Ia Aminoglycoside-2''-*O*-phosphotransferase type Ia

ATP Adenosine triphosphate

cAPK Cyclic-AMP dependent protein kinase, also known as protein kinase A

CCD Charge-coupled device

CoA Coenzyme A

CoASH Coenzyme A (free sulfhydryl form)

DTDP 4,4'-dithiodipyridine

DTT Dithiothreitol

ePK Eukaryotic-type protein kinase

GDP Guanosine diphosphate

GMPPNP	Guanosine-β,γ-imidotriphosphate
GMPPCP	Guanosine-β,γ-methylene triphosphate
GTP	Guanosine triphosphate
GTP-γ-S	Guanosine γ-thiotriphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	kiloDalton (1000 grams/mol)
LB	Luria-Bertani lysogeny broth
NADH	Nicotinamide adenine dinucleotide hydride
NaCl	Sodium chloride
NCS	Non-crystallographic symmetry
NDP	Nucleoside diphosphate
NTP	Nucleoside triphosphate
R_G	Radius of gyration
SAXS	Small-angle X-ray scattering
TCEP	*tris*-Carboxyethylphosphine
TLS	Torsion, libration, screw model for anisotropic distortions of rigid bodies

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1 Introduction

1.1 Antibiotics and resistance

Antibiotics are some of the most important compounds used in modern medicine and single-handedly drove a revolution in the field (Aminov, 2010). These compounds, which kill or inhibit the growth of bacteria, allow medical practitioners to easily treat bacterial infections, and facilitate other treatments like surgery, dialysis, and cancer treatments with greatly reduced risk of infection. Following their discovery and development, antibiotics have become a ubiquitous feature of the contemporary medical establishment. Antibiotics also find widespread use in veterinary medicine, agriculture, sanitation, and food preservation (Meek et al., 2015).

In addition to their use as medically active reagents, antibiotics changed our view of the world. Antibiotics first demonstrated that a chemical compound – a drug – could be therapeutic. Antibiotic molecules helped birth the modern pharmaceutical industry, and continue to influence how we think about drug development and drug policy. These compounds, with origins in the microbial environment, have shaped the world we live in today.

At the same time, bacterial resistance has the potential to erode or eliminate the use of antibiotics. Compounds that have become life-saving interventions and permit many advanced medical interventions are at risk of obsolescence (Piddock, 2012). Resistance can appear to have emerged in response to clinical antimicrobial use, but it actually has an intricate and complicated natural history, developed alongside antibiotics long before we used them to treat infections. While antibiotics are a gift from nature, antibiotic resistance is nature’s curse.

1.1.1 The history of antibiotics: From novelty to necessity

Since ancient times, we have sought to rid ourselves of the scourge of infectious disease. Prior to the advent of modern medicine, treatment for infection relied mostly on symptomatic relief, or invasive, risky, and often crippling procedures like amputation (Burnet and White, 1972). While some infections could be treated, many conditions were a death sentence. If a patient survived, the effects of the infection could still condemn the patient to a lifelong, chronic condition. A bacterial infection was rarely trivial.

This changed over the course of the last 100 years. Paul Ehrlich first demonstrated that a chemical compound could be used to treat an infectious disease (Williams, 2009). By searching for chemical compounds that selectively interact with microbes and not eukaryotic cells, Ehrlich identified the first molecule to selectively kill bacteria while remaining relatively nontoxic to the patient. Named Salvarsan, this compound was particularly effective against *Treponema pallidum*, the bacterial cause of syphilis. Salvarsan was the first chemical to successfully cure a bacterial infection and demonstrated that infectious disease could be treated with a chemical compound – in Ehrlich's terms, a “chemotherapeutic” (Gensini et al., 2007).

Salvarsan was effective in treating syphilis, but was not without drawbacks. It was limited in scope toward *Treponema* and similar microbes, while other bacteria were not affected. In addition, while less toxic to patients than to bacteria, this arsenic-based compound still showed considerable toxicity (Ebright, 1913). Following Ehrlich's breakthrough, other researchers searched for safer compounds active toward a wider array of bacteria. Gerhardt Domagk discovered and synthesized sulfonamides (Sneader, 2001), more useful and broadly applicable compounds, resulting in the first commercially available antimicrobial drug, Prontosil (Domagk, 1957).

These early successes in antimicrobial discoovery were made by chemists. However, many of the most important antimicrobial compounds were discovered by biologists. Antimicrobial research came to life with a landmark discovery by Alexander Fleming. Famously, Fleming discovered a compound on plates of *Staphylococcus aureus* that were contaminated with *Penicillium* mould (Fleming, 1929). A zone of clearing about the mould suggested to Fleming that the mould was producing an antimicrobial chemical that killed the *S. aureus* cells, which he

isolated and named penicillin[1]. Penicillin demonstrated its true value when Chain and Florey scaled up production and purification to generate medically useful amounts (Quirke, 2001).

Penicillin was a breakthrough compound that was nontoxic and extremely effective in treating infections caused by a wide variety of bacteria. While it drove a revolution in medicine it also drove a scientific revolution. Penicillin is scientifically important because of its origin: is is produced by a micro-organism. This finding indicated that microbes deliberately produce chemicals to act upon other microbes. Penicillin showed us that microbes in nature engage in an antagonistic relationship, producing chemicals that act as weapons toward each other. In his discovery of penicillin, Fleming identified microbes as a useful source of antimicrobial compounds, the chemical capacity to produce these compounds was not limited to the chemistry lab. The natural phenomenon of microbial chemical antagonism could be exploited by chemists, microbiologists, and clinicians in the search for medically useful antimicrobial compounds.

The discovery of penicillin was followed by a gold rush in antimicrobial discovery. The years between 1940 and 1960 are frequently referred to as the “golden age” of antimicrobial discovery (Thomson et al., 2004). Realizing that microbes produced antagonistic compounds, researchers scoured the Earth in search of microbes that produced antimicrobial compounds. Some families of microbe types proved extremely useful – *Streptomyces* bacteria becoming an exceptionally valuable source of these compounds. Selman Waksman, a Russian-American *Streptomyces* specialist, became one of the most prominent names in antimicrobial discovery by enriching and screening streptomycetes for antimicrobial compounds. Waksman coined the term “antibiotic” to refer to a compound produced by microbes that blocks the growth of or kills bacteria (Pringle, 2013). Waksman and Albert Schatz discovered streptomycin, the first aminoglycoside antibiotic. This compound was also the first antibiotic compound with good activity toward *Mycobacterium tuberculosis* (Schatz et al., 1944), for which no good treatment existed at the time. Following penicillin, streptomycin was the second “blockbuster” antibiotic, a drug upon which much of the paradigm of the modern pharmaceutical industry was built.

Following streptomycin, multiple successful classes of antimicrobials were discovered from natural sources, including the tetracyclines, macrolides, rifampicins, and glycopeptides (Aminov, 2010). Many of these compounds still find widespread use in the current medical repertoire.

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1 The first of many penicillins, this compound is also known as penicillin G or benzylpenicillin.

1.1.2 Antibiotics in medicine – the “magic bullet”

While antibiotics are used in nature between antagonistic microbes, we use them to our advantage to treat bacterial infections. Most current medically useful antibiotics come from environmental microbes. These “magic bullets” kill bacteria but don’t cause undue toxicity to the host (Amyes, 2003). Several extremely useful antibiotics have been developed that make the treatment of an infection as easy as taking a few pills. This simplicity of treatment for life-threatening infections was unthinkable before the discovery of antibiotics.

The value of antibiotics in medicine should not be underestimated. Antibiotics are used to treat infectious disease, but also for widespread prophylactic purposes (Enzler et al., 2011). Antibiotics are necessary to prevent infections in the immunosuppressed, for medical devices like stents and dialysis machines, and are critically important for surgical procedures, from biopsies to invasive major surgeries.

Some of the most valuable antibiotics are broad-spectrum compounds. They act equally well in the treatment of both Gram-positive and Gram-negative bacteria. The use of these compounds has been popular because a single antibiotic can effectively prevent and treat infections caused by a broad swath of infectious bacteria (Kollef, 2008). Traditionally, the broadest spectrum antibiotics have been the most desirable. However, antibiotics with broad activity and limited toxicity are rare, and following the early discovery of major antibiotic families, very few new broad-spectrum compounds have been identified.

The limited repertoire of effective antibiotics and their declining efficacy in treating infections drives a search for new, effective antimicrobials. However, there have been few effective antimicrobials discovered since the 1960's. This antibiotic drought has been driven both by the difficulty of screening for novel antimicrobial compounds, as well as challenges that emerge from the chemical complexity of many antibiotic compounds.

1.1.3 Antibiotics are no ordinary drugs

Antibiotics are difficult to speak of in general terms – they are not a monolithic group of compounds. Antibiotics are defined functionally as compounds that kill or inhibit the growth of bacteria, but they are a chemically diverse group of compounds that bind to multiple physiological targets and exhibit diverse mechanisms of action. However, antibiotics do share some chemical trends that distinguish them from other types of pharmaceutical compounds (Payne et al., 2007).

Antibiotics are often large compounds, with many stereogenic centres and rotatable bonds. They often have many chemically similar polar functional groups. These properties make antibiotics good, tight-binding and specific compounds toward their respective target, and can aid in their ability to cross the bacterial cell membrane. However, this chemical complexity also presents a problem for chemists that might want to synthesize an antibiotic *de novo* to develop new compounds. Chemical complexity makes chemical synthesis of many antibiotics prohibitively challenging.

Perhaps as a result of the complicated chemistry of antibiotics, many of our most effective compounds come from natural sources, where we take advantage of the complex biochemical synthesis systems present in microbes. While some fully synthetic antibiotics exist, a majority of the most successful antibiotics are either natural-source antibiotics or semi-synthetic: modified natural antibiotics (Table 1.1). Traditionally, we have searched nature for new compounds – or at least for inspiration. This is a problem when we consider that antibiotics come from a microbial war-zone, where resistance also lives.

Antibiotic	Class	Date of Discovery	Source
Penicillin	β-lactam	1929	*Penicillium sp.*
Streptomycin	Aminoglycoside	1943	*Streptomyces griseus*
Chlortetracycline	Tetracycline	1947	*Streptomyces aureofaciens*
Erythromycin	Macrolide	1952	*Streptomyces coelocolor*
Vancomycin	Glycopeptide	1964	*Amycolatopsis orientalis*
Rifamycin	Rifamycin	1957	*Amycolatopsis rifamycinica*
Clindamycin	Lincosamide	1970	*Streptomyces lincolnensis*
Ciprofloxacin	Fluoroquinolone	1980	Synthetic
Daptomycin	Lipopeptide	1988	*Streptomyces roseosporus*
Linezolid	Oxazolidinone	1996	Synthetic

Table 1.1: Common antibiotics and their source

Most antibiotics are discovered from environmental microbial isolates. In recent years some synthetic antibiotics have been developed, but most effective antibiotics are still of microbial origin.

1.1.4 The environmental origins of antibiotics

Fleming realized that most antibiotics are produced by a microbial “arms race” between organisms fighting to get the upper hand in the wild. This complicated dance happens throughout the natural world, where microbes produce antibiotics to get ahead of their competition, while others develop countermeasures to compete. Played out over millions of years, these microbes have developed incredibly sophisticated mechanisms of producing and defending against these microbial “swords” with microbial “shields”. As we have learned more about the microbial environment, we appreciate that antimicrobial interactions are not just beneficial to the producer, they also appear to contribute to long-term stability of microbial ecosystems (Abrudan et al., 2015; Kelsic et al., 2015), and so they form an integral, and possibly inevitable part of the microbial biosphere. Antibiotics, and antimicrobial resistance, are forces of nature.

The presence of antibiotics in the environment is part of a diverse chemical network dubbed the “parvome” (Davies and Ryan, 2012). In this model, antibiotics constitute an essential part of the environment where they can even behave as metabolic signalling compounds (Yim et al., 2007), or be involved in the stable maintenance of bacterial populations (Kelsic et al., 2015). Antibiotics and other bioactive small molecules have affected bacteria for millions, if not

billions, of years, providing selective evolutionary pressures on environmental bacteria. This situation has selected not just for antibiotics, but also their counterpart: resistance.

1.2 Antibiotic resistance: From the wild to the clinic

Like antibiotics, antibiotic resistance is an ancient phenomenon that predates the medical use of antibiotics and human civilization altogether (Bhullar et al., 2012; D'Costa et al., 2011). Penicillin resistance had already been observed before penicillin was available to the public, (Abraham and Chain, 1940) - Fleming himself observed that some bacteria became resistant to penicillin in the lab. Resistance to streptomycin, the second major antibiotic, was first discovered in 1948 (Crofton and Mitchison, 1948). All new antibiotics have been followed by the discovery of resistance, often within only a few years of antibiotic use. Troublingly, the time between clinical use of an antibiotic and the discovery of resistance has become shorter in recent years (Ventola, 2015), further indicating that antibiotic resistance is not only widespread, but inevitable.

Antibiotic resistance is acknowledged as one of the great challenges to the future of modern medicine. The loss of useful antibiotics has been described as a return to the "post-antibiotic era" (Alanis, 2005), where simple infections can once again be deadly. In addition to the loss of effective treatment options for resistant infections, the loss of reliable antibiotic prophylaxis makes surgical procedures and treatment of vulnerable populations become much more risky as well. Antibiotic resistance threatens to undermine one of the major pillars of modern medicine. Resistance is a healthcare menace.

Resistance occurs by many mechanisms, some of the most widespread of which are contained within mobile genetic elements such as plasmids and transposable elements (Frost et al., 2005). These mobile elements often concentrate multiple mechanisms of resistance together, so a recipient bacterium gains access to multiple forms of resistance at once. Use of broad-spectrum antimicrobial compounds in the clinic have selected for the spread of these mobile genetic elements, as even off-target action of an antimicrobial can enrich the resistant populations (Levy, 2002).

The human environment has also shaped the conditions in which antibiotics and resistance interact. Widespread use of antibiotics in agriculture for treatment of infection, preemptive medical use, and for non-specific growth promotion have greatly increased local concentrations of the antibiotics that we add to animal feed. This dramatically alters the microbial environment in and around farms and feedlots. This selects for new emergent resistance, and also helps drive the spread of resistance elements already present in the environment (Davies and Davies, 2010).

1.2.1 Origins of antimicrobial resistance

The microbial environment is rich with a complexity of small molecules that interact with and exert effects on neighbouring bacterial cells (Aminov, 2009; Davies, 2013). Central to this understanding is also antimicrobial resistance, the means by which a target bacterium escapes the toxic effects of an antibiotic.

Natural antimicrobials are part of a complicated network of antagonism and co-operation between environmental microbes (Martínez, 2008). A microbe that is susceptible to an antibiotic can gain a selective advantage by developing or acquiring resistance to the antibiotic. Antibiotic producers can in turn modify the antibiotic restore its effectiveness against competitors. This produces an ever-moving “arms race” of microbes producing new antimicrobials and new mechanisms of resistance (Hede, 2014). It also fine-tunes existing means of antibiotic production and resistance to optimize their respective activity for efficiency and efficacy. The collective pool of antibiotic resistance factors and proto-resistance factors form the global antibiotic resistome (Wright, 2007).

Biological molecules evolve to reflect their function. Antibiotic resistance factors are subject to extreme selective pressures and present particularly notable cases of adaptive evolution. Antibiotic resistance enzymes can serve as excellent model systems for the study of molecular evolution (Oz et al., 2014). However, there is still a lot unknown about how these resistance factors evolve and develop new or altered functions.

The original functions of proto-resistance enzymes are not well understood, although there are several possible original sources (Wright, 2007). A prominent hypothesis is that some resistance enzymes began as mechanisms of self-protection in antibiotic-producing organisms (Benveniste and Davies, 1973). However, promiscuous proteins with functions unrelated to

antibiotics other functions could also be co-opted for antibiotic resistance functions (Olivares et al., 2013). In either case, the *de novo* emergence of resistance eventually evolves to effective, dedicated resistance.

1.2.2 *De novo* and dedicated resistance

Antibiotic resistance can be loosely categorized into two extremes: *de novo* resistance that emerges in response to new antibiotic exposure, and dedicated resistance that has evolved and developed alongside antibiotics for extended periods of time.

De novo resistance arises through random mutations of bacterial genes, which then become enriched in a population if they confer a selective advantage (Woodford and Ellington, 2007). Mutation of these proto-resistance genes to resistance genes and selection in bacterial populations leads to clonal resistance. *De novo* resistance has been studied in laboratory setting (Toprak et al., 2011), but is difficult to predict outside of such highly controlled environments and infer how it occurs in the natural environment. Some *de novo* adaptations can be accelerated by increasing the background rate or mutation in bacteria, a common response to treatment by some antibiotics (Chopra et al., 2003), suggesting that antibiotics could accelerate the emergence of new resistance activities. Resistance of this type is often achieved through mutation of the antibiotic target site, changes that reduce a factor that confers susceptibility, or through changes in an existing form of resistance that expand the antibiotic range of the resistance factor. As these forms of resistance are newly developed, they typically carry some negative consequences for the bacterium, which have not yet been offset by evolutionary adaptation (Angst and Hall, 2013). *De novo* resistance mechanisms tend to be sloppy, inefficient, and carry a high penalty for a bacterium expressing this resistance in the absence of antibiotic.

The second form of resistance is dedicated resistance, in some cases referred to as microbial R genes (Davies and Davies, 2010). In this case, a bacterial resistance gene developed long ago, in some cases over geological timescales alongside antibiotics in the environment. Long periods of mutation and natural selection have refined the activities of these resistance factors. Some dedicated resistance mechanisms are thought to have their origins in antibiotic producing bacteria, where originally protected a bacterium from the toxicity of its own compounds, although other sources of dedicated resistance factors are also possible. Wild microbial

populations serve as a reservoir of these mature resistance genes (Riesenfeld et al., 2004), which can then jump to clinical antibiotic-resistant pathogens by horizontal gene transfer (Martínez, 2013). The most effective forms of dedicated resistance include the optimized resistance factors that are present on transferable elements that allow them to pass easily between bacteria. These transferable resistance factors are optimized resistance machines (Magnet and Blanchard, 2005).

Of course, all heavily-evolved antibiotic resistance mechanisms began once as a spontaneous case of *de novo* resistance. *De novo* and dedicated antibiotic resistance mechanisms are two ends of a spectrum. All forms of resistance begin as a novel function and are eventually optimized to produce the maximum resistance with minimum negative effects for the host. The difference that distinguishes these forms of resistance is that evolved resistance mechanisms have had time to accumulate further mutations and genetic changes that help mitigate the negative effects of the initial resistance mutation. The inefficiency of *de novo* mutations is eventually replaced by specificity. Compensatory mutations accrue to offset the negative cost of resistance factors. This negative “fitness cost” is central to our understanding of resistance as it exists in environmental microbes.

1.2.3 The fitness cost of antimicrobial resistance

Any evolutionary change carries both a benefit and a cost to the fitness of the organism. The change may make the organism more or less fit in its environment, depending upon the nature of the genetic change, and the nature of the environment (Orr, 2009). In the presence of antibiotic resistance, the fitness benefit of resistance factors is very large. In the absence of a resistance factor, the bacterium dies, so antibiotics drive a strong selection for antibiotic resistance (MacLean et al., 2010). Bacteria that gain a resistance factor reap enormous fitness benefits in the presence of antibiotics. However, with no antibiotic present, the fitness cost of resistance may become more prominent (Andersson, 2006). The amount of energy a microbe spends maintaining resistance, or growth deficits that result from the resistance factor may make a microbe less competitive than those without it.

Fitness costs underlie efforts to control antibiotic use and drive back resistance. It was believed that reducing the use of antibiotics in the clinical setting will reduce the spread of antibiotic resistance by selecting against antibiotic resistance (Andersson and Hughes, 2010). If the fitness cost to resistance is strong enough, the removal of antibiotics was thought to place the microbe in an environment where there is no benefit to the resistance factor, but still an appreciable cost. This has driven efforts to curtail antibiotic usage, in the hopes that resistance will subside in the absence of antibiotics. This has been some success using this strategy, but it is a slow process and has not worked nearly as well as might have been hoped (Lenski, 1998).

The cost of antibiotic resistance can vary based upon the mechanism of resistance and even the precise amino acid substitution, in the case of resistant mutations (Enne et al., 2004a). Laboratory studies of resistance evolution have found that in many cases, changes that offset the fitness cost of resistance completely remove the selective pressure against resistant microbes (Schrag et al., 1997), limit it to the production of the resistance factor polypeptide itself (LaMarre et al., 2011), or in some cases even make resistant pathogens more fit than non-resistant counterparts (Enne et al., 2004b; Luo et al., 2005).

So, there is not a zero-sum interaction between the selective benefit and fitness cost of resistance. In cases of well-established antimicrobial resistance strategies, the mechanism of resistance has been optimized over long time scales to provide effective resistance but mitigate fitness costs as much as possible (Gillespie and McHugh, 1997). This occurs through compensatory mechanisms that offset the cost of resistance for the microbe that expresses the resistance factor (Björkman et al., 2000; Levin et al., 2000).

Forces of selection and costs shape an economy of design in antibiotic resistance. Resistance factors that more efficiently mitigate the evolutionary fitness cost are more adaptive in both the presence and absence of antibiotics (Aminov and Mackie, 2007). The most successful antibiotic resistance factors will confer a high level of resistance when an antibiotic is present, but impose very low fitness cost when it is absent. The longer a resistance factor is subjected to selection, the more likely it will have developed adaptive means of mitigating fitness costs. These mechanisms by which resistance factors reduce fitness cost are diverse corresponding to the diversity of mechanisms of resistance, and study of the means by which bacteria offset the cost of resistance remains an important challenge in counteracting antibiotic resistance.

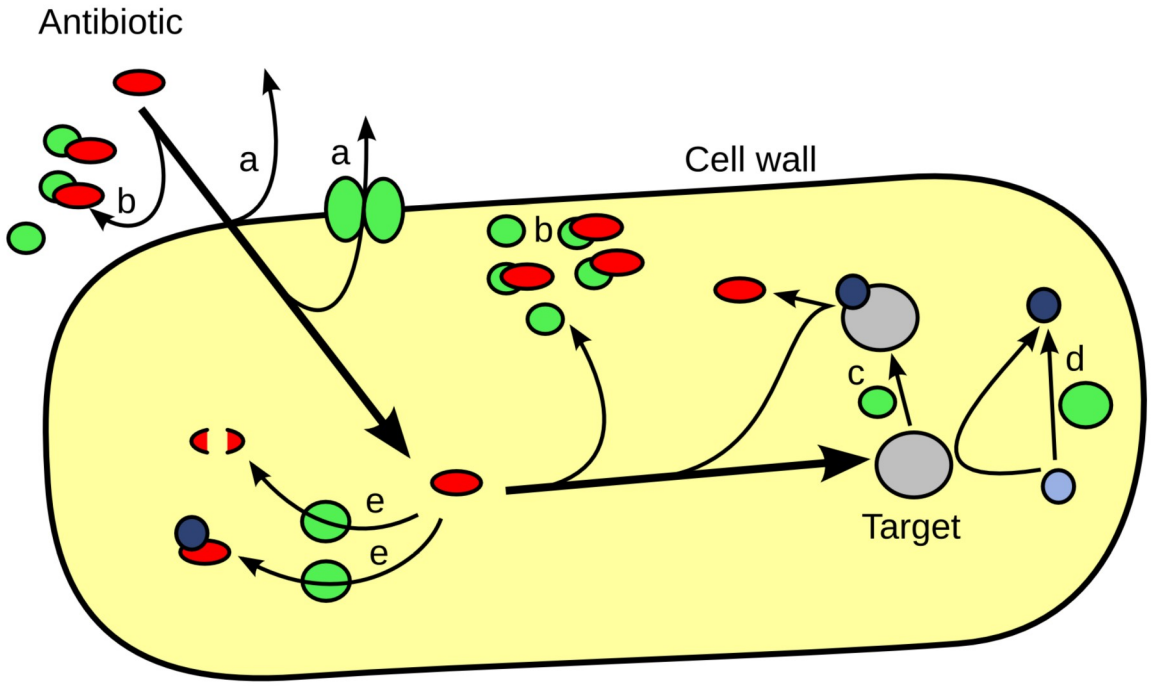

Figure 1.1: Mechanisms of antibiotic resistance

Antibiotic resistance can occur by multiple mechanisms. An antibiotic is presented in red, while its site of action is gray. Yellow represents the intracellular environment. Resistance factors are shown in green, while chemical changes conferred by resistance factors are indicated in blue. a) Changes to the membrane permeability reduces the effective concentration of antibiotic in the cell interior. This can occur *via* passive permeability changes or by active efflux of the antibiotic by dedicated efflux proteins. b) Sequestration can reduce the effective intracellular concentration of antibiotic by removing it from bulk cytosol through binding. Intra- and extracellular factors can both be involved in this form of resistance. c) Modification of the antibiotic target can block binding to an antibiotic's site of action. d) Factors that replace the function of the antibiotic target can bypass the antibiotic by finding alternative solutions to the blocked function. e) Enzymes can directly interact with an antibiotic and inactivate it *via* degradation or chemical modification.

1.2.4 Mechanisms of antibiotic resistance

In order for an antibiotic to be effective, it must reach its site of action, be capable of binding or exerting its effect on that site of action, and the bacterium must be negatively impacted by this action. Any change that interferes with these three arms of antibiotic action will confer resistance (Blair et al., 2015). Many different mechanisms of resistance exist, diverse strategies that lead to resistance, corresponding to the chemical diversity of antibiotic molecules themselves. They can be grouped into 5 categories.

1.2.4.1 Resistance by altered membrane transport

Changes that reduce the concentration of antibiotic in the cytosol confer effective antibiotic resistance (Kumar and Schweizer, 2005). Alterations to bacterial membranes, or to the proteins within that membrane, can confer resistance to antibiotics that act on intracellular targets. Changes that physically block the passage of the antibiotic into the cell confer resistance (Nikaido, 1994). This is especially true of Gram-negative bacteria, where the outer membrane provides an extra permeability barrier for the antibiotics to cross (Delcour, 2009). Alterations that reduce permeability across the outer membrane or the plasma membrane can lead to effective, broad antibiotic resistance.

In addition to changes that prevent the antibiotic from crossing membranes, there are also antibiotic resistance factors that actively export antibiotics, using energy from the hydrolysis of ATP to remove the compounds from the cell, against the concentration gradient (Levy, 1992; Poole, 2005a). These proteins are often found as transferable antibiotic resistance factors located on plasmids (Köhler et al., 1999; Ruiz et al., 2012).

1.2.4.2 Resistance by sequestration

Binding of an antibiotic by non-target molecules can remove enough antibiotic from solution that it no longer reaches and interacts with its target. This can happen through non-specific or very specific mechanisms. In bacterial biofilms, carbohydrate polymers can confer resistance by sequestering the antibiotic away from its site of action (Beaudoin et al., 2012; Mah et al., 2003). Overproduction of dummy targets for antibiotics can also overcome the action of the antibiotic (Reynolds, 1989; Thom and Prescott, 1997). Tight binding to a specific protein, sometimes referred to as an immunity protein, can also provide resistance (Dumas et al., 1994).

Overproduction of a target protein can also achieve this effect (del Castillo et al., 1991). Lastly, proteins that normally have other functions have also been seen to confer antibiotic resistance when over-expressed (Magnet et al., 2003; Menard et al., 1993).

1.2.4.3 Resistance by target modification

For an antibiotic to be active, it needs to effectively bind its target and exert a biochemical change upon that target. Modifications of the target that prevent antibiotic binding thus lead to resistance (Lambert, 2005; Leclercq and Courvalin, 1991). This modification can occur directly through mutation of the target itself, or by the action of transferable resistance factors that enzymatically modify the antibiotic target. This is particularly effective when an antibiotic binds a single site (Spratt, 1994), and in cases where changes to the bacterial target do not negatively impact bacterial physiology.

1.2.4.4 Resistance by metabolic bypass

In cases where an antibiotic blocks an important metabolic process, any factor that allows the cell to survive in the presence of this block will confer resistance. In some instances, new enzymes are introduced to perform the same function as an inhibited enzyme or an alternate macromolecule is substituted for the normal antibiotic target (McManus, 1997; Wright, 2011). These mechanisms tend to be gained from horizontal gene transfer from other bacteria where this alternate system is already in place.

1.2.4.5 Resistance by enzymatic degradation or modification

A final mechanism of antibiotic resistance involves direct chemical change to the antibiotic (Wright, 2005). This is accomplished by enzymes that chemically change the antibiotic, either by degradation or by chemical modification. The widespread β-lactamase enzymes are perhaps the best known of these factors and break down penicillins and other β-lactam antibiotics. Many enzymes with this function are known (Bush et al., 1995). These enzymes hydrolyse the central β-lactam ring of these compounds, rendering them inactive (Poole, 2004). Lyase enzymes can also confer resistance to some antibiotics that are broken down without the requirement of water molecules (Korczynska et al., 2007).

In contrast to the degradative enzymes that require only water or no additional substrates, antibiotic-modifying enzymes use co-substrates to chemically modify the antibiotic, rendering it inert (Wright, 2005). These enzymes can inactivate antibiotics by acetylation (Shaw, 1992), phosphorylation (Paradiso et al., 1987), thiolation (Thompson et al., 2013), nucleotidylation (Bozdogan et al., 1999), ADP-ribosylation (Quan et al., 1997), glycosylation (Cundliffe, 1992), and through redox reactions (Yang et al., 2004b).

Possibly the best-known antibiotic-modifying enzymes are those that act on aminoglycosides (Azucena and Mobashery, 2001; Haas and Dowding, 1975; Ramirez and Tolmasky, 2010). These include aminoglycoside acetyltransferases, phosphotransferases, and nucleotidyltransferases. They will be covered in more depth in Section 1.4.

1.2.5 Specificity and breadth of antibiotic resistance

Like antibiotics themselves, antibiotic resistance factors can be broad or narrow in spectrum. Some resistance factors act on a small group of antibiotics, while others confer resistance to many individual compounds. The broader the resistance factor, the more useful it is for a bacterium. A single gene that confers resistance to many different compounds confers an enormous selective advantage, especially for microbes that are likely to encounter many different compounds such as those that cause hospital infections.

At the same time, a protein that interacts specifically with many different compounds can be very effective and reduce the negative side effects of resistance. The trade-off between entropic and enthalpic contributions in binding (Chodera and Mobley, 2013) means that a more broadly-specific enzyme is frequently a “sloppier” enzyme. In order for an enzyme to become both broadly-binding and an effective resistance factor, considerable lengths of time and adaptive evolution are required. Effective broad-profile enzymes do not spontaneously emerge, they are sculpted over countless generations.

1.2.6 Strategies to counteract antibiotic resistance

Antibiotic resistance is an enormously challenging problem for clinicians and public health officials (Sheldon, 2005). This problem is complicated by many factors, including agricultural antibiotic use, prolific worldwide transportation and distribution networks, and the clinical challenge of balancing needs of individual patients against needs of the population at large (Laxminarayan et al., 2013). Many different strategies have been suggested to counteract antibiotic resistance, and they are as variable as the researchers proposing the methods. It is likely that antibiotic resistance will only be countered by a synergy of many such strategies (Bush et al., 2011). For most of these strategies to be effective, we need as much information as possible about the mechanisms of antibiotic resistance.

The spread of resistance is driven by antibiotic use, and so careful use of antibiotics and control of resistance will be critical moving forward. Important areas of antibiotic resistance management include changes to agricultural and veterinary antibiotic usage (Teuber, 2001), systems of patient care, rapid diagnostics and targeted treatment (Rice, 2011), antibiotic stewardship (Bartlett, 2011), and careful surveillance of resistance (Bax et al., 2001).

In addition to these systems-level approaches, there are many avenues of potential new therapeutic development:

1.2.6.1 Development of new antibiotics

Ever since initial discoveries of antibiotic resistance, we have looked for new antibiotics that are not subject to resistance. To deal with antibiotic resistance, we have historically searched for new compounds to which resistance has not had time to emerge and spread. While this strategy worked exceptionally well in the early days of antimicrobial discovery, the number of new antimicrobials developed has steadily declined since, and the pipeline of new antibiotics has all but dried up (Cooper and Shlaes, 2011). The resulting innovation gap (Walsh, 2003) has left us with few options for treatment of some serious microbial infections and the spectre of the truly untreatable infection looms large if many new antibiotics are not discovered soon.

While some approaches to screening for antibiotics have not been fruitful (Walsh and Wencewicz, 2014), there is some movement toward the development of new antibiotics, most notably in the use of novel culture techniques to find new environmental antibiotics (Ling et al., 2015). In the present day, we need to search for antimicrobials that are effective against multi-drug resistant or pan-drug-resistant microbes (Chopra et al., 1997; O'Connell et al., 2013). This is an enormously difficult challenge, especially in the absence of good mechanistic understanding of some resistance mechanisms.

1.2.6.2 Antibiotic adjuvants

An approach to improve the activity of existing antibiotics, rather than replacing them, is to introduce additional compounds alongside the antibiotic. Broadly termed adjuvants, these compounds could to modulate antibiotic activity (Kalan and Wright, 2011). Adjuvants could be compounds that amplify the bacterial toxicity of an antibiotic (Allison et al., 2011), increase its effective intracellular concentration, or alter the physiology of a microbe in other means to improve the effect of antibiotics (Brackman et al., 2011). Alternatively, inhibitors of antibiotic resistance factors can be deployed alongside antibiotics to restore their activity in the presence of resistance factors (De Pascale and Wright, 2010). This strategy has seen great success in the treatment of β-lactam resistant bacteria (Drawz and Bonomo, 2010).

Resistance enzymes are particularly amenable to antibiotic-inhibitor combinations, where the resistance factor can be blocked, leaving the unaltered antibiotic to exert its toxic effect on the bacterium. This strategy even carries the promise of restoring old antibiotics that have been retired due to extensive resistance (Burk and Berghuis, 2002).

1.2.6.3 Novel therapies

Many other promising avenues of treatment towards antibiotic resistant bacteria exist. These therapies include vaccines (Mishra et al., 2012), bacteriophage therapy (Keen, 2012), metal-based compounds (Lemire et al., 2013), antimicrobial peptides (Hancock and Sahl, 2006), antivirulence strategies (Allen et al., 2014; Rasko and Sperandio, 2010), and macromolecules that target bacteria (Oleksiewicz et al., 2012). All of these therapies benefit from increased knowledge and understanding of the mechanisms of antibiotic resistance, as they can be combined with antibiotic therapies as well (Lindgren and Sjöstedt, 2016).

1.3 Aminoglycoside antibiotics

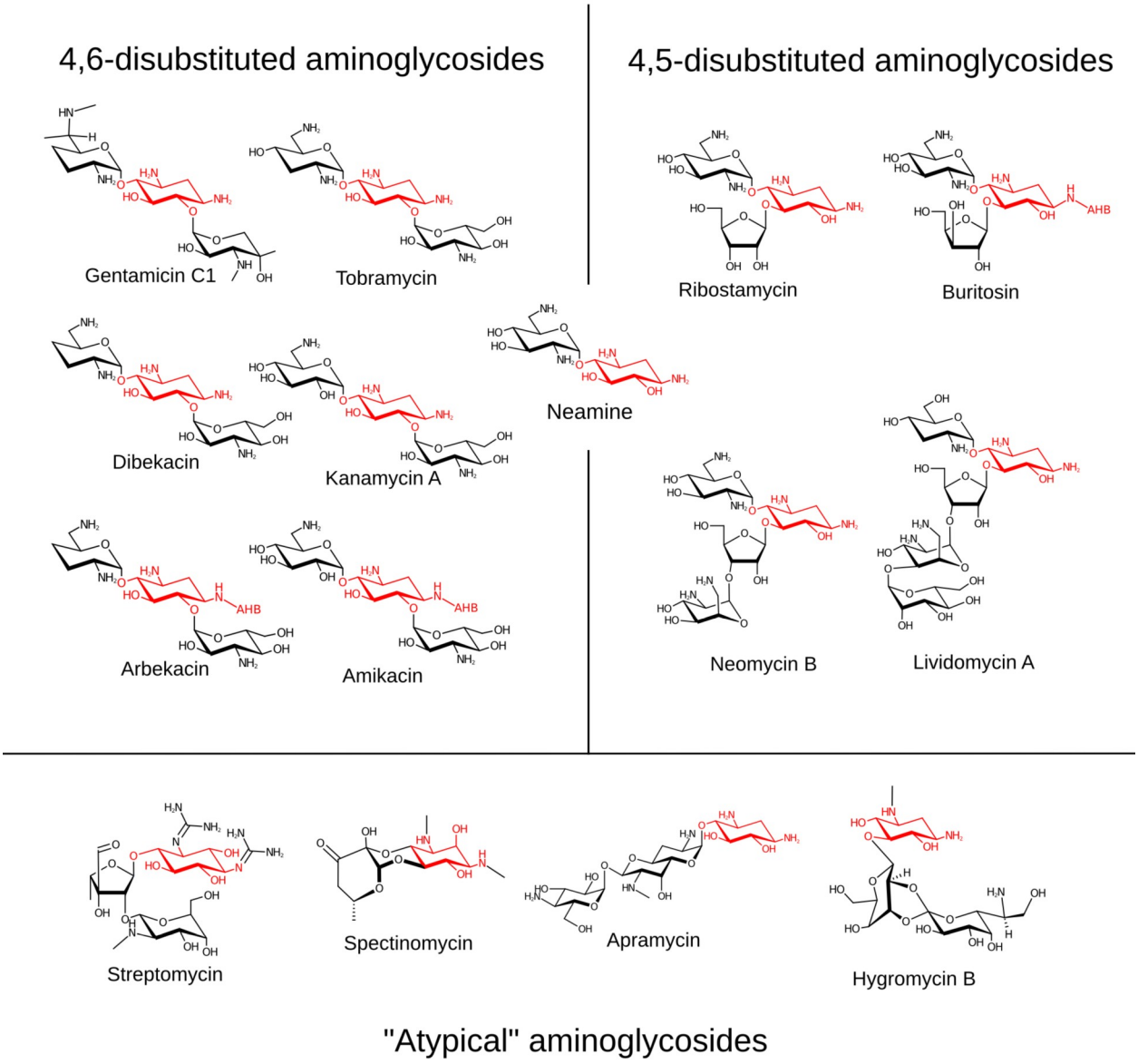

***Figure 1.2*: Aminoglycoside antibiotics**

Aminoglycoside antibiotics discussed in this thesis are indicated. The aminocyclitol ring of each compound is indicated in red.

After penicillin emerged as the first β-lactam antibiotic, streptomycin formed the second major class of antibiotics – the aminoglycosides (Arya, 2007). Dubbed a “wonder drug”, streptomycin made great gains in treating patients in a war-torn Europe, and helped birth the modern pharmaceutical industry (Pringle, 2013). This compound was exceptionally important as it was the first chemical compound active toward tuberculosis. Waksman's group went on to find many additional antibiotics, including the aminoglycoside neomycin B (Waksman and Lechevalier, 1949). Aminoglycosides are broad-spectrum compounds that act on Gram-positive and Gram-negative bacteria (Poulikakos and Falagas, 2013) and are effective in the treatment of serious systemic bacterial infections (Gonzalez and Spencer, 1998). While there are aminoglycosides in development, no new compounds in this family have gained approval in North America since amikacin, in 1976. This class of antibiotics is due for a renaissance (Houghton et al., 2010).

Several prominent aminoglycosides are used in the treatment of clinical infection (Becker and Cooper, 2013). Tobramycin, gentamicin complex, and amikacin are commonly used to treat infections, while other compounds like arbekacin, sisomicin, and isepamicin are also effective toward some bacterial strains. Some aminoglycosides are also important research tools, like kanamycin, a laboratory staple for molecular cloning experiments (Pridmore, 1987).

1.3.1 Chemical structure of aminoglycosides

Aminoglycosides are synthesized in actinomycete bacteria. The synthetic pathway to create these compounds take many steps, with many independent enzymes to form these compounds from metabolic precursors (Kudo and Eguchi, 2009). In addition to the enzymes that produce the antibiotic, some of these organisms also contain enzymes that chemically modify the antibiotics to block their toxicity, protecting the producer bacterium (Cundliffe, 1989; Cundliffe and Demain, 2010). If antibiotics are weapons forged by microbial blacksmiths, these enzymes are the blacksmith's glove. They are thought to be a possible source for some aminoglycoside resistance enzymes that have spread to pathogenic bacteria.

The central feature of all aminoglycosides is an aminocyclitol ring – a six-membered carbon ring decorated with hydroxyl and amino groups, to which additional rings, usually amino sugars, are linked (Figure 1.2). In most compounds, this aminocyclitol is a 2-deoxystreptamine (2-DOS) ring (Busscher et al., 2005), to which other rings are connected by glycosidic linkages. Aminoglycosides are all positively charged compounds (Blagbrough et al., 2011), which aids in their interaction with their target site, the bacterial ribosome (Carter et al., 2000).

4,6-disubstituted	4,5-disubstituted	“Atypical”
Kanamycin A, B, C	Neomycin B	Streptomycin[§]
Tobramycin	Paromomycin	Hygromycin
Dibekacin	Ribostamycin	Spectinomycin
Gentamicin C1, C1a, C2	Butirosin A, B	Fortimicin
Sisomicin	Lividomycin A	Neamine*
G418/geneticin		Apramycin
Netilmicin		
Amikacin		
Arbekacin		
Plazomicin		
Isepamicin		

Table 1.2: Aminoglycoside antibiotics

4,5-disubstituted, 4,6-disubstituted, and atypical aminoglycosides. Semisynthetic aminoglycosides are highlighted in yellow.

* Neamine forms the foundation for the 4,5-disubstituted and 4,6-disubstituted aminoglycoside families.

[§] As described elsewhere, spectinomycin is technically an aminocyclitol, but included due to chemical, biosynthetic and physiological similarity to aminoglycosides.

Aminoglycosides are categorized into three groups (Figure 1.2), depending on how additional amino sugars are linked to the aminocyclitol ring of the compound. Two of these are built upon a 2-deoxystreptamine-4-aminohexose core structure, which we will refer to as the neamine-like core after the two-ringed compound isolated from the breakdown of neomycin B (Leach and Teeters, 1951). These “typical” aminoglycosides link additional sugars to the 5- or 6-position of the 2-deoxystreptamine ring. This produces the 4,5-disubstituted and 4,6-disubstituted subgroups of aminoglycosides. The third group consists of aminoglycoside

compounds that do not contain these rings, collectively referred to as “atypical” aminoglycosides – the protists of the aminoglycoside world.

1.3.2 Streptomycin and the “atypical” aminoglycosides

As the aminoglycoside family has grown, streptomycin, the first and most famous compound in this group, has been relegated to the atypical aminoglycoside group. It is joined by other misfits like spectinomycin[2], hygromycin, and apramycin. Defined by exclusion, these compounds share little in common besides some common synthetic pathways. They have different properties and mechanisms of action.

While neamine-based aminoglycosides are bactericidal and act through a conserved binding site on the bacterial ribosome, atypical aminoglycosides do not necessarily obey the same mechanisms. Spectinomycin and hygromycin inhibit ribosomal translocation (Borovinskaya et al., 2007a, 2008). Streptomycin binds near the interface of ribosomal subunits, leading to inhibition of initiation and termination, and also increasing the error rate of the ribosome (Abad and Amils, 1994). Apramycin shows a similar mode of binding to neamine-based aminoglycosides, but extends in the opposite direction in the binding site (Han et al., 2005).

However, despite diverse mechanisms of action, the predominant mechanisms of resistance to atypical aminoglycosides are similar to those that confer resistance to neamine-based compounds: covalent modification (Covered in more detail in Section 1.4). It appears that a limited set of effective resistance mechanisms to aminoglycosides have been been the most successful for these compounds, irrespective of their chemical structure.

2 Spectinomycin is technically an aminocyclitol antibiotic, containing three fused rings, and not an aminoglycoside. However, it shares the same mechanisms of synthesis and resistance as aminoglycosides, so is frequently considered aminoglycoside for the sake of comparison.

1.3.3 Neomycin and neamine-based aminoglycosides

Neomycin B (hereafter referred to simply as “neomycin”) was first reported in 1949 (Waksman and Lechevalier, 1949). This compound was the first clinically useful aminoglycoside built on 2-deoxystreptamine 2-DOS. Many of the effective aminoglycosides that followed were also based upon 2-deoxystreptamine. In fact, streptomycin, which is not built on this scaffold, is now considered an “atypical” aminoglycoside in contrast to the “typical” 2-DOS based compounds.

2-DOS aminoglycosides make excellent antibiotics because they are bactericidal at therapeutic concentrations, have a broad spectrum of activity, and exhibit synergism with other antibiotics (Krogstad et al., 1978). Most aminoglycosides that are used in the clinic fall into this group, in one of two sub-categories: 4,5-disubstituted and 4,6-disubstituted aminoglycoside antibiotics. These families are both built upon a shared two-ring core.

These compounds can all be considered modifications of neamine, a pseudo-disaccharide compound isolated from the breakdown of the larger neomycin (Leach and Teeters, 1951). Neamine has weak antibiotic activity, but serves as a synthetic precursor to the more effective 4,5- and 4,6-disubstituted compounds. Neamine contains a 2-deoxystreptamine (2-DOS) ring, as well as an amino sugar linked to the 4-position of the 2-DOS ring. The addition of sugar or amino-sugar rings to the 5- or 6- positions of neamine forms 4,5- and 4,6-disubstituted aminoglycosides (Figure 1.3).

Addition of a ribose ring at the 5-position of 2-deoxystreptamine generates ribostamycin (originally SF-733) (Akita et al., 1970). This compound is the simplest 4,5-disubstituted aminoglycoside, with only three rings. Neomycin (Leach et al., 1951) and paromomycin (Davidson et al., 2009) are further modifications of this compound, with an additional hexose ring linked to the ribose. Lividomycin A (Machiyama, 1971) contains another ring, creating a 5-ring aminoglycoside compound, the largest used. All of these compounds are effective antimicrobials, and paromomycin is also an effective anti-parasitic.

Addition of a hexose ring at the 6-position of 2-deoxystreptamine creates 4,6-disubstituted aminoglycosides. These compounds are highly efficacious and cheap to produce, and have reduced toxicity relative to 4,5-disubstituted compounds like neomycin (Owada, 1962). As resistance to some aminoglycosides was identified, researchers searched for and identified more new 4,6-disubstituted aminoglycosides with modified substituents. These include tobramycin and gentamicin, which remain some of the most useful aminoglycosides in clinical use.

With the 2-DOS and 4-aminohexose rings in common, the neamine-based core forms the central, functional unit of the aminoglycosides (Kulik et al., 2015). Because of this shared core, modifications of groups on the 2-DOS or 4-aminohexose rings can confer resistance to both classes of compound. The additional rings add specificity and tune other pharmacological properties of the compounds. In addition to these natural aminogycosides, some semi-synthetic compounds have also been built from neamine-based aminoglycosides.

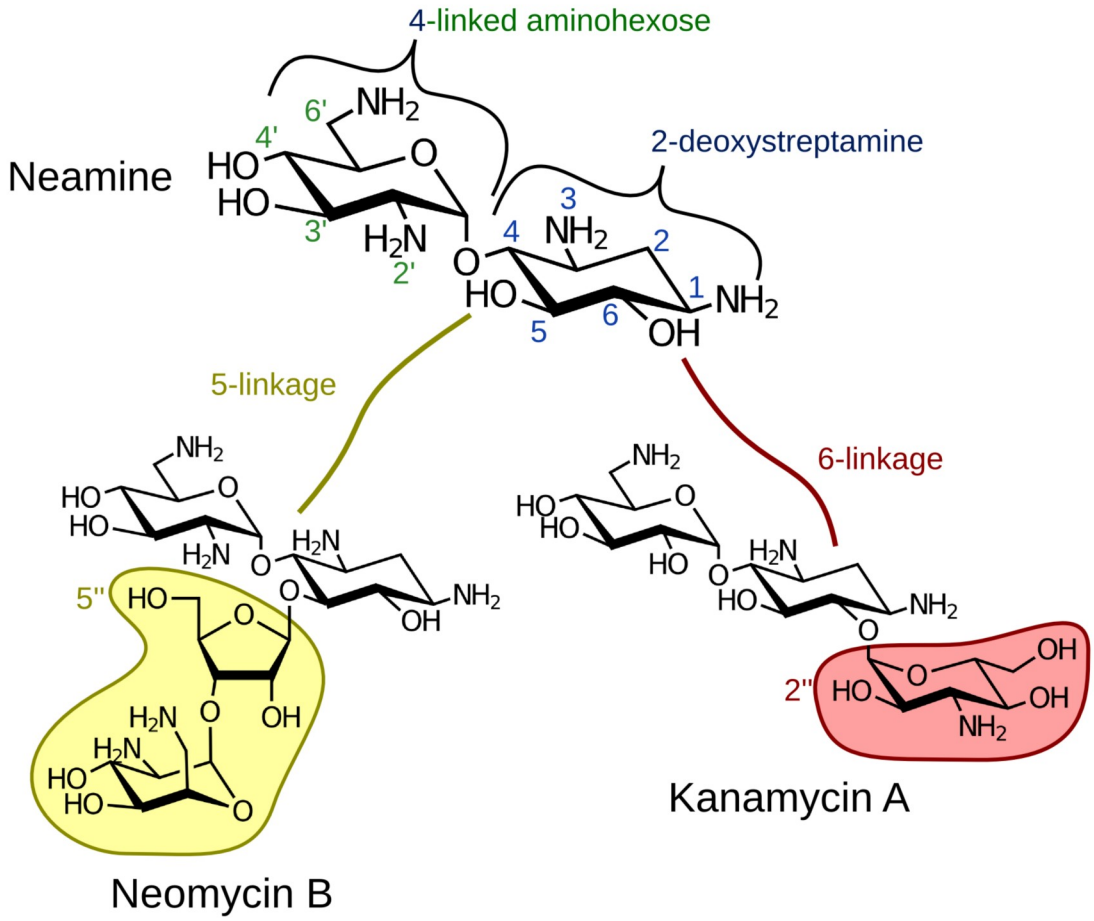

***Figure 1.3*: Neamine and its derivative aminoglycoside antibiotics.**

Neamine is the core functional unit of 2-deoxystreptamine aminoglycosides. 4,5- and 4,6-disubstituted aminoglycosides are based upon this scaffold. Neomycin, a 4,5-disubstituted compound, links a pentose and subsequent aminohexose to the 5-position of neamine. Kanamycin A, a 4,6-disubstituted aminoglycoside, links an aminohexose ring at the 6-position.

1.3.4 Semi-synthetic and resistance-evading aminoglycosides

The most useful aminoglycosides in medicine are the 4,6-disubstituted compounds. Kanamycin A (referred to simply as kanamycin unless otherwise indicated) is the simplest example of these compounds, with a hydroxyl or amino group at every carbon. This compound is effective and still finds use in research laboratories, but the many modifiable groups on this

compound leave it susceptible to resistance by chemical modification. Tobramycin was identified as a compound similar to kanamycin with the 3' hydroxyl group removed (Koch and Rhoades, 1970). This makes this compound invulnerable to resistance factors that would modify kanamycin at the 3' position.. Gentamicin complex compounds (gentamicin C1, C1a and C2) were also identified, which lack both 3' and 4' groups and are similarly not subject to resistance due to 3' or 4' site modification (Weinstein et al., 1963).

Dibekacin was the first semisynthetic antibiotic synthesized. This compound removes both the 3' and 4' hydroxyl groups from kanamycin B, a close relative of kanamycin A (Umezawa et al., 1971). This modification confers the same resistance benefit as seen for gentamicin – the compound evades resistance caused by modifications on the 3' and 4' hydroxyl groups, but remains an effective antibiotic.

Other semisynthetic aminoglycosides have been made by addition of groups, rather than removal. Inspired by the discovery of butirosin (Howells et al., 1972), which contains an N1-(*S*)-2-hydroxy-4-aminobutyrate (AHB) group, other aminoglycosides were modified to add this bulky group. Amikacin is produced by this modification of kanamycin A (Price et al., 1976), while arbekacin is made by adding AHB to dibekacin (Kondo et al., 1973). Some other aminoglycosides carry similar modifications, including plazomicin, the first new aminoglycoside to enter clinical trials in over 3 decades (Zhanel et al., 2012).

1.3.5 Mechanism of action of neamine-based aminoglycosides

Neamine-based aminoglycosides cause bacterial cell lysis and death – they are bactericidal antibiotics. This is in contrast to bacteriostatic antibiotics, which block bacterial growth but don't kill the cell under most conditions studied. Aminoglycosides exert these toxic effects by binding to the bacterial ribosome (Davies and Davis, 1968). Both 4,5- and 4,6-disubstituted aminoglycosides interfere with the decoding process of the ribosome, which results in aberrant translation of proteins (Lando et al., 1973). This miscoding is achieved by selectively stabilizing the paired form of the tRNA as it binds the mRNA and checks for fidelity (François et al., 2005).

Both 4,5- and 4,6-disubstituted aminoglycosides bind this site in the same mode, where their 5- or 6-linked rings form additional contacts within the ribosomal site (Demeshkina et al., 2012; Kulik et al., 2015; Vicens and Westhof, 2003). The N1-modified group of semisynthetic aminoglycosides is also accommodated in the active site, where it contributes to binding (Kondo et al., 2006). Stabilizing this reaction leads to a reduced coding fidelity and an increased incidence of mistranslated amino acids inserted in the growing polypeptide chain (Demeshkina et al., 2012). In contrast to bacteriostatic antibiotics that bind their target site in a competitive manner, aminoglycosides affect the flow of information from mRNA to protein, potentially dys-regulating many downstream processes within the cell.

Because of this mechanism of action, the action of aminoglycosides is not stoichiometric. One antibiotic molecule can lead to cascading effects that alter many downstream molecules. The ultimate mechanism of cell death caused by aminoglycosides remains a subject of debate (Kohanski et al., 2007; Liu and Imlay, 2013), but it is not controversial that aminoglycosides start a positive feedback cycle where small initial effects become amplified, ultimately terminating in bacterial cell death (Davis, 1987). This makes the rapid sequestration and removal of aminoglycoside antibiotics of critical importance for any bacterium to avoid cell death conferred by these antibiotics.

1.3.6 Resistance to aminoglycosides

Aminoglycoside resistance can occur through several mechanisms (Garneau-Tsodikova and Labby, 2016). Because their mechanism of action can lead to direct alteration of many cellular molecules, there has not been any example of aminoglycoside resistance by metabolic bypass, but other forms of resistance previously described (Section 1.2.4) have all been observed to confer resistance to aminoglycosides. Any means of preventing aminoglycoside binding to the ribosome can confer resistance.

Changes to the cell envelope that reduce uptake of aminoglycosides can confer resistance, including alterations that decrease the resting potential of the plasma membrane (Taber et al., 1987). In Gram-negative pathogens, alterations to the outer membrane permeability can increase aminoglycoside resistance (Nikaido, 1989; Poole, 2005b). In addition to changes that alter the membrane permeability, molecules that actively remove aminoglycosides from the cytosol can

also lead to high aminoglycoside resistance. The MexXY system (Fraud and Poole, 2011) actively pumps aminoglycosides out of the cell after they have crossed the cell envelope. The charged and hydrophilic nature of aminoglycosides require a specific and specialized system of proteins.

Alterations to the bacterial ribosome can also lead to antibiotic resistance. In bacteria like mycobacteria with a single gene for ribosomal RNA, mutations to the aminoglycoside binding site can directly escape antibiotic action (Finken et al., 1993; Georghiou et al., 2012). Transferable resistance factors can also chemically modify the ribosome by methylation using S-adenosylmethionine as a co-substrate (Wachino and Arakawa, 2012). These enzymes are thought to share evolutionary roots with enzymes involved in self-protection in the actinomycetes that produce aminoglycosides, although considerable divergence has converted these methyltransferase enzymes into evolutionarily optimized aminoglycoside resistance factors.

An under-appreciated means of resistance to aminoglycosides is through sequestration of the antibiotic away from its site of action. In bacteria that form biofilms as a form of resistance, negatively charged polysaccharides bind aminoglycosides (Sadovskaya et al., 2010), providing some resistance. Binding of aminoglycosides by catalytically inert enzymes has also been observed to confer some resistance to aminoglycosides (Magnet et al., 2003; Menard et al., 1993).

Despite these diverse means of resistance, by far the most common form of aminoglycoside resistance is *via* enzymatic modification. There are three different forms of aminoglycoside-modifying enzyme, with distinct evolutionary origins and chemical mechanisms. Despite these divergent origins, these enzymes also share common features in their interactions with aminoglycoside antibiotics. As a group, these resistance enzymes are responsible for the vast majority of clinical aminoglycoside resistance.

1.4 Aminoglycoside-modifying enzymes (AMEs)

The most widespread mechanism of resistance to aminoglycosides is chemical modification of the antibiotic (Davies and Wright, 1997). Addition of a chemical group to the aminoglycoside blocks binding to the ribosome, which renders the aminoglycoside completely ineffective (Llano-Sotelo et al., 2002). These enzymes transfer a group from a donor molecule, usually a

metabolic co-substrate, to the aminoglycoside. All exhibit a Bi-Bi reaction chemistry (Cleland, 1963), with a Michaelis complex containing both donor and acceptor substrates. The enzyme facilitates direct chemical transfer from the donor substrate to the aminoglycoside.

Three families of aminoglycoside-modifying enzymes have been identified, and these enzymes are classified in a systematic nomenclature (Vanhoof et al., 1998). In this system, a three-letter abbreviation is used for the chemical class of the enzyme: aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC) or aminoglycoside nucleotidyltransferase (ANT). This name is followed by identifiers that reflect the substrate regiospecificity (in Arabic numerals with prime symbols indicating the modified aminoglycoside ring and substituent), substrate profile (in Roman numerals), and individual gene sequence of the enzyme, in order of discovery (as lower-case letters). For example, APH(3')-IIIa is an aminoglycoside phosphotransferase that modifies the 3' position of aminoglycosides. This enzyme has the third characterized profile of substrates (III), and was the first gene identified to do so (a). Naturally, this discontinuous characterization of continuous characteristics of compounds is sometimes inadequate (Ramirez and Tolmasky, 2010), illustrated by cases such as APH(3')-IIIa that also acts upon the 5" position of some aminoglycosides (Thompson et al., 1996), making APH(3', 5")-IIIa a more descriptive name.

Many of these aminoglycoside-modifying enzymes were identified on transferable antibiotic resistance plasmids in the 1960s and 1970s. Their presence on these factors indicate that they have been optimized for effective activity toward aminoglycoside antibiotics because they exist within the pool of optimized antibiotic resistance factors that are rapidly shared in this way. The origins of these resistance enzymes remain somewhat obscure, although an early and compelling hypothesis is that they originate from enzymes that act as self-protection factors that modify aminoglycosides in producing organisms (Benveniste and Davies, 1973; Kirby, 1990), as described in section 1.3.1. An alternative possibility is that these enzymes originated as enzymes that acted on other substrates and were co-opted to act on aminoglycosides (Piepersberg et al., 1988). Irrespective of source, at some point these enzymes developed from these proto-resistance elements (Perry et al., 2014), and have become independently optimized to their new role as high-level antibiotic resistance factors. These three families of aminoglycoside-modifying enzyme carry out different chemical reactions, but all can confer high-level antibiotic resistance.

1.4.1 Aminoglycoside acetyltransferase (AAC)

Aminoglycoside acetyltransferase enzymes are prototypical enzymes in the large Gcn5-related acetyltransferase (GNAT) enzyme superfamily (Vetting et al., 2005). These enzymes transfer acetyl groups from acetyl-CoA directly to a substrate amine. This family includes the AAC enzymes, as well as histone acetyltransferases and aromatic amine acetyltransferases like serotonin acetyltransferase.

GNAT enzymes consist of a mixed β-sheet, with a distortion in backbone hydrogen bonding (“β-bulge”) where the acetyl group of the acetyl-CoA is bound and stabilized (Dyda et al., 2000). Aminoglycoside, amino acid, or protein substrates are bound by loops and helices surrounding this beta sheet, where they are coordinated in a fashion that guides them toward the activated thioacetate group of acetyl-CoA for catalytic transfer. Variations in this architecture yield a remarkably versatile fold in both substrate spectrum and in functional variability.

The AAC enzymes are GNAT enzymes that act on aminoglycosides. Many different AAC enzymes exist that modify aminoglycosides on the 6', 2', 3, 1, and 3" positions (Garneau-Tsodikova and Labby, 2016), binding the antibiotic in completely different orientations to modify structurally distinct sites of the aminoglycoside. The multimerization capabilities of these enzymes are also diverse including multiple dimerization forms (Burk et al., 2003), a monomeric enzyme (Vetting et al., 2008), and a complex multifunctional complex with 6 subunits per particle (Chen et al., 2011).

Acetylation of aminoglycosides at the 6' position by AAC(6') enzymes is the predominant form of resistance by acetylation. At this writing, more than 30 distinct enzymes with this activity have been catalogued (Garneau-Tsodikova and Labby, 2016; Ramirez and Tolmasky, 2010). The myriad variations in the sequence and activity of this enzyme activity indicate a plastic resistance factor that has radiated into many different forms as necessary in different evolutionary niches. These enzymes modify a site on aminoglycosides conserved between both 4,5-disubstituted and 4,6-disubstituted aminoglycosides, so AAC(6') enzymes can frequently inactivate both of these families of aminoglycosides. These characteristics can lead to broad aminoglycoside resistance, a serious challenge in antimicrobial resistant isolates.

1.4.2 Aminoglycoside phosphotransferase (APH)

A second class of enzymes that confers aminoglycoside resistance are the aminoglycoside phosphotransferase enzymes (Davies and Wright, 1997). These enzymes were first identified in plasmids involved in the spread of antibiotic resistance between bacteria (Kondo et al., 1968). These enzymes use ATP or other NTPs to phosphorylate the antibiotic, using magnesium ions to stabilize the transition state (Ozanne et al., 1969). Phosphorylation completely abrogates binding of the aminoglycoside to the ribosomal target, conferring complete resistance.

The first aminoglycoside phosphotransferase to have a structure determined was APH(3')-IIIa (Hon et al., 1997). This structure indicated that APH enzymes are members of the eukaryotic protein kinase superfamily. While this family of enzymes is named for those that phosphorylate proteins on serine/threonine and tyrosine residues, it also includes those that phosphorylate small molecules, and others that have diverged to non-catalytic functions as well, for which the group of enzymes is now instead called the eukaryotic-like protein kinase (ePK) superfamily. These enzymes have a bi-lobal structure that stabilize the substrates between and carry out catalysis with the aid of magnesium ions coordinated in this cleft.

Conserved motifs in this family centre around residues that coordinate magnesium ions and that stabilize a phosphate group transferring from the NTP to the acceptor substrate hydroxyl group (Hanks and Hunter, 1995). Outside of this catalytic architecture, the enzymes can deviate considerably. N-and C-terminal regions, as well as insertion regions on the basic kinase architecture introduce regulatory capabilities in these enzymes. Additional non-conserved regions lead to subfamily-specific functions in branches of the kinase family. More detail on the comparison between protein kinase enzymes and APH enzymes will be covered in Chapter 4.

APH enzymes have become prototypical enzymes in the eukaryotic protein kinase-like superfamily that do not modify proteins. Enzymes that modify neamine-based aminoglycosides at the 3' position and 4,6-disubstituted compounds at the 2'' position are commonly found. Phosphotransferases specific for the atypical aminoglycosides streptomycin, spectinomycin, and hygromycin have all been characterized. While these enzymes all bind different substrates in different modes, the core kinase catalysis elements are largely conserved throughout all enzymes.

1.4.3 Aminoglycoside nucleotidyltransferase (ANT)

The third family of aminoglycoside-modifying enzymes is the aminoglycoside nucleotidyltransferase or ANT family. These enzymes use ATP to transfer AMP to the aminoglycoside using magnesium ions, yielding adenylated antibiotic. In contrast to the aminoglycoside phosphotransferase enzymes which activate the γ-phosphate for transfer, these enzymes stabilize the α-phosphate from ATP to transfer to the aminoglycoside, releasing pyrophosphate as a byproduct. Correspondingly, these enzymes are completely divergent from the phosphotransferase enzymes, unrelated in sequence and structure.

Until very recently, the only characterized member of this family was the ANT(4') enzyme (Sakon et al., 1993). More recent studies on ANT(2") have led to greater insight about enzymes that use this mechanism (Bassenden et al., 2016; Cox et al., 2015), and in this case these enzymes are much more dissimilar than the AAC and APH families, although all three families show some conserved features in spite of considerable divergence in sequence and structure.

1.4.4 Common and divergent features of AMEs

There are three chemically distinct types of aminoglycoside-modifying enzyme, and each type has been suggested to have emerged more than once (Oruganty et al., 2016; Salipante and Hall, 2003; Stogios et al., 2015). This indicates that aminoglycoside modification has developed many independent times as a mechanism of resistance, illustrating convergent evolution toward aminoglycoside modification. Multiple proto-resistance factors independently arrived at the aminoglycoside-modifying activity as evolutionary forces select for aminoglycoside-inactivating function. Correspondingly, these enzymes share common and divergent features that reflect independent origins but shared function. These enzymes have solved some problems in unique ways, specific to their origins, resulting in unique features among specific subgroups of AME.

Aminoglycosides are aliphatic, positively charged compounds with many hydrogen bond donors and acceptors. Interactions between these compounds and macromolecules depend on charge interactions and geometrically constrained hydrogen-bond networks. Aminoglycosides also carry substantial positive charges (Kulik et al., 2015). Aminoglycoside-modifying enzymes all have negatively-charged enzyme active sites to bind their substrate antibiotics (Romanowska et al., 2013). Aminoglycoside binding to these enzymes is typically very exothermic with the

formation of ionic interactions and hydrogen bonds dominating the interactions (Norris et al., 2010; Ozen and Serpersu, 2004; Wright and Serpersu, 2006).

Aminoglycoside-modifying enzymes trade off specificity of binding in order to be active toward a spectrum of antibiotic substrates. Accommodation of multiple aminoglycosides in broadly specific enzymes is achieved using multiple different strategies. Some enzymes becoming more ordered overall in response to substrate binding, while others use transitions of specific loops to facilitate aminoglycoside binding (Serpersu and Norris, 2012). In some cases, water molecules are also used to stabilize binding of these compounds to the aminoglycoside-binding site (Jing and Serpersu, 2014).

Aminoglycoside-modifying enzymes also show variation in their degree of regulation. While they have been traditionally treated as rigid and inflexible enzymes active at the maximal activity, some have a more nuanced character, indicating potential for adaptive regulation (Freiburger et al., 2011; Jing et al., 2012).

1.4.5 Multimeric state of aminoglycoside-modifying enzymes

Most known aminoglycoside-modifying enzymes are monomeric or dimeric. The one notable exception to this is the multimeric acetyltransferase *Eis* from Mycobacterium *tuberculosis*, which forms hexamers (Green et al., 2015). The role of quaternary structure in these enzymes is not well understood, but there are multiple instances where the formation of dimers in aminoglycoside-modifying enzymes is accompanied by improved antibiotic modifying function.

In AAC(6')-Ii, dimerization facilitates a complex allostery between domains of the enzyme (Draker et al., 2003; Freiburger et al., 2011). This allows for modulation of the activity of each monomer in response to the activity of the other. In ANT(4')-Ia, dimerization is dynamic and only occurs following aminoglycoside binding, regulating the enzyme, which is inactive in the monomeric form (Jing et al., 2012).

In contrast to AAC and ANT enzymes, APH enzymes appear to all be monomeric. If there is any dynamic regulation of activity in these enzymes, it must be self-contained within a single protein chain.

1.4.6 The antibiotic resistance profile of AMEs

Aminoglycoside-modifying enzymes can be conserved or hyper-variable, the most extreme variability demonstrated by the widespread AAC(6')-Ib enzyme (Ramirez et al., 2013). AMEs can also be specific to one or two compounds, or they can be broadly active and bind many different aminoglycosides.

In this thesis I will define broadly active aminoglycoside-modifying enzymes to describe those that bind and act upon both 4,5-disubstituted and 4,6-disubstituted aminoglycoside antibiotics. These compounds share structural elements, so modification of these shared elements confer resistance to both. However, an enzyme must both act upon the shared modification site, and also bind the chemically distinct elements of the compound. This introduces a need for diversity and flexibility in any resistance enzyme. For a resistance enzyme to be active toward a compound, it must be capable of chemically modifying the necessary site on the antibiotic, but also binding the antibiotic in a productive fashion, without clashes.

Some resistance enzymes do this remarkably well – they act on a broad swath of aminoglycosides by accommodating their divergent structures in binding, allowing them to act upon both 4,5- and 4,6-disubstituted aminoglycosides. AAC(6')-Ib (Vetting et al., 2008), APH(3')-IIIa (Fong and Berghuis, 2002), and AAC(6')-Ii (Draker et al., 2003) are well-studied antibiotic resistance enzymes that are widely distributed in clinical isolates. As a result, these enzymes have been studied thoroughly, and we have learned a great deal about their mechanisms of substrate binding. Other resistance enzymes such as ANT(2")-Ia (Bassenden et al., 2016) and AAC(3)-Ia (Wohlleben et al., 1989) are less tolerant of variation in their substrates, they act on only some of the 2-DOS based aminoglycosides.

1.4.7 Bifunctional AMEs

Perhaps as a means of expanding the substrate profile of resistance enzymes, some resistance factors have fused into bifunctional proteins with two domains in a single polypeptide. This effect has been predominantly observed in aminoglycoside-modifying enzymes, although a bifunctional enzyme that acts upon β-lactam antibiotics has also been observed (Zhang et al., 2009).

These bifunctional enzymes all contain separable domains with homology to free, monomeric domains. While all contain an acetyltransferase domain, an ANT-AAC fusion (Kim et al., 2006a), two AAC-AAC fusions (Dubois et al., 2002; Mendes et al., 2004), and an AAC-APH fusion enzyme (Culebras and Martínez, 1999) have all been discovered. These enzymes do not share a common origin, so their existence suggests a driving force to produce bifunctional aminoglycoside-modifying enzymes. They will be discussed in more detail in section 2.1.1.

The best studied bifunctional aminoglycoside-modifying enzyme is AAC(6')-Ie/APH(2")-Ia, one of the most prolific and clinically challenging aminoglycoside resistance factors.

1.5 AAC(6')-Ie/APH(2'')-Ia

AAC(6')-Ie/APH(2'')-Ia is the prototypical bifunctional aminoglycoside-modifying enzyme. With a worldwide distribution and a high degree of efficacy toward gentamicin and other aminoglycosides, AAC(6')-Ie/APH(2'')-Ia confers high resistance in pathogens that also carry other resistance factors. The enzyme is found within a mobile transposable element (Lyon et al., 1984), which facilitates movement between plasmid and chromosomal sites. While predominantly found in Gram-positive isolates like *Staphylococcus aureus* and *Enterococcus* sp., it has also been observed in genetic islands of Gram-negative isolates (Qin et al., 2012), alongside resistance factors for many other compounds.

Comprehensive information on the epidemiology of AAC(6')-Ie/APH(2'')-Ia spread is not easily obtained. However, in hospitals where there are breakouts of drug resistant *Enterococcus faecium*, bacteria carrying the gene for the bifunctional enzyme are frequently present (Ardic et al., 2006; Gad et al., 2011; Rosvoll et al., 2012; Yadegar et al., 2009). AAC(6')-Ie/APH(2'')-Ia is often the most frequent aminoglycoside resistance factor identified.

This protein contains two activities that make it not only an extremely broad resistance factor, but also a difficult factor to anticipate, inhibit, or escape. Targeting and dealing with the AAC(6')-Ie/APH(2'')-Ia protein is a unique challenge as this bifunctional resistance enzyme is more complicated in structure, mechanism, and antibiotic-binding profile than monomeric aminoglycoside-modifying enzymes.

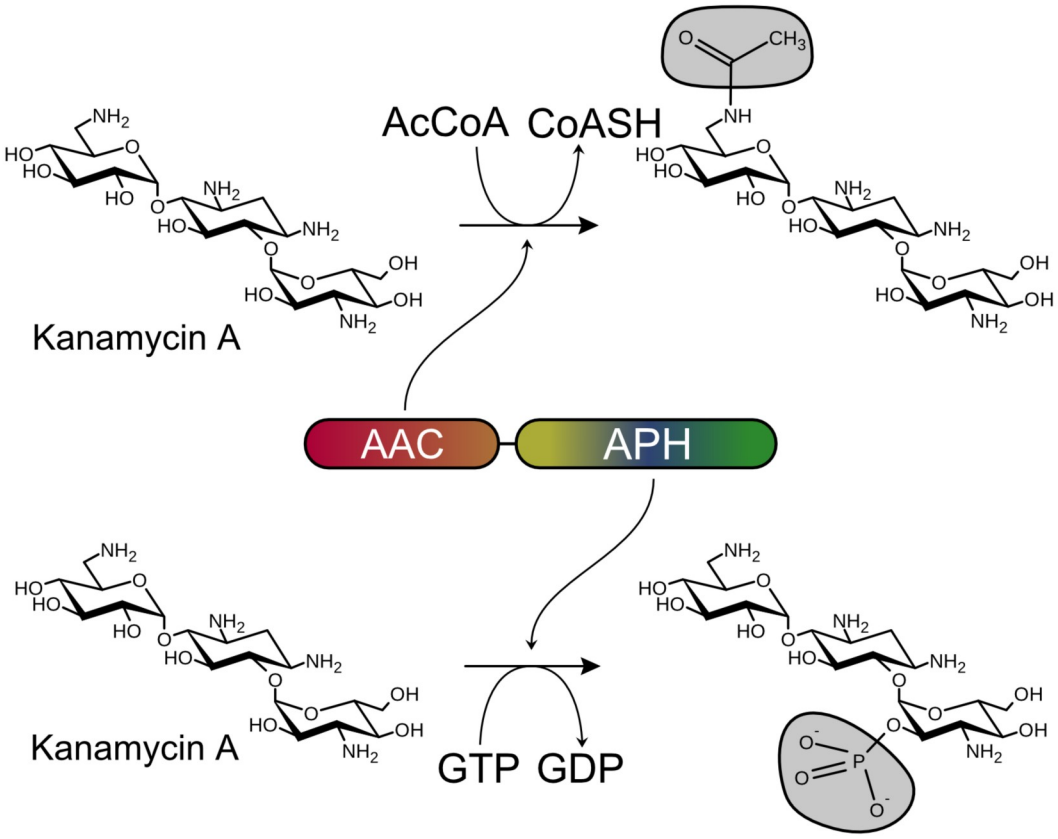

Figure 1.4: Resistance to aminoglycosides conferred by AAC(6')-Ie/APH(2'')-Ia

The AAC(6')-Ie/APH(2")-Ia bifunctional protein confers resistance through two independent enzymatic activities: acetyl-CoA-dependent acetylation from the N-terminal domain, and GTP-dependent phosphorylation from the C-terminal domain.

1.5.1 Discovery of a bifunctional resistance enzyme

AAC(6')-Ie/APH(2")-Ia was first discovered from a strain of gentamicin-resistant *Staphylococcus aureus* (Dowding, 1977). Initial biochemical characterizations were complicated by the co-incidence of another resistance factor in the extract, but comparative phosphorylation of different aminoglycosides indicated that the extract had 2"-*O*-phosphotransferase activity, which could be separated by chromatography from known 3'-phosphotransferase activity.

Functional clues about the bifunctional nature of this enzyme were already seen in the inability to separate acetyltransferase and phosphotransferase activities using chromatography.

After it became clear that the enzyme probably possessed both acetyltransferase and phosphotransferase activities, an early model hypothesized that the enzyme active site was capable of binding and interacting with both the nucleoside triphosphate (at that time, ATP was expected) and coenzyme A in the same fashion (Le Goffic et al., 1977a). It was not unreasonable at the time to expect that perhaps a single active site accommodated both donor substrates and bound the aminoglycosides in the same way, as the understanding of structural and functional domains was not as sophisticated at the time. The demonstration that this enzyme was truly a bifunctional particle with independent domains came with genetic sequencing studies. Later, the sequence of APH(6')-Ie/APH(2")-Ia was determined simultaneously by two groups, from enterococcal (Ferretti et al., 1986) and staphylococcal (Rouch et al., 1987) sources. These sequences were identical, showing that the enzyme has moved between these clades recently, and leaving the origins of the resistance factor ultimately murky.

Sequences and some biochemical studies demonstrated that the activities of the enzyme are spatially separated in the enzyme. It became clear that this bifunctional enzyme had separable activities in two different enzyme active sites. The enzyme consists of two domains[3]: an N-terminal acetyltransferase domain, and a C-terminal phosphotransferase domain.

1.5.2 Relationship between domains in AAC(6')-Ie/APH(2")-Ia

The acetyltransferase and phosphotransferase domains of AAC(6')-Ie/APH(2")-Ia were mapped by truncation experiments – acetyltransferase at the N-terminus, phosphotransferase at the C-terminus. Substrates bound to the APH(2")-Ia domain were shown to protect the AAC(6')-Ie domain from thermal denaturation, suggesting a structural association between activities (Martel et al., 1983). These functions can be expressed independently, although they seem to pay a catalytic penalty upon separation (Ferretti et al., 1986).

3 Throughout this thesis, the term “domain” will be used to refer to either the AAC(6')-Ie or APH(2")-Ia enzyme portions of the protein. The term “lobe” and “subdomain” will be used for sections within these respective domains.

Very little conclusive information is available about the relationship between the AAC(6')-Ie and APH(2")-Ia domains of the bifunctional enzyme. The two domains of AAC(6')-Ie/APH(2")-Ia contain two enzymatic activities, which have not been shown to depend on the other domain to any meaningful degree. These enzymes are independently powerful antibiotic resistance factors, and they have non-mutual profiles of substrates, expanding the range of the enzyme to almost all neamine-based aminoglycosides (Daigle et al., 1999a).

Much of our biochemical understanding of the enzyme comes from Boehr *et al*'s experiments on the protein (Boehr et al., 2004). An overlapping region of amino acids was found to be necessary for the function of both enzymatic domains, which suggested shared structural associations using this overlapping region. Mutants that disrupted the secondary structure in this region negatively influenced the activity of both enzymatic domains. These findings implied a potential structural interaction between the domains, though not conclusive proof. The structural relationship between the N-terminal AAC(6')-Ie and C-terminal APH(2")-Ia domains will be the subject of Chapter 2.

1.5.3 AAC(6')-Ie

Like other AAC enzymes, the AAC(6')-Ie acetyltransferase domain of AAC(6')-Ie/APH(2")-Ia uses acetyl-coenzyme A to acetylate aminoglycosides on the 6' position. This position is part of the conserved neamine rings of the compounds, and modification at this site confers high-level resistance to most compounds with 6' amino groups. Studies also indicated that the enzyme is even more broadly active toward compounds with 6' hydroxyl groups, indicating a separate chemistry is possible within this acetyltransferase domain, albeit at low rates (Daigle et al., 1999a). The closest homologue of this enzyme domain is the aminoglycoside acetyltransferase AAC(6')-Im (Chow et al., 2001), but the closest homologue with structure determined is the broad-profile monomeric AAC(6')-Ib (Vetting et al., 2008), discussed above.

1.5.4 APH(2")-Ia

The APH(2")-Ia domain of AAC(6')-Ie/APH(2")-Ia is the prototypical aminoglycoside 2"-kinase (Daigle et al., 1997). Other enzymes in this family include APH(2")-IIa (Chow et al., 2001), APH(2")-IIIa (Chow et al., 1997), APH(2")-IVa (Tsai et al., 1998), APH(2")-IVb (Mahbub Alam et al., 2005), and APH(2")-If (Toth et al., 2013). APH(2")-Ia serves as the model for this

family, although subgroups in this family share only ~30% sequence identity. Despite structural characterization of members of all four subfamilies, much of the mechanism of these enzymes remains unstudied.

Kinases all use a nucleoside triphosphate co-substrate as the phosphate donor in their reactions, most frequently ATP. A distinguishing feature of APH(2") enzymes is their relationship to their nucleoside substrate. Some of these enzymes use ATP as the substrate, some facultatively use either ATP or GTP, while still others are restricted to GTP. APH(2")-Ia exclusively uses GTP as its triphosphate co-substrate (Toth et al., 2009).

The APH(2")-Ia domain of AAC(6')-Ie/APH(2")-Ia was the first discovered phosphotransferase enzyme that acted on gentamicin, and the first to phosphorylate aminoglycosides at the 2" position. This position is opposite the 3' position that APH enzymes known at the time phosphorylated. This alternate location of phosphorylation makes the enzyme effective toward compounds that are not subject to resistance by APH(3') enzymes, such as tobramycin, dibekacin, and gentamicin.

The mechanism of catalysis in these enzymes has not been thoroughly studied. This topic will be covered in more depth in chapter 4. This enzyme domain binds aminoglycosides of both classes, although conflicting reports of its activity have generated uncertainty about the nature of aminoglycoside binding in this enzyme. The binding of aminoglycosides by APH(2")-Ia will be elaborated in chapters 3 and 5.

1.5.5 Evolution of function and resistance in AAC(6')-Ie/APH(2")-Ia

The AAC(6')-Ie/APH(2")-Ia enzyme is a remarkably resilient resistance factor, with two independent resistance activities that inactivate aminoglycosides with high effectiveness. The evolution of these functions and their combination in a single polypeptide present interesting questions about evolution of function in antibiotic resistance in this enzyme.

First, what is the structural relationship between domains in the full-length enzyme? Aminoglycosides need only a single modification for effectively complete resistance (Llano-Sotelo et al., 2002), so the addition of an additional attached aminoglycoside resistance enzyme does not serve an obvious purpose. Furthermore, the two domains are active independently, and there is no apparent reason for them to fuse into a single polypeptide. In order for this domain arrangement to be maintained through evolutionary selection, there must be some kind of selective benefit to formation of the full-length bifunctional protein in comparison to independent domains. The emergence of additional bifunctional aminoglycoside-modifying enzymes indicate that bifunctionality could be a common trait, and so structural study of this protein may influence our understanding of the larger class of bifunctional aminoglycoside-modifying proteins.

Secondly, what is the relationship of the APH(2")-Ia domain in binding to aminoglycosides, especially 4,5-disubstituted compounds? The APH(2")-Ia domain of AAC(6')-Ie/APH(2")-Ia is the prototype APH(2") enzyme, and while important studies have examined structural changes (Shi et al., 2011) and nucleoside binding (Bhattacharya et al., 2013; Shakya and Wright, 2010; Shi and Berghuis, 2012; Toth et al., 2009) in these enzymes, the aminoglycoside-binding site is less well studied. There is contradictory information in the literature about aminoglycoside binding by APH(2")-Ia, and a structural approach can help resolve these contradictions.

Thirdly, how well-conserved is the mechanism of phosphotransfer in the APH(2")-Ia domain? Despite an understanding of APH(2")-Ia as a member of the eukaryotic protein kinase superfamily, the mechanism of phosphotransfer in this enzyme has not been examined from a structural perspective. The distantly related APH(3')-IIIa enzyme has been well-characterized (Burk et al., 2001), but it is not known if the same mechanistic details are also conserved in the APH(2") family of enzymes. A structural study of catalysis in APH(2")-Ia would help understand mechanisms of resistance in the greater family of aminoglycoside phosphotransferases.

Finally, how much can the AAC(6')-Ie/APH(2")-Ia enzyme adapt to new conditions? The wildtype AAC(6')-Ie/APH(2")-Ia enzyme appears to interact with all neamine-based aminoglycosides without an N1 modification. This semisynthetic addition to aminoglycosides allows antibiotics to escape modification by AAC(6')-Ie/APH(2")-Ia under normal circumstances. Experiments designed to determine clinical resistance mutations that expand the

binding profile of APH(2") enzymes have had limited success (Lee et al., 2002; Toth et al., 2010). It has been observed that several cases of clinical resistance to semisynthetic aminoglycosides are conferred by mutations in the AAC(6')-Ie/APH(2")-Ia gene (Fujimura et al., 2000; Ishino et al., 2004). The impact of the S376N mutation on binding of aminoglycosides is an interesting case study of a new function emerging in the aminoglycoside-binding site of APH(2")-Ia.

This thesis will study these questions as they pertain to the bifunctional aminoglycoside-modifying enzyme AAC(6')-Ie/APH(2")-Ia. These multiple questions give us new insight into the development of resistance and the changes that can occur to drive function in antibiotic-modifying enzymes. They also provide new potential means of tackling the problems of clinical antibiotic resistance, a problem of pressing public health concern.

1.6 Summary of Experiments

This thesis describes studies of the structure and mechanisms of aminoglycoside resistance conferred by the bifunctional aminoglycoside-modifying enzyme AAC(6')-Ie/APH(2")-Ia. Small-angle X-ray scattering and X-ray crystallography were used to probe the ultrastructure, aminoglycoside binding profile, catalytic mechanism of the enzyme, as well as the structure of a clinical mutant of the enzyme with altered substrate profile. All of the experiments described in this thesis were carried out independently, with the assistance of undergraduates Yolanda Huang who helped with crystal optimization trials for APH(2")-Ia, and Manjot Sangha, who piloted some early phosphate release assays. Robert Reiss and Lucy Yu assisted with molecular cloning projects that ultimately did not lead to data included in this thesis. This is a traditional-format thesis so chapters are written independently of published journal articles, but the data presented in Chapter 2 was published in the journal Antimicrobial Agents and Chemotherapy (Caldwell and Berghuis, 2012), while data from Chapters 3 and 4 were combined to form a manuscript published in Structure (Caldwell et al., 2016). At the time of submission, data from Chapter 5 and some sections of Chapters 3 and 4 are unpublished.

Chapter 2: Solution-based small-angle-X-ray scattering (SAXS) was used to probe the overall solution structure of the enzyme and changes upon binding of substrates. The full-length AAC(6')-Ie/APH(2")-Ia enzyme was subjected to scattering in the *apo* form over a series of concentrations in order to construct a model of the full-length protein. Following the construction of this model, multiple substrates were mixed with the protein by dialysis at the same concentration and the scattering of the resulting mixtures was examined to track changes to the protein upon ligand binding.

Chapter 3: The APH(2")-Ia domain of the protein was purified and crystallized with GMPPNP and saturating concentrations of magnesium. Following the solution of this structure, crystals of the enzyme were soaked with 4,5-disubstituted and 4,6-disubstituted aminoglycosides to directly observe binding interactions of these compounds in the protein. The structures were aligned to compare binding of aminoglycosides to the enzyme, and changes to the protein that followed aminoglycoside binding.

Chapter 4: Following from analysis of the GMPPNP structure of APH(2")-Ia, this domain was also crystallized with GDP, and an additional structure was determined with GDP and soaked gentamicin. These structures revealed open and closed conformations of the enzyme, and allow modelling of the Michaelis complex of the enzyme with both substrates bound. Analysis of protein motions and changes in the nucleoside triphosphate conformation in the GMPPNP co-substrate drove me to co-crystallize with another GTP analogue GMPPCP, as well as to generate the S214A and Y237F mutants of the enzyme through site-directed mutagenesis and crystallize these mutants with GMPPNP. Taken with aminoglycoside-bound structures, these structures collectively delineate major transitions involved in activating the enzyme for catalysis upon antibiotic binding. A malachite green-based assay for determination of free phosphate released from the enzyme also helped track the shift of the enzyme from an inactive to catalytically active form which results in increased hydrolysis of the GTP co-substrate.

Chapter 5: The S376N mutant of APH(2")-Ia was generated by site-directed mutagenesis, expressed, and purified, and crystallized with GMPPNP. The structure of this mutant was determined and the crystals were also used for crystallographic soaking experiments with N1-substituted and N1-unsubstituted aminoglycosides. Crystallographic soaks of the wildtype protein with the semisynthetic aminoglycosides dibekacin, and arbekacin were also conducted, and co-crystals were grown in the presence of GMPPNP and the N1-substituted aminoglycoside amikacin. These structures illustrate the interaction of APH(2")-Ia with semisynthetic aminoglycosides and suggest means by which the S376N mutant confers increased resistance toward N1-substituted aminoglycosides.

1.7 Original Knowledge Contributions

The studies described in this thesis reiterate that antibiotic resistance is a multifaceted phenomenon, with competing forces shaping resistance factors to be highly effective versus broadly active proteins. Features of the AAC(6')-Ie/APH(2")-Ia resistance factor indicate both that it has been selected for high activity toward its native substrates, but also exhibits the propensity for change as the demands of resistance are altered. AAC(6')-Ie/APH(2")-Ia is antibiotic resistance in microcosm.

This thesis describes four independent knowledge contributions:

Chapter 2: AAC(6')-Ie/APH(2'')-Ia has a rigid structure suggesting adaptive change in the bifunctional protein

Solution scattering analysis of the AAC(6')-Ie/APH(2")-Ia particle indicates a close structural association of the AAC(6')-Ie and APH(2")-Ia domains within the protein. This close association implies that the fusion of these domains occurred long ago in order to allow sufficient time for the enzyme to develop a stable interface between domains. AAC(6')-Ie/APH(2")-Ia is an ancient antibiotic resistance factor.

Introduction of ligands to the protein exhibited very limited changes to the enzyme scattering profile, indicating a lack of large-scale induced structural changes, and that the protein maintains the same global architecture with only local adaptations to ligand binding.

These findings suggest that the fusion of these two domains into a single protein could confer a selective benefit for bifunctionality in the protein, which may include allosteric mechanisms or resistance through improved aminoglycoside binding behaviour.

Chapter 3: APH(2'')-Ia binds the neamine nucleus of aminoglycosides of 4,5- and 4,6-disubstituted aminoglycosides

Crystallization of the APH(2")-Ia domain of the enzyme followed by soaking of aminoglycoside substrates into the crystals allowed the comparison of the binding interactions of 4,5- and 4,6-disubstituted aminoglycoside antibiotics. The enzyme is active even in the crystal as seen by the phosphorylation of tobramycin *in crystallo*. Both 4,5-disubstituted and 4,6-

disubstituted aminoglycosides are captured in the active site using the conserved neamine-based rings. This occurs even though this binding mode precludes chemical modification of the 4,5-disubstituted compounds, which suggests that the enzyme could provide some low-level resistance to these compounds by sequestration from solution.

The binding of both of these compounds in the same manner highlights a conservation of strategy between broad-profile aminoglycoside-active resistance factors, that occurs even in the absence of catalysis of non-substrate aminoglycosides like neomycin and ribostamycin. Broad profile aminoglycoside resistance factors bind the neamine-based nucleus, while accommodating the variability in 5- and 6-linked rings. This tolerance and variability breaks down for larger compounds like lividomycin A, which exhibits binding to the enzyme but no consistent binding mode, possibly due to steric effects arising from its large size.

Chapter 4: APH(2'')-Ia regulates phosphotransfer in a catalytic switch flipped by enzyme closure and the Gly-loop

The nucleoside-bound APH(2")-Ia revealed a novel conformation of the triphosphate group in the enzyme active site. Disruption of this conformation activates the enzyme and loop rearrangements complete the activation for catalysis. The equilibrium between the novel stabilized conformation and the activated state of the triphosphate indicates that the enzyme exhibits control over its catalytic activation. This equilibrium between states represents a catalytic switch that converts between inactive and active states of the enzyme.

The conversion between these two states is responsive to the bound aminoglycoside, which drives the triphosphate to activate. Movement of the distal helical subdomain toward the core subdomain occurs upon aminoglycoside binding and brings it into contact with loops that influence catalysis. Two residues are involved in converting this structural change to catalytic modification, comprising a latch on the enzyme's catalytic switch.

Chapter 5: Structural studies on binding of N1-substituted aminoglycosides to wildtype and S376N mutant APH(2'')-Ia

N1 modification of aminoglycoside antibiotics block the neamine-based binding interaction observed for other antibiotics bound to the enzyme. This confirms that this modification leads to greatly reduced efficacy of the enzyme toward these compounds. The clinical arbekacin-resistant S376N mutation also precludes this mode of binding, for both N1-substituted and unsubstituted compounds. This mutant does not introduce new structural changes that would accommodate the addition of a group to these compounds, and no soaked compounds indicated improved binding to the mutant enzyme.

Dibekacin, the N1-unmodified precursor to arbekacin, is bound to the enzyme in the same fashion as other 4,6-disubstituted aminoglycosides, indicating that changes to the 4-linked aminohexose of this compound and arbekacin do not facilitate alternate binding to the enzyme either.

An additional mode of interaction between amikacin and APH(2")-Ia was identified, and this binding mode conserves an interaction of the 2" hydroxyl group in the enzyme catalytic site. This mode of binding is compatible with the S376N mutation of the enzyme, which may play a role in stabilizing this novel, weak binding interaction. This indicates a potential for adaptive change and emergence of *de novo* function in APH(2")-Ia.

2 AAC(6')-Ie/APH(2'')-Ia has a rigid structure suggesting adaptive change in the bifunctional protein

2.1 Background

The bifunctional AAC(6')-Ie/APH(2'')-Ia was formed by fusion of AAC and APH enzymes into a single polypeptide, first reported in 1977 (Le Goffic et al., 1977b). This protein was the first of several bifunctional aminoglycoside-modifying enzymes identified, and as such, it serves as a prototype for this group of proteins. The existence and evolutionary success of AAC(6')-Ie/APH(2'')-Ia implies that this bifunctional character has given the enzyme some adaptive function that improves upon that of free, independent enzymes. Whatever this function is, it must confer a selective advantage for the protein, although it is currently unclear what this selective advantage would be.

Structural studies can help probe the relationship betweeen the AAC(6')-Ie and APH(2'')-Ia domains of this protein – are they flexibly linked, rigidly associated, or involved in any higher-order structures? This information will help us understand the function of the two-domain fusion. In addition, findings that help us understand this enzyme may also inform the study of other bifunctional resistance factors.

2.1.1 Bifunctional aminoglycoside-modifying enzymes

Most aminoglycoside-modifying enzymes are single-domain, monofunctional enzymes (Ramirez and Tolmasky, 2010). These enzymes all carry out a simple transformation – transfer of a chemical group from a donor to the aminoglycoside acceptor. This chemical transformation can be accomplished with a single enzymatic domain. As a result, most known aminoglycoside-modifying enzymes contain a single, monofunctional domain. Many of these proteins have become widespread, successful antibiotic resistance factors like APH(3')-IIIa, ANT(2'')-Ia, and AAC(6')-Ib (Wright, 1999).

At the same time, several bifunctional aminoglycoside-modifying enzymes have also been identified from clinical samples (Zhang et al., 2009). In these proteins, multiple antibiotic resistance enzymes are combined within a single multifunctional polypeptide. These enzymes include a nucleotidyltransferase-acetyltransferase fusion, two acetyltransferase-acetyltransferase fusions with distinct activity spectra, and AAC(6')-Ie/APH(2")-Ia, the acetyltransferase-phosphotransferase fusion. These bifunctional enzymes, including AAC(3)-Ib/AAC(6')-Ib' (Dubois et al., 2002), AAC(6')-30/AAC(6')-Ib' (Mendes et al., 2004), and ANT(3")-Ii/AAC(6')-IId (Centrón and Roy, 2002), have arrived at bifunctionality independently, with different chemical functions and evolutionary origins. The combination of unrelated chemical functions and regiospecificities in these architectures indicates a common force driving the formation and spread of these factors separate from their chemical activity, sequence, or structure.

AAC(6')-Ie/APH(2")-Ia was the first bifunctional aminoglycoside-modifying enzyme identified, and is the most widely spread in clinical resistance to aminoglycosides. Despite being known for decades, the role of bifunctionality in this enzyme remains unclear. The individual domains appear to be catalytically independent, with some reduction in activity, but remaining highly active when separate (Boehr et al., 2004). Homologues of both domains exist and are active as independent enzymes, and the APH(2")-Ia domain is even translated independently from the same gene *via* an internal initiation of translation (Daigle et al., 1999a). As a result, the role of bifunctionality in AAC(6')-Ie/APH(2")-Ia is an interesting subject of study. What brings these domains together? What is their structural relationship? Do they influence each other? How does it compare to other multidomain enzymes?

2.1.2 Emergent functions in multidomain enzymes

Multidomain proteins are common in biological systems, where 40-65% of all proteins contain more than one functional domain (Ekman et al., 2005). Multidomain proteins provide opportunity for the generation of new function by fusion of individual proteins into a single molecule (Todd et al., 2001). The combination of two or more protein domains can introduce more complexity and tighter specificity compared to single-domain proteins, as this fusion of domains into a single polypeptide provides more opportunity for mutation and the selection of emergent functions (Hardie and Coggins, 1986). Combination of domains can modify substrate

binding, or distribute a function to specialized domains, leading to greater efficiency in the recognition of ligands (Bashton and Chothia, 2007).

Multidomain proteins made from enzyme domains are less common than other types of multidomain proteins (Traut, 2014), although they are found more frequently in bacteria than in archaea or eukarya. These types of enzymes are overwhelmingly involved in metabolic pathways, frequently catalysing subsequent steps (Cheng et al., 2012). Through direct channelling of substrate between domains through a tunnel or cleft, or simply control of the spatial diffusion of compounds, metabolic efficiency is improved (Yon-Kahn and Hervé, 2009). The multidomain property also introduces the possibility for regulation of the enzyme domains through their influence upon each other. Through multiple different mechanisms, domains in a multifunctional enzyme can communicate and influence each other (Nagradova, 2003). The relationship of two domains in a multifunctional enzyme contribute to the overall fitness of the combined enzyme, and in cases where it confers a selective benefit, the bifunctional enzyme can be remarkably successful, especially as we observe in antibiotic resistance.

2.1.3 The benefits of bifunctionality in antibiotic resistance

What selective advantages does a bifunctional antibiotic-modifying enzyme gain? There are multiple possible explanations that might be explored. Of course, these explanations aren't mutually exclusive, and they could together contribute to the bifunctional nature of the protein.

One possibility is that fusion of two enzyme activities into a single gene could be driven solely by co-expression of two activities in a similar genetic context. In this way, two enzyme activities could be regulated as a single unit of transcriptional control. In a population genetics context, this could also allow the co-selection of two activities at once in bacterial populations. In this case, we do not expect any structural association of these domains, as there is no selective benefit to drive these enzymes toward a structural interaction.

Another possibility is channelling of substrates, as seen in some bifunctional metabolic enzymes. In these cases, the active sites of adjacent enzymes are placed in close proximity to pass the products of one enzyme as substrates to the next. However, resistance to aminoglycosides does not require sequential modification of a compound. It is possible that an antibiotic could become dual-modified, and this has been shown several times (Azucena et al.,

1997; Green et al., 2011; Kondo et al., 1993), but this dual modification has not been seen to be physiologically important for improved resistance, as a single modification typically confers complete resistance (Llano-Sotelo et al., 2002). This dual modification also does not necessitate combining the enzymes into a single polypeptide, unless a higher efficiency is necessary to ensure a ligand does not return to bulk solution.

A bifunctional enzyme also act as an “aminoglycoside sink”, binding to bulk aminoglycoside, even if it is not productively modified. Despite the lack of modification, the antibiotic is still removed from solution. There is precedent for this effect playing a role in aminoglycoside resistance as multiple aminoglycoside-modifying enzymes have been shown to convey aminoglycoside resistance even when the catalytic residues are removed, killing the enzyme activity (Magnet et al., 2003; McGann et al., 2014). Bifunctional enzymes could increase this ability by binding aminoglycoside in two active sites, whether or not it is modified. Incorporating the protein's enzymatic activities, it could also form a reciprocal antibiotic sink where apprehension of the antibiotic first occurs before limited diffusion between domains.

A final compelling mechanism for bifunctionality is co-operativity between domains. The concentration of activity in one polypeptide could facilitate higher-order activity through co-operative binding or allosteric interactions. It is possible that the enzyme activates in response to aminoglycoside binding in allosteric or hysteretic fashion. This could allow cross-talk between the different enzymes: domain one could act as a modulator of domain two, and *vice versa*. Antibiotic resistance enzymes have historically not been thought to have co-operativity, but studies of dimeric aminoglycoside-modifying enzymes (Freiburger et al., 2014, 2011; Porter et al., 2010) have illustrated complicated co-operativity between domains in homodimers. This could also be the case for a bifunctional enzyme with two distinctly functional but related domains. Fused domains in a bifunctional enzyme could exhibit adaptive allosteric behaviour. Structural data could provide great insight into such a mechanism.

2.1.4 Fusion of two enzymes to form AAC(6')-Ie/APH(2'')-Ia

Antibiotic resistance enzymes can emerge as *de novo* functions of existing enzymes, or they can be ancient, catalytically-optimized resistance factors (Section 1.2.2). Evolutionary analyses have indicated that both the APH(2'')-Ia (Oruganty et al., 2016) and AAC(6')-Ie (Vetting et al., 2005) domains are members of ancient enzyme families, but the time of fusion into a single bifunctional polypeptide is unclear. The first substrate of AAC(6')-Ie/APH(2'')-Ia, neomycin, first entered clinical use in the 1950s. The AAC(6')-Ie/APH(2'')-Ia enzyme was first found only 20 years later, in 1977 (Le Goffic et al., 1977b). This enzyme has been widespread throughout the world and it is possible that the fusion to form this new enzyme occurred following clinical use of aminoglycosides, although it could also be an enzyme that was optimized in the pre-antibiotic era to act on these compounds.

AAC(6')-Ie/APH(2'')-Ia was originally identified as a source of gentamicin resistance, through its phosphotransferase activity. Based upon its substrate profile, the phosphotransferase activity was hypothesized (Dowding, 1977) and then deduced (Le Goffic et al., 1977b) to be a 2''-directed activity. An inability to separate this new 2''-phosphotransferase activity from 6'-acetyltransferase activity by chromatography indicated that both enzymes might exist in a single polypeptide. It hypothesized for a time that both activities might take place in a single, polyfunctional active site (Le Goffic et al., 1977a). GTP co-substrate protected the acetyltransferase activity of the enzyme from thermal denaturation (Martel et al., 1983), indicating that the protein was indeed a single bifunctional enzyme and suggesting the activities were structurally interrelated.

Truncation experiments demonstrated that a version of the protein missing the N-terminal 137 amino acids showed only APH activity, indicating the activities were spatially separated (Ferretti et al., 1986), followed by sequence analysis that confirmed the enzyme contained independent acetyltransferase and phosphotransferase domains (Ferretti et al., 1986; Rouch et al., 1987). This sequence work found an N-terminal domain with homology to acetyltransferase enzymes, and a C-terminal enzyme with the 2''-phosphotransferase activity.

The gene encoding AAC(6')-Ie/APH(2")-Ia lies in a transposable element, making it capable of quick movement between plasmid and chromosome (Lyon et al., 1984). As a result, this resistance factor exists in an environment where it is subject to rapid evolutionary pressures. The protein could be more subject to faster selective cycles, and have more opportunity for mutation and selection than chromosomal genes. The mobile nature of the genes also increases the chance of genetic rearrangement, making the possibility of genetic fusion more accessible to parental genes. This resistance factor is mobile, must be versatile, and optimized for multiple hosts. Whether old or new, bifunctionality would seem to impart some physicochemical benefit to the enzyme.

2.1.5 What is the role of bifunctionality in AAC(6')-Ie/APH(2")-Ia?

There are a number of examples that illustrate that activity of either AAC(6')-Ie or APH(2")-Ia enzymes confer reasonable antibiotic resistance. Homologous AAC(6') (Costa et al., 1993; Ramirez et al., 2013) and APH(2") (Chow et al., 1997; Kao et al., 2000; Tsai et al., 1998) enzymes have long been known to be stable and active without a fused domain. Very close homologues of the AAC(6')-Ie and APH(2")-Ia domains have been studied more recently (Toth et al., 2012, 2013). Dissection of AAC(6')-Ie/APH(2")-Ia into constituent domains showed that the respective domains do not require each other to be active (Boehr et al., 2004) (although they are reduced in activity). Upon expression of the bifunctional protein, a 35 kDa protein is also synthesized, which is believed to be the independent translation of the APH(2")-Ia domain from an internal initiation of translation (Daigle et al., 1999a). These observations all argue against the importance of a bifunctional fusion protein.

It would seem that the bifunctional character of the AAC(6')-Ie/APH(2")-Ia enzyme is not necessary for resistance. However, if a feature of a molecule confers limited benefit to balance the additional metabolic burden it imposes, that feature is typically lost. Competition, especially under strong selective pressure from antibiotics, selects for efficiency and efficacy. So, in order for this bifunctional arrangement to be maintained in bacterial populations, there must be some selective benefit to bifunctionality in this protein. A structural approach to probing this protein will give insight toward the function of the bifunctional protein.

2.1.6 Experimental approach to study multidomain interactions in AAC(6')-Ie/APH(2'')-Ia

Small-angle X-ray scattering (SAXS) was deployed to probe the flexibility and domain-domain interactions between AAC(6')-Ie and APH(2'')-Ia domains of AAC(6')-Ie/APH(2'')-Ia. This technique allows for the analysis of particles in solution, removing sample preparation biases inherent to other structural biology techniques such as crystallography or electron microscopy. SAXS returns information about the dispersity, flexibility, and structure of particles suspended in solution.

Using maximum-likelihood algorithms employed in the ATSAS analysis package (Konarev et al., 2006), it has now become tractable to construct *ab initio* models of a particle by scattering alone, and also to model the interaction of independent rigid bodies of a protein. I determined the SAXS scattering profile of apo AAC(6')-Ie/APH(2'')-Ia, and measured the radius of gyration (R_G) of the protein in complex with nucleoside, coenzyme, and aminoglycoside substrates in order to test for structural changes upon substrate binding in the enzyme. This information allows us to narrow the possible explanations for the fusion of AAC(6')-Ie and APH(2'')-Ia into a single bifunctional polypeptide and helps better understand the evolution of antibiotic resistance as carried out by this resistance factor.

2.2 Methods

2.2.1 Protein production and purification

A plasmid containing the N-terminal histidine-tagged bifunctional AAC(6')-Ie/APH(2")-Ia enzyme was obtained from Dr. G. D. Wright (McMaster University, Hamilton, Ontario). This plasmid was transformed into competent *E. coli* BL21 (λDE3) cells by a standard heat shock protocol. The cells were screened on ampicillin-LB-agar plates, and allowed to grow overnight.

Cells were grown in auto-induction media, by the Studier protocol (Studier, 2005). 2.5 mL of ZYP-0.8G media with ampicillin was inoculated from a single colony of bacteria containing the pET-15b NHis-AAC(6')-Ie/APH(2")-Ia plasmid. This culture was allowed to incubate, shaking at 37˚C, overnight. 100 µL of this "starter culture" was used to inoculate a 2.5 mL starter culture of ZYP-0.8G + Ampicillin, which was incubated shaking at 37˚C for 1 hour. This full 2.5mL starter culture was used to inoculate 500 mL of ZYP-5052 auto-induction culture media + 100 µg/mL ampicillin, shaking at 37˚C in a Fernbach flask. After 2.5 hours growth at 37˚C, the temperature was reduced to 22˚C and allowed to continue incubation overnight. This culture was harvested by centrifugation at 5000 g for 15 minutes. Pellets were re-suspended in a small volume of spent media and transferred to 50 mL conical tubes and centrifuged again for 30 minutes at 3200 g. The supernatant was removed and the pellets flash frozen in liquid nitrogen before storage at -20˚C.

Protein was purified from these cell pellets. The cells were thawed to room temperature and re-suspended in a buffer containing 1 M NaCl, 10 mM imidazole, and 25 mM HEPES pH 7.5. They were subjected to ultrasonication on ice using a Model 500 Sonic Dismembrator (Fisher Scientific) for 15 minutes total pulse time, in intervals of 10 seconds on with 20 seconds off. The lysate from this process was clarified in an Avanti J-26XP centrifuge with JA-25.5 rotor in 50 mL polycarbonate tubes, at 50 000 x g for 30 minutes.

The lysate was filtered through a 0.45 µm pore size syringe filter (Millipore) and loaded to a pre-equilibrated Ni-NTA HiTrap column (Qiagen), and the flowthrough was collected. The protein was eluted using a gradient of 10-250mM imidazole, with constant 1 M NaCl and 25 mM HEPES pH 7.5. 1 mM β-mercaptoethanol was included as a reducing agent.

The protein was exchanged into 10 mM HEPES 7.5 and 5% glycerol by successive concentration and dilution in an Amicon concentrator (Millipore) with 50 kDa cutoff. At final state, less than 0.3% of the original buffer would be carried over.

2.2.2 Activity validation enzyme assays

Purified enzyme was assayed for acetyltransferase and phosphotransferase activities. The acetyltransferase assay is a real-time spectrophotomeric assay that couples the production of coenzyme A byproduct to the reduction of DTDP, which generates an absorbant aromatic thiol byproduct that absorbs at 324 nm. This allows real-time tracking of the AAC reaction, as free coenzyme A is produced as a byproduct of the acetylation reaction.

The APH enzyme assay was conducted by a standard protocol coupling the phosphorylation of substrate to the reduction of NADH. This coupled reaction uses pyruvate kinase, lactate dehydrogenase, and phosphoenol-pyruvate to couple the production of nucleoside diphosphate (NDP) throughout the reaction to a decrease in NADH, which is measured spectrophotometrically at 340 nm.

These assays indicated that the bifunctional enzyme was active as both acetyltransferase and phosphotransferase. This confirmed the enzyme was active and that it productively bound substrates for both enzymatic domains.

2.2.3 SAXS data collection for the apoenzyme

The exchanged AAC(6')-Ie/APH(2")-Ia was concentrated to 50 mg/mL in Amicon concentrators (Millipore) with 30 kDa molecular weight cutoff. This protein was in turn diluted by factors of two in the filtrate buffer, producing a concentration series of the protein from 50-3.12 mg/mL.

This concentration series of protein was loaded into the capillary of an Anton Paar SAXSess mc^2 instrument with CCD detection. The most concentrated samples were exposed for 30 minutes, while the more dilute samples experienced exposures up to 24 hours. Buffer scattering and instrumental noise were collected for each sample, and subtracted to determine the scattering from each concentration of enzyme. The samples showed identical scattering patterns with the exception of inter-particle interference at low q values (Figure 2.1). Merging of these curves in

PRIMUS (Konarev et al., 2003) eliminated this effect and generated a concentration-independent scattering profile for the protein (Figure 2.2a).

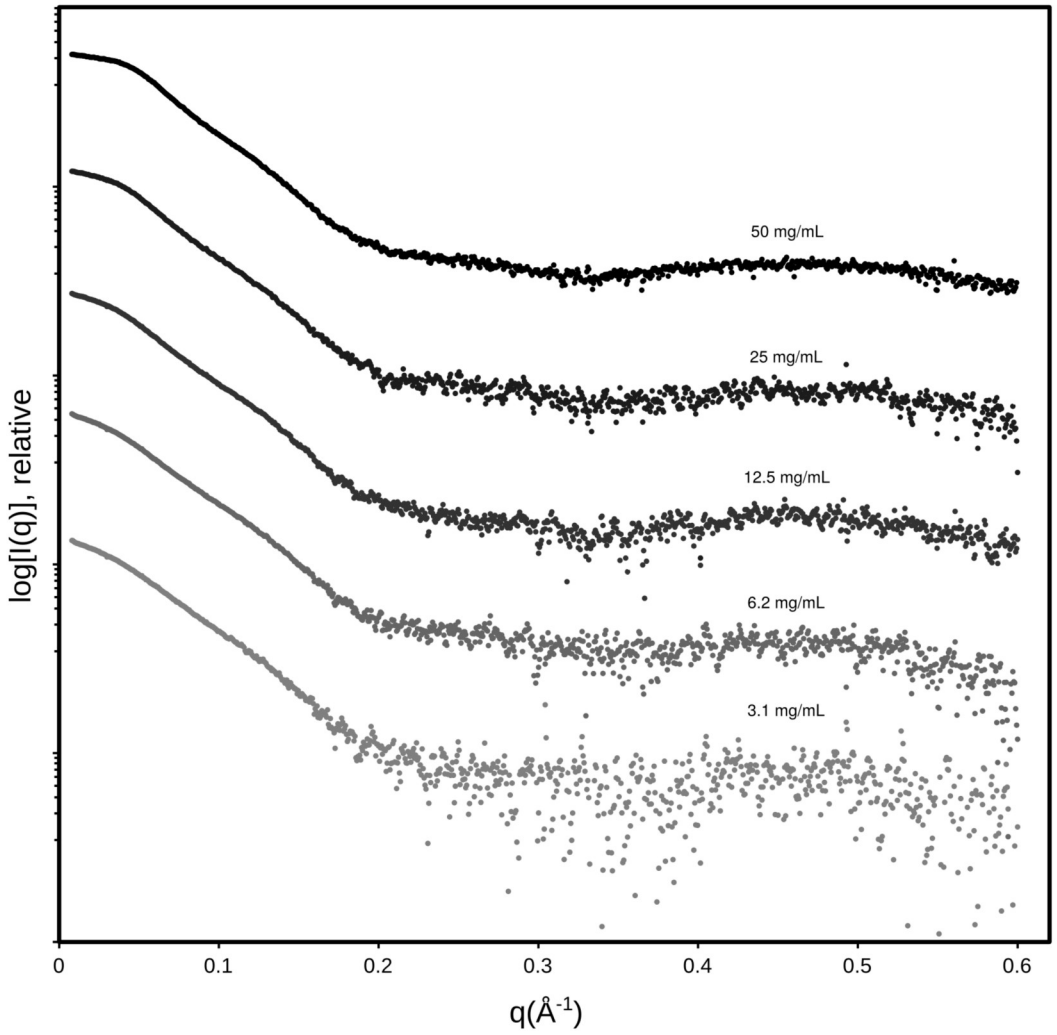

Figure 2.1: SAXS scattering profile of AAC(6')-Ie/APH(2'')-Ia

Dilution series of scattering profiles from AAC(6')-Ie/APH(2")-Ia. SAXS scattering was measured for solutions of 50-3.12 mg/mL protein concentration. Measured scattering for these samples was in turn corrected for buffer scattering and normalized. Exposures of samples were 30 minutes (50 mg/mL), 60 minutes (25 mg/mL), 120 minutes (12.5 mg/mL), 240 minutes (6.2 mg/mL) and 720 minutes (3.1 mg/mL).

2.2.4 Substrate dialysis and SAXS analysis

NHis-AAC(6')-Ie/APH(2'')-Ia was prepared at 10 mg/mL and incubated with various substrates individually and in combination. These substrates were prepared in the sample buffer, and allowed to equilibrate across a 30 kDa MWCO dialysis membrane using the caps of plastic laboratory tubes as the vessel and the collar of the tube as a clamp. The protein was placed inside this vessel, and equilibrated with buffer containing respective ligand molecules. Dialysis was carried out for 24-48 hours at 4˚C.

These samples were subjected to 30-minute exposures in the SAXSess capillary, with accompanying buffer samples, also subjected to the same amount of scattering. Buffer-subtracted scattering profiles of the enzyme were analyzed by Guinier transformation to measure the R_G of respective ligand-soaked protein samples .

2.2.5 Structural modelling of the AAC(6')-Ie/APH(2'')-Ia particle

The processed scattering curve was used as input for the GASBOR *ab initio* modelling algorithm (Svergun et al., 2001), which calculates scattering envelopes using simulated annealing from an initial randomly displaced ensemble of atoms. This algorithm was run 50 times to generate multiple independent models. These models showed good agreement, and were averaged using DAMAVER (Volkov and Svergun, 2003) to obtain a single, averaged *ab initio* model of AAC(6')-Ie/APH(2'')-Ia.

Structures of homologous enzymes APH(2'')-IIa (Young et al., 2009) and AAC(6')-Ib (Vetting et al., 2008) were selected to construct homology models of AAC(6')-Ie/APH(2'')-Ia. Crystal structures of these enzymes (PDB 3UZR and 1V0C, respectively) were used to generate homology models of the domains in AAC(6')-Ie/APH(2'')-Ia in MODELLER version 9v4 (Fiser and Sali, 2003). These homology models of AAC(6')-Ie and APH(2'')-Ia were combined with a 17-residue linker of residues omitted from both homology models. This model was refined 20 times against the scattering profile of AAC(6')-Ie/APH(2'')-Ia using the program BUNCH. These models all refined against the SAXS scattering profile with χ^2 between 1.1 and 1.4.

2.3 Results

2.3.1 Small-angle X-ray scattering of AAC(6')-Ie/APH(2'')-Ia

The AAC(6')-Ie/APH(2'')-Ia enzyme proved to be an excellent subject for SAXS analysis. The enzyme tolerates concentrations up to 50 mg/mL for extended periods of time and along with 5% glycerol and reducing agents like DTT or TCEP, tolerated the exposure to X-ray with no apparent degradation. SAXS is notoriously sensitive to aggregation effects, and so the experiments were repeated multiple times under different conditions, with a reproducible scattering profile.

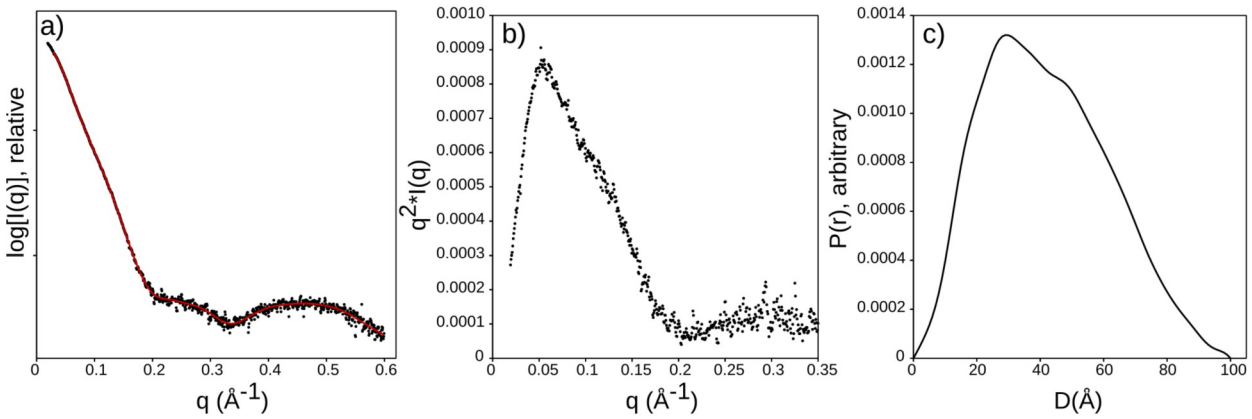

***Figure 2.2*: Transformations of AAC(6')-Ie/APH(2'')-Ia scattering profile**

a) Merged concentration-independent scattering profile of AAC(6')-Ie/APH(2'')-Ia and fit of curve using truncated Fourier operation (red). b) Kratky transformation of scattering profile of AAC(6')-Ie/APH(2'')-Ia. c) P(r) plot representing the Fourier transformation of the SAXS profile, with a specified maximum dimension (D_{max}) of 100 Å.

A dilution series of the protein from 50 mg/mL by half down to 3.1 mg/mL was used to correct for concentration-dependent effects in the SAXS scattering profile of the protein. The merged scattering profile for the protein exhibits features to the detection limit of the instrument at 0.6 $Å^{-1}$, but also shows structure in the low-q regime, which is most useful for analyzing the structure of proteins (Figure 2.2a). The Kratky transformation of the scattering profile drops to near zero at q = 0.2, indicating a folded, rigid particle (Figure 2.2b). Beyond this value there is

some increase, which could indicate some residual flexibility, or the scattering impact of flexible regions such as the histidine-tag in the disordered N-terminus of the protein.

Guinier analysis of the protein indicated an R_G of 32 Å for the bifunctional particle, while Fourier transformation of the data using GNOM (Svergun, 1992) returns an R_G of 31.6 Å, with a maximum dimension of 100 Å. The P(r) distribution determined by this method reveals a bi-modal distribution, indicative of a rigid two-domain protein (Figure 2.2c).

2.3.2 *ab initio* model of the AAC(6')-Ie/APH(2'')-Ia particle

Following establishment of the rigid character of the AAC(6')-Ie/APH(2'')-Ia particle, it was possible to undertake *ab initio* modelling of the protein. 50 models of the particle were generated with the GASBOR simulated annealing algorithm, which converged on a bi-lobal particle with some asymmetry. Aligning and averaging of multiple models in the DAMAVER suite showed that most models independently generated from the data were consistent (one model of the 50 was excluded from further analysis by DAMAVER). Averaging of these models generated a single model that more closely reflected the true character of the protein.

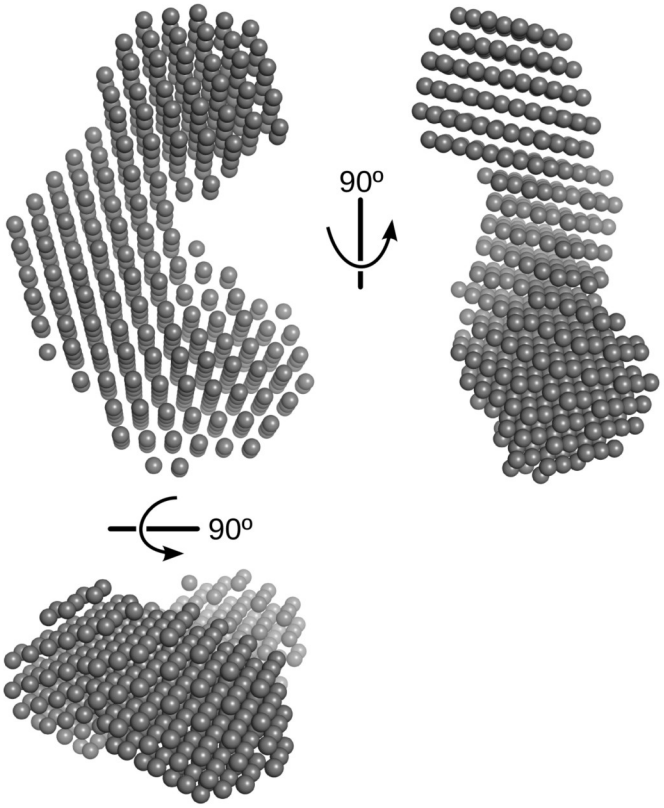

***Figure 2.3*: *ab initio* model of AAC(6')-Ie/APH(2'')-Ia**

Averaged envelope of 49 independently determined *ab initio* envelopes generated in GASBOR, averaged with default parameters in DAMAVER.

2.3.3 Homology modelling and rigid-body fitting of AAC(6')-Ie and APH(2'')-Ia models

The homologous AAC enzyme AAC(6')-Ib and homologous APH APH(2'')-IIa were used to construct homology models of AAC(6')-Ie and APH(2'')-Ia using MODELLER. Residues that could not be unambiguously assigned to homologous residues in AAC(6')-Ib or APH(2'')-IIa near the interface of the domains were omitted, leaving 17 residues of unmodelled “linker”. This starter model was used to run independent modelling runs in the program BUNCH (Petoukhov and Svergun, 2005). 20 models were produced by this method, 19 of which superimpose with normalized spatial discrepancy (NSD) of 0.9-1.1, and one model rejected. Of the remaining

models, the majority showed a conserved form, placing the AAC(6')-Ie domain directly against the N-terminus of the APH(2")-Ia domain (Figure 2.4). However, there are multiple equivalently consistent orientations of the AAC(6')-Ie domain in these structures. These orientations could not be resolved, leaving some speculation as to the relative spatial arrangement of the AAC(6')-Ie and APH(2")-Ia domains.

The rigid-body models of AAC(6')-Ie/APH(2")-Ia superimpose well with the *ab initio* models of the enzyme, providing good agreement between methods (Figure 2.5). While I cannot present an unambiguous arrangement of the two domains in the full-length AAC(6')-Ie/APH(2")-Ia particle, there are some global features of the enzyme that can be remarked upon. First, evident from qualitative inspection of transformation plots of the raw scattering data: the particle is rigid. This observation is important because the genetic and molecular context of AAC(6')-Ie/APH(2")-Ia does not require a rigid structure for the enzyme's function. A rigid particle implies additional function above and beyond expressing two activities in a single polypeptide.

Secondly, the active sites of AAC(6')-Ie and APH(2")-Ia are not directly connected to each other in a fashion that would allow direct transfer of substrates. None of the rigid-body models calculated by BUNCH placed the active sites of the enzymes in contact. This means that there can be no direct channelling of substrate between the domains, although it does not rule out this channelling through more indirect mechanisms.

Lastly, the AAC(6')-Ie domain is held close to the N-terminal region of the APH(2")-Ia domain. This region of the protein contains important loops that play a regulatory role in the enzyme activity (more in Chapter 4) and could provide a means by which the two domain activities could influence each other, overtly or subtly.

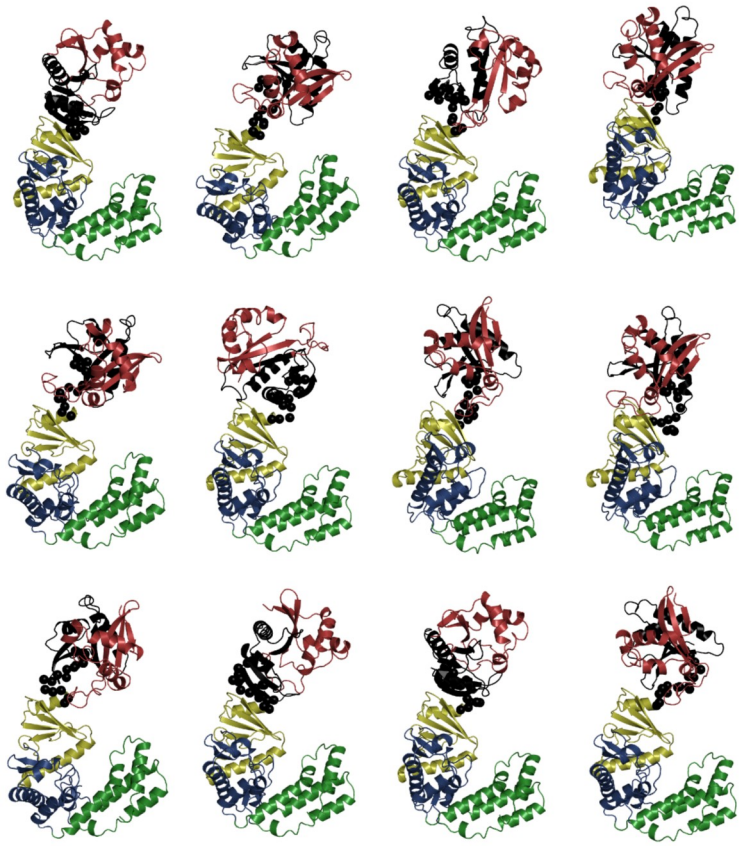

***Figure 2.4*: Selected rigid-body models refined against scattering data for AAC(6')-Ie/APH(2'')-Ia**

In these models, the AAC(6')-Ie domain (maroon, black) is refined to a similar position adjacent to the N-terminal lobe of the APH(2")-Ia domain. While the orientation of the AAC domain is not ambiguously determined, the location of the domains relative to each other is conserved.

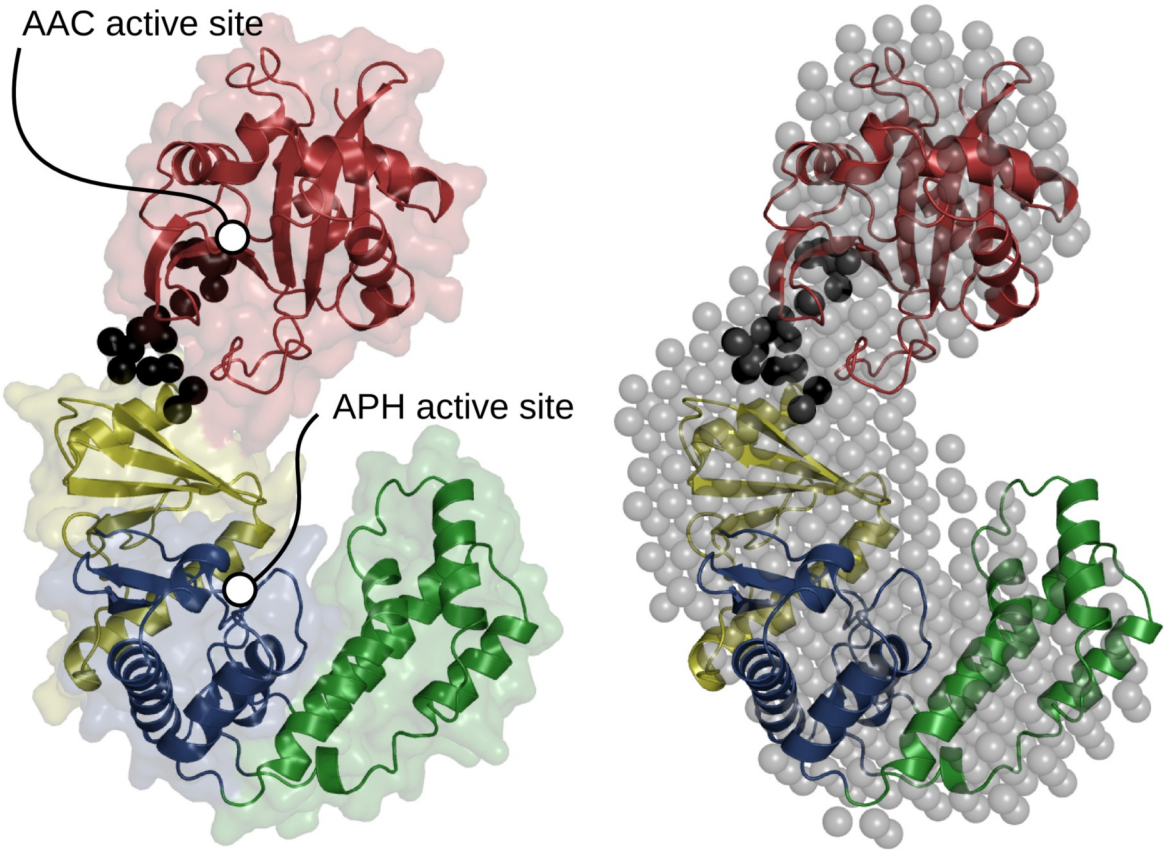

***Figure 2.5*: Rigid body model of AAC(6')-Ie/APH(2'')-Ia**

A selected rigid-body model of the enzyme determined using BUNCH is illustrated with the AAC domain (maroon) connected by a linker (black spheres) to the APH(2")-Ia domain (yellow, blue, green). This model superimposes extremely well with the independently determined *ab initio* model of the protein (gray spheres).

2.3.4 Introduction of co-substrates to the enzyme generate small compaction, limited structural changes

In addition to modelling and determination of low-resolution structural models of AAC(6')-Ie/APH(2")-Ia, a series of substrate incubation experiments were also carried out in order to assess the structural impact of substrate binding to the protein. These scattering experiments were all carried out at 10 mg/mL, which allowed for the collection of many samples with minimal SAXS beam time, but carried some inter-particle effects, precluding any structural modelling of the particles.

Comparison of the apo protein with that of protein bound with coenzyme A, acetyl-coenzyme A, guanosine-β,γ-imidodiphosphate (GMPPNP), and GDP, showed that all of these ligands induced minor change in the protein. This is evident in the nearly-coincident scattering curves (Figure 2.6). Determination of the radius of gyration for these samples indicated that each exhibited small compactions of the particle, indicating either a physical change of size of the whole particle, or a reduction in flexibility of individual domains, resulting in a smaller apparent size as measured by R_G.

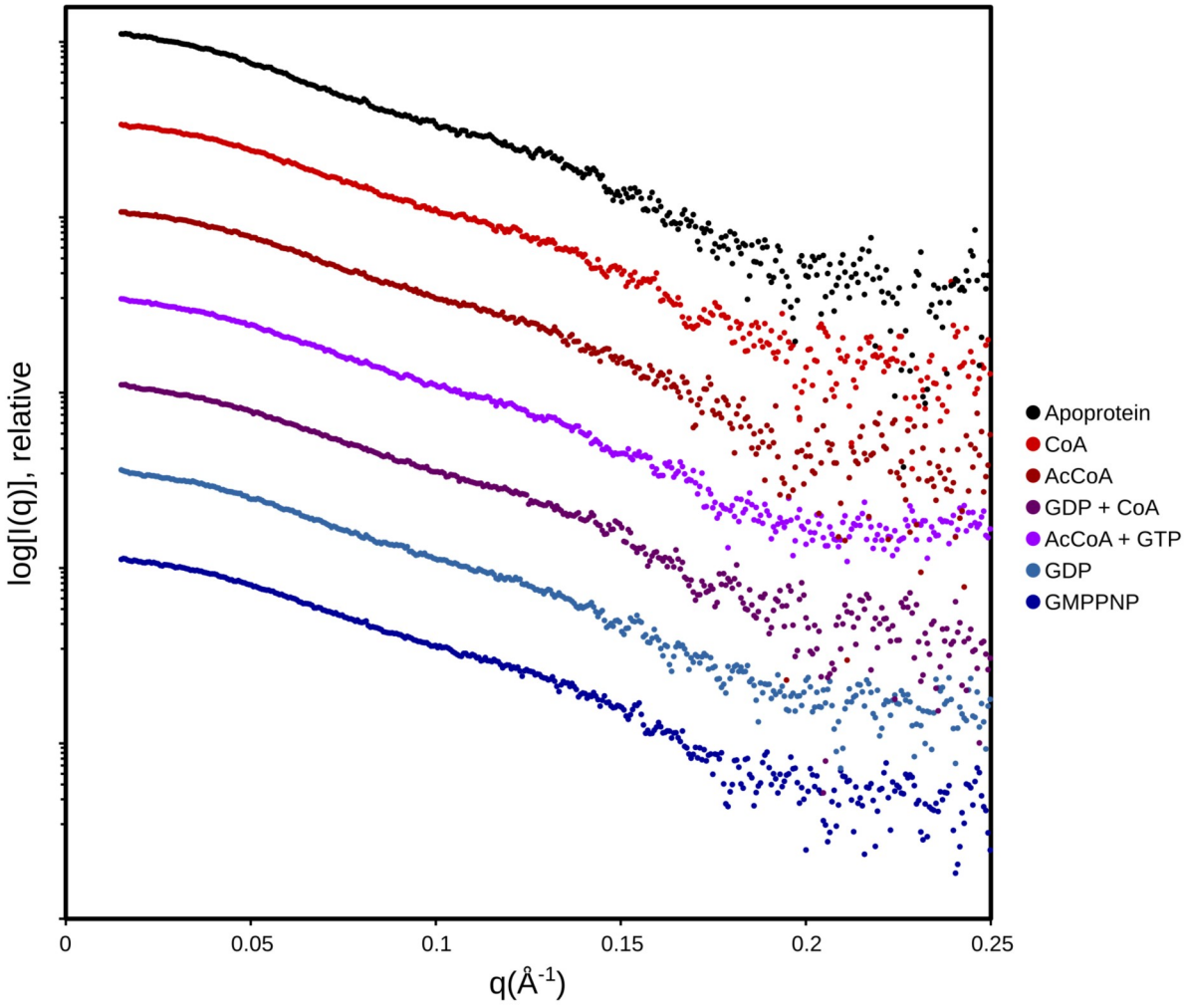

***Figure 2.6*: Scattering profiles of AAC(6')-Ie/APH(2'')-Ia in complex with donor substrates and reaction products**

Addition of the aminoglycoside kanamycin showed an increase in apparent particle size, which could be due to intra-particle effects like a change in structure or flexibility, or an increase in inter-particle effects such as multimerization or aggregation. Some turbidity was observed when preparing AAC(6')-Ie/APH(2")-Ia + aminoglycoside samples for other experiments, but the protein solution loaded into the SAXS instrument was always visibly clear.

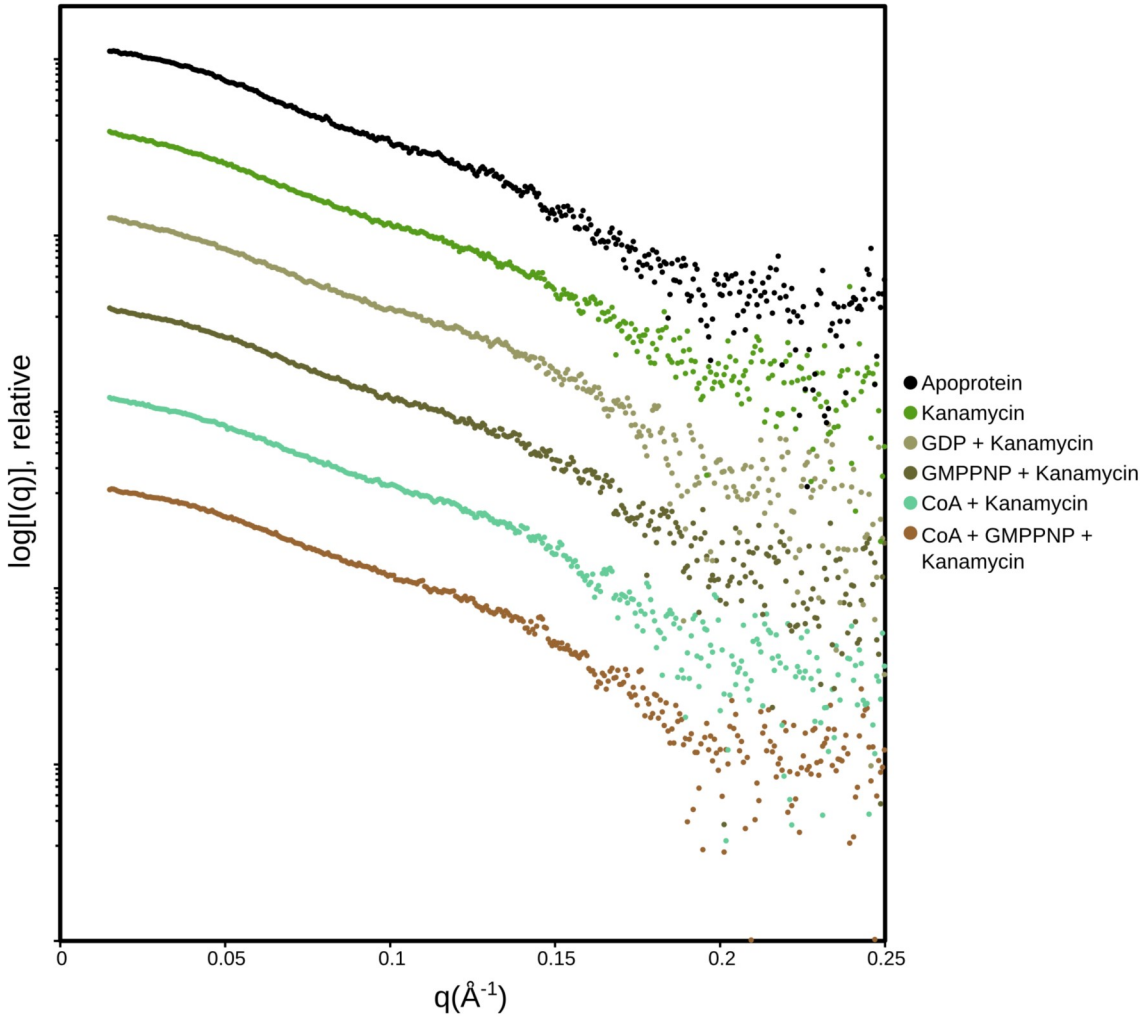

***Figure 2.7*: Scattering profiles of AAC(6')-Ie/APH(2'')-Ia in complex with kanamycin and donor substrates**

Very interestingly, co-incubation with CoA, AcCoA, or GMPPNP blocked this change in apparent R_G increase. This observation indicates that the change in scattering profile from kanamycin involves interaction of kanamycin with the protein specifically, in a manner that is cancelled out by the binding of co-substrates. GDP did not prevent this change, further suggesting that this change in the protein depends on the nature of the bound co-substrate ligand. This effect certainly merits further investigation, especially given the role of nucleoside in apparent co-operative behaviour in the enzyme (Chapter 4).

Content	**Apparent R_G (Å)**
AAC(6')-Ie/APH(2")-Ia (Apoprotein)	30.2 ± 0.2
+ Coenzyme A	29.4 ± 0.2
+ Acetyl-CoA	29.0 ± 0.2
+ GDP (Mg)	29.0 ± 0.2
+ GMPPNP (Mg)	29.4 ± 0.2
+ GDP (Mg) and coenzyme A	29.1 ± 0.4
+ GDP (Mg) and acetyl-CoA	28.6 ± 0.3
+ Kanamycin	33.9 ± 0.2
+ Kanamycin and GDP	33.3 ± 0.2
+ Kanamycin and GMPPNP	30.5 ± 0.2
+ Kanamycin and coenzyme A	30.6 ± 0.2
+ Kanamycin, GDP (Mg) and coenzyme A	30.1 ± 0.2
+ Kanamycin, GMPPNP (Mg) and coenzyme A	29.4 ± 0.2

Table 2.1: Apparent R_G calculated from SAXS scattering curves of AAC(6')-Ie/APH(2")-Ia in complex with various ligands

R_G determined by Guinier analysis of the processed scattering curve for each sample. Errors represent uncertainty in Guinier transformation as reported by SAXSQuant analysis package.

2.4 Discussion

2.4.1 AAC(6')-Ie/APH(2'')-Ia has a rigid solution structure

The scattering patterns determined from solutions of AAC(6')-Ie/APH(2'')-Ia showed that the protein exhibits limited flexibility in solution. With this established, I modelled the three-dimensional structure of the protein using both *ab initio* and rigid-body modelling methods. These methods both converged on a consistent shape, resembling a bent peanut or butternut squash. This model places the AAC(6')-Ie domain directly against the N-terminus of the APH(2'')-Ia domain. Unfortunately, I cannot estimate the relative orientation of the AAC(6')-Ie domain with much confidence. Multiple consistent rigid-body models of the full-length enzyme were generated, all with equivalent overall shape, but distinct AAC domain orientation.

Many of the aminoglycoside substrates for AAC(6')-Ie/APH(2'')-Ia can bind to both enzyme domains. A potential model of the protein would place the AAC(6')-Ie and APH(2'')-Ia domains in an orientation where their respective active sites could come together to form a convergent, double-active site where an aminoglycoside can potentially bind to both. Our models rule out this possibility, in order for the protein to generate a model consistent with the SAXS scattering, there must be some spatial separation of these active sites.

2.4.2 Ligand binding drives modest compaction of the protein

AAC(6')-Ie/APH(2'')-Ia shows a reduced size when substrates are added, but the change is limited. The modest shrinkage of R_G is easily explained by restriction of local flexibility upon ligand binding. With this being the case, what does this tell us about the enzyme itself?

The addition of ligands to AAC(6')-Ie/APH(2'')-Ia, like many other particles, leads to compaction and induced fit, but unfortunately, SAXS only gives us a very high-level view of this change. This modest change is easily explained by binding to the enzyme driving compaction of loops and reduced flexibility of the respective domains. This observation tells us that the enzyme remains relatively rigid in all states, and there is no dramatic structural rearrangements or order-disorder transitions that would imply functional transition in the enzyme.

This modest compaction of the enzyme on coenzyme A or GMPPNP binding is consistent with some early studies on the enzyme. These studies demonstrated that binding of GTP to the protein drove thermal protection of the enzyme (Martel et al., 1983), so the binding of ligand drives a thermodynamic tolerance in the enzyme that protects it from denaturation. This aligns well with our findings of a rigid particle that has structural transitions that feed from one domain to the other.

2.4.3 Adaptation and development of a AAC(6')-Ie/APH(2'')-Ia interface

Interfaces in bi-domain proteins are typically smaller than that of dimeric proteins, which is thought to be a results of proximity effects requiring less specific and weaker interactions to form a stable and functional unit (Jones et al., 2000). So, the arrangement of AAC(6')-Ie and APH(2'')-Ia domains in the bifunctional particle may not necessarily be extensive to still generate a stable and rigid particle.

The structural model of AAC(6')-Ie/APH(2'')-Ia presented has been corroborated by researchers who modelled the full-length protein using crystal structures of the individual AAC(6')-Ie and APH(2'')-Ia domains (Smith et al., 2014). These researchers identified a single orientation of the AAC(6')-Ie domain, although further studies are still needed to validate this model with different techniques. The rigid interface between domains is still present in this model, which actually places the domains even closer together with smaller R_G and D_{max}, recapitulating our model but even more compact. This rigid interface between domains is important to our understanding of the evolution of function in AAC(6')-Ie/APH(2'')-Ia.

The packing of two domains directly adjacent to each other in a single rigid particle influences our understanding of the development of this protein as a bifunctional enzyme. Placement of two domains immediately next to each other in a fusion protein generates clashes and incompatibility, the adaptation to form a stable interface takes evolutionary time to develop. Most fusion proteins form through genetic accident, then adapt over time to accommodate the change. Formation of a rigid particle in AAC(6')-Ie/APH(2'')-Ia implies this adaptation to form a stable interface. The adaptation of two monomeric enzymes into a functional particle would not happen spontaneously, but instead would be the process of successive rounds of selection

towards an interface. It is unlikely that this adaptive evolution could have occurred within the 20 years between clinical use of aminoglycosides and the identification of AAC(6')-Ie/APH(2")-Ia in resistant microbial strains. This resistance factor almost certainly must have developed as a bifunctional particle long before human use of aminoglycosides, as an optimized form of antibiotic resistance. The selective forces driving this association must have acted on this protein for a very long time, driving selection toward a functional rigid interface in the protein.

2.4.4 Emergent function in a bifunctional protein

An intriguing implication of the rigid interaction observed is that it implies a selective evolutionary force to form the rigid interface. What could this selective force be? We can look to dimeric aminoglycoside-modifying enzymes for ideas. In these cases the interaction between domains can be dynamically assembled (Wright and Serpersu, 2005), or in other cases involved in complicated allosteric regulation mechanisms (Freiburger et al., 2011). The multiple aminoglycoside-modifying activities in these proteins serve to regulate each other. It is possible similar effects go on in this bifunctional protein. AAC(6')-Ie/APH(2")-Ia could exhibit some form of allostery where the two proteins affect the activity of the opposite domain. In this case, binding of substrate to one domain could affect the activity of the other enzymatic domain.

Another possibility is the production of a collaboratively productive electrostatic field to more efficiently bind aminoglycoside compounds. Aminoglycosides are very positively charged compounds (Blagbrough et al., 2011), while the active sites of aminoglycoside-modifying enzymes are very negatively charged in order to bind these compounds efficiently (Romanowska et al., 2013; Thompson et al., 1999). Aminoglycosides are also unique antibiotics in that they exert their effect non-stoichiometrically, and the most important step in an antibiotic resistance enzyme is to bind the antibiotic and remove it from solution (see section 1.3.5). With two negatively charged active sites in a single bifunctional enzyme, constructive electrostatic interactions could combine to increase the binding capacity of the bifunctional protein compared to either domain alone, and even provide some limited resistance through antibiotic binding alone, with enzymatic activity as secondary function.

It has been suggested that for active enzymes, the most important function of the enzyme is apprehension of the antibiotic over its modification (Gates and Northrop, 1988a). A possible explanation of the rigid character of AAC(6')-Ie/APH(2")-Ia is that by fusing two aminoglycoside binding enzymes into a single polypeptide, the binding of aminoglycoside, and its removal from solution, is greatly enhanced. The model reported by Smith, et al., (Smith et al., 2014) challenges the potential for the enzyme to surround a single negatively-charged binding cavity, but the co-operative electrostatic effects in aminoglycoside binding could indeed still exist.

A third possibility is that the rigid association of domains allows for passage of product from one domain to another, in a form of substrate channelling. The combination of two such domains in a rigid particle could lead to more efficient aminoglycoside binding and possibly the passage of substrate from one domain to the other in a type of “electrostatic highway” like that seen in some other bifunctional enzymes (Knighton et al., 1994; Stroud, 1994). Co-operative electrostatic effects could then ensure the aminoglycoside does not leave the enzyme, even if it is not effectively modified by one domain and requires the other activity for inactivation.

2.4.5 Does AAC(6')-Ie/APH(2")-Ia predict a common characteristic of bifunctional AMEs?

While AAC(6')-Ie/APH(2")-Ia exhibits a rigid arrangement of domains, other bifunctional aminoglycoside-modifying enzymes may or may not adopt the same structural arrangement. The other bifunctional aminoglycoside-modifying enzymes are not studied as thoroughly as AAC(6')-Ie/APH(2")-Ia, but sequence analysis allows us to compare them to other homologous enzymes, and we can observe that these enzymes typically show much higher identity to monomeric enzymes, suggesting these gene fusions may have formed more recently.

The only other bifunctional enzyme to be tested in a similar way is the bifunctional ANT(3")-Ii/AAC(6')-IId enzyme. In this enzyme, in contrast to AAC(6')-Ie/APH(2")-Ia, researchers found that the enzymes could be cleanly dissected with almost identical activity recovered (Green and Garneau-Tsodikova, 2013). Clearly more work is needed to better understand the multiple bifunctional aminoglycoside-modifying enzymes and the forces shaping

their evolution and development, and whether consistent or divergent forces are involved in bifunctionality in these proteins.

2.5 Conclusions

SAXS analysis of AAC(6')-Ie/APH(2")-Ia indicates that the protein is a rigid particle, with a tight association of AAC(6')-Ie and APH(2")-Ia domains. The scattering profile of the protein indicates a single conformation, while R_G and D_{max} estimates from this data are compatible with a rigid model of the protein. Rigid-body models of the enzyme leave too little space between domains for a stable flexible arrangement compatible with the protein's X-ray scattering profile, and suggest an interface between domains that facilitates this rigid packing arrangement.

Because these domains are packed closely together, they must have co-evolved to form a rigid particle. This structural association requires that the domains have developed in the presence of each other over a long time period to offset the negative consequences of this close association. This implies an ancient origin for domain fusion in this protein.

The arrangement of domains in AAC(6')-Ie/APH(2")-Ia implies an evolutionary benefit that has selected for a stable interface over the flexible linkage that would result from simple fusion. This benefit might be by one of several mechanisms, including co-operativity, constructive electrostatic effects, or sequestration and tight binding of the antibiotics. Interactions between domains in this rigid particle could facilitate co-operative interactions between the domains or other beneficial features of bifunctionality in the protein.

Incubation of the enzyme with substrates induces modest compaction, consistent with ligand binding that brings greater order to the protein, but does not produce dramatic structural changes that can be observed in SAXS scattering profiles. The possible exception is the aminoglycoside substrate, where the scattering profile indicates either a structural change or inter-particle interactions. Binding of co-substrates for either domain prevent this effect, indicating both that it is a change brought on by aminoglycoside action, and that there is some cross-talk between domains of the bifunctional protein.

These findings collectively indicate that this protein formed by fusion long before the advent of antibiotic use to treat bacterial infections. The co-evolution of two enzymatic domains to form a rigid arrangement indicates that a selective pressure drove the formation of a rigid interaction between these domains. The selective pressure to do so is not clear, but could be

improved catalytic activity through regulatory mechanisms, improved binding through co-operative electrostatic effects, or the combination of binding modules in nearby domains to more efficiently remove aminoglycoside from solution. Study of unrelated bifunctional enzymes may help to determine what common features drive the development of bifunctionality in aminoglycoside resistance.

3 APH(2'')-Ia binds the neamine nucleus of aminoglycosides of 4,5- and 4,6-disubstituted aminoglycosides

3.1 Background

The most problematic aminoglycoside-modifying enzymes are broad profile enzymes that act upon and inactivate both 4,5-disubstituted and 4,6-disubstituted aminoglyosides. AAC(6')-Ie/APH(2")-Ia is one of these enzymes, unique for its broadly active AAC(6')-Ie acetyltransferase domain, and the APH(2")-Ia phosphotransferase domain that expands the substrate range beyond the compounds inactivated by AAC(6')-Ie. Most aminoglycosides that do not bind to the AAC(6')-Ie domain are bound and modified by the APH(2")-Ia domain, and *vice versa*. This makes the protein a powerful resistance factor because it fuses two enzymes with remarkably broad substrate spectra.

In contrast to the AAC(6')-Ie domain which has well-defined range of substrates, there are uncertainties in the aminoglycoside activity of APH(2")-Ia. The bifunctional enzyme was identified as a factor that conferred resistance to the 4,6-disubstituted aminoglycoside gentamicin, although it also carries activity toward other compounds. It is active against most 4,6-disubstituted aminoglycosides, but its activity toward 4,5-disubstituted compounds has been subject to conflicting reports. Without a high-resolution structure of the enzyme, the binding mode of these compounds could not be unambiguously defined.

3.1.1 Broad and narrow-profile aminoglycoside-modifying enzymes

As discussed in section 1.3.3, the 4,5- and 4,6-disubstituted aminoglycoside groups share a common nucleus of two rings that are critical to binding of the compounds to their site of action in the ribosome. This neamine nucleus of the compounds is the minimal functional unit of these aminoglycosides. Additional rings linked at the 5- or 6-positions of neamine form the 4,5-disubstituted and 4,6-disubstituted classes of compounds. These additional rings change their properties, and also alter their susceptibility to resistance through interactions of these rings with resistance factors. These rings can alter steric interactions and change binding to resistance factors, or they can add or remove modifiable groups altogether.

In a comprehensive survey of aminoglycoside resistance enzymes, Ramirez and Tolmasky catalogued the known enzymes that act on aminoglycosides (Ramirez and Tolmasky, 2010). These enzymes vary from specific enzymes that act on only a subfamily of aminoglycosides or a single compound, to those that act on nearly all 4,5- and 4,6-disubstituted aminoglycosides (Davies and Wright, 1997). The distinction between broad and narrow profile enzymes is difficult to predict, as even closely related enzymes can be different in their substrate binding propensities (Norris and Serpersu, 2013).

Broad-profile aminoglycoside modifying enzymes that act on both groups are overrepresented in clinical isolates, likely due to their success in spreading and persevering in the presence of many compounds. These enzymes bind and modify both the 4,5-disubstituted compounds and the 4,6-disubstituted compounds, typically on the shared 4-aminohexose ring. These enzymes tend to be found in clinical resistance settings, where their broad resistance profile allows them to spread with minimal restrictions.

In addition to these extremes, other enzymes are intermediate between narrow and broad profile. Enzymes with high activity toward some compounds can have low activity toward others, providing strong resistance toward some compounds and intermediate resistance toward others. The properties and breadth of an aminoglycoside-modifying enzyme are dictated both by its ability to bind compounds, and the ability to modify aminoglycosides once bound. Structural features of enzymes govern both of these steps.

3.1.2 Aminoglycoside-macromolecule interactions

Aminoglycoside compounds have 4 to 7 amino groups and carry many positive charges as a result (Clouet-d'Orval et al., 1995; Szczepanik et al., 2002). Correspondingly, they interact with negatively-charged target sites in nucleic acids (Cho and Rando, 1999; Wang and Tor, 1997), negatively-charged binding sites in proteins (Matesanz et al., 2012; Thompson et al., 1999) and even negatively-charged membrane surfaces (Brasseur et al., 1984). In addition to charge interactions, aromatic rings also play important roles in the recognition and binding of aminoglycosides (Vacas et al., 2010). These interactions rely on the negatively-charged electron cloud of the aromatics, and also the positive charge and polarized C-H bonds of the 2-deoxystreptamine ring.

Binding of aminoglycosides to their ribosomal site of action has been shown to conserve the binding of the 2-deoxystreptamine and 6-linked aminohexose rings (François et al., 2005; Kondo et al., 2006), which in turn interfere with the ribosomal decoding process (Demeshkina et al., 2012; Pape et al., 2000). This mode of interaction with the ribosome even remains consistent with the atypical aminoglycoside apramycin (Han et al., 2005), which contains no 6-linked ring and unique substituents linked to the 5-position of 2-deoxystreptamine. The binding of aminoglycosides to the ribosome depend on this 2-deoxystreptamine ring and its interactions at the ribosomal decoding site.

4,5- and 4,6-disubstituted aminoglycosides bind the decoding site in equivalent fashion, conserving interactions with the neamine rings of the compounds (Figure 3.1a). This conserved binding mode of the 2-DOS and 6-aminohexose rings forms a minimal active unit of the compounds, and many of the most effective aminoglycosides are all based upon this neamine scaffold. The 5- or 6-linked rings of these compounds are accommodated and make additional contacts in the ribosomal site (François et al., 2005; Kulik et al., 2015; Vicens and Westhof, 2003). Differences in these rings lead to subfamily-specific properties such as secondary binding effects (Borovinskaya et al., 2007b). They also dramatically influence the susceptibility of modification of these compounds by antibiotic resistance enzymes.

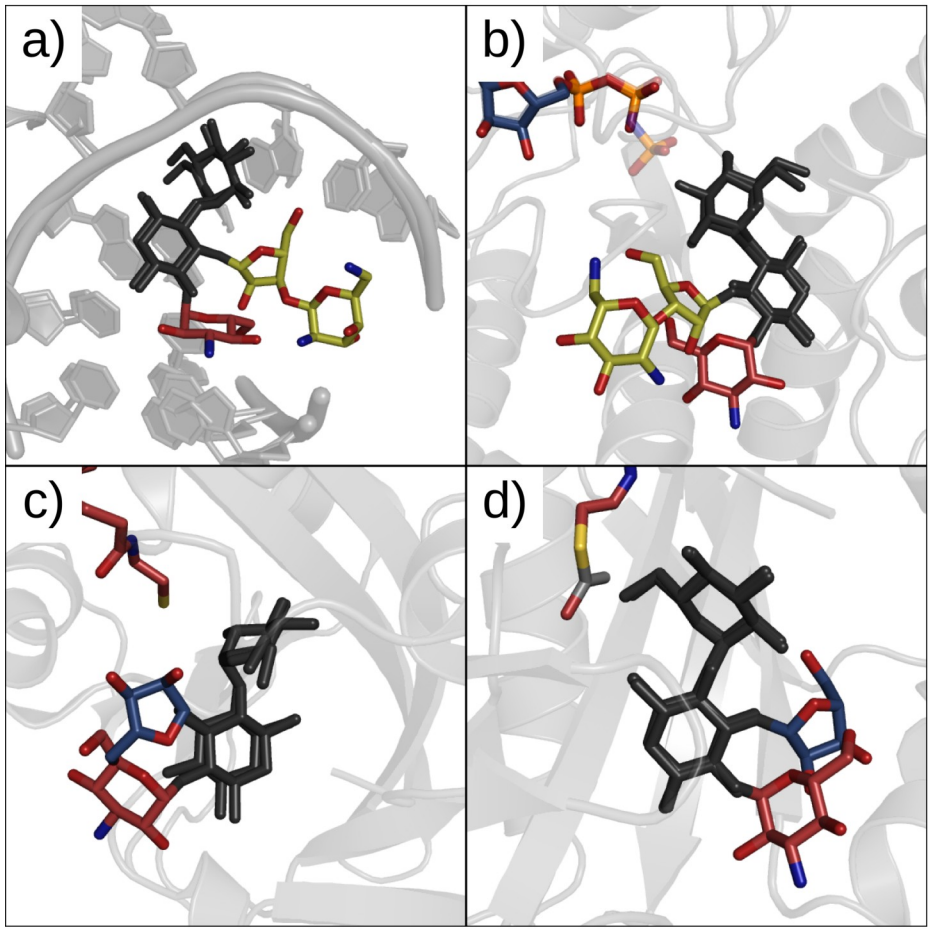

***Figure 3.1*: Binding of 4,5- and 4,6-disubstituted aminoglycosides to macromolecular sites**

a) Binding of 4,6-disubstituted kanamycin A (maroon) and 4,5-disubstituted neomycin B (yellow) to the ribosomal decoding centre. Models from PDB 2ESI and 2ET4 (François et al., 2005). b) Phosphotransferase APH(3')-IIIa in complex with nucleoside donor substrates, kanamycin A and neomycin B. Models are PDB 1L8T and 2B0Q (Fong and Berghuis, 2002) with superimposed triphosphate substrate from PDB 1J7U (Burk et al., 2001). c) Binding of kanamycin A and 4,5-disubstituted ribostamycin (blue) to AAC(2')-Ic in complex with coenzyme A. Models from PDB 1M4I and 1M4G (Vetting et al., 2002). d) The acetyltransferase AAC(6')-Ib in complex with kanamycin C and ribostamycin. Models are PBD 1V0C and 2BUE (Vetting et al., 2008).

3.1.3 Target mimicry in aminoglycoside resistance enzymes

Comprehensive structural studies have been conducted on several broad-profile aminoglycoside-modifying enzymes that bind and modify 4,5- and 4,6-disubstituted aminoglycosides. These enzymes have independent evolutionary origins, with different chemical activities and function (Section 1.4.4). However, they have converged on some common features in their modes of interaction with aminoglycoside antibiotics. These enzymes exhibit a degree of "target mimicry" (Fong and Berghuis, 2002) where they form an active binding pocket that resembles the aminoglycoside binding site in the microbial ribosome.

These enzymes, like the ribosome, selectively interact with the neamine-based rings of the aminoglycosides, while accommodating interactions with the remaining rings. While they have converged on strategy, they diverge in the means by which they achieve it (Wright, 2003). In a textbook example of molecular promiscuity (Copley, 2003), APH(3')-IIIa binds both groups of compounds using a loop that facilitates binding to both 4,5-disubstituted and 4,6-disubstituted aminoglycosides (Fong and Berghuis, 2002). The result is that both compounds are held in position where the 3' group can be modified by an appropriately positioned γ-phosphate of ATP (Figure 3.1b).

Aminoglycoside acetyltransferases AAC(6')-Ib and AAC(2')-Ic also bind both of these families of aminoglycoside, although differences in their site of action require that they bind the aminoglycosides in a different orientation (Figure 3.1c, d). Like in binding to APH(3')-IIIa, the neamine rings of the aminoglycosides superimpose while the additional rings are accommodated. In AAC(2')-Ic the antibiotics make very few contacts with the protein itself, instead mediating most binding interactions through ordered water molecules (Vetting et al., 2002). In AAC(6')-Ib, the neamine rings bind a conserved platform of aromatic side chains, while the additional rings are more loosely held (Vetting et al., 2008).

Not all aminoglycoside-modifying enzymes adopt this strategy. Some bind a single compound specifically, like the spectinomycin phosphotransferase APH(9)-Ia (Fong et al., 2010). Others, like the nucleotidyltransferase ANT(4')-Ia has been crystallized with 4,6-disubstituted kanamycin, but also shows activity at alternative sites on 4,6-disubstituted compounds, and also shows activity toward the 4,5-disubstituted aminoglycoside neomycin (Jing and Serpersu, 2014).

The possibility of alternative binding modes and other means of binding aminoglycosides will be discussed in more detail in Chapter 5.

The most prominent aminoglycoside resistance enzymes confer resistance by binding the conserved neamine rings of 4,5- and 4,6-disubstituted aminoglycosides. This convergence toward binding of the mechanistically important neamine group ensures that modifications to aminoglycosides do not easily escape resistance from the enzymes, as the elements necessary to bind the ribosome are also selected by the resistance enzyme. An interesting question arises when we then look at a resistance enzyme that modifies a location outside the neamine core of aminoglycosides. In the nucleotidyltransferase ANT(2")-Ia, 4,6-disubstituted aminoglycosides are bound selectively, without a means of binding 4,5-disubstituted compounds (Bassenden et al., 2016). APH(2")-Ia is another enzyme which acts on a non-conserved region of aminoglycosides, how does it interact with neamine-based aminoglycosides?

3.1.4 The aminoglycoside binding range of APH(2")-Ia

The APH(2")-Ia domain of AAC(6')-Ie/APH(2")-Ia has a curious relationship toward aminoglycoside substrates. Originally characterized as a domain for gentamicin resistance, the domain is active against other 4,6-disubstituted aminoglycosides like tobramycin and kanamycin. At the same time, there has been evidence of interaction of the enzyme with 4,5-disubstituted compounds (Daigle et al., 1999a). Measured interactions with 4,5-disubstituted aminoglycosides are puzzling because the 2"-OH group that the enzyme modifies on gentamicin and others has no structural equivalent in 4,5-disubstituted compounds. Studies before (Le Goffic et al., 1977b) and afterwards (Frase et al., 2012) have argued that these compounds are not substrates for APH(2")-Ia. Further complicating the analysis is that the adjacent AAC(6')-Ie domain is active toward both 4,5-disubstituted and 4,6-disubstituted aminoglycosides, so it becomes difficult to study the effect of aminoglycoside binding in the full-length protein. Structural study through crystallization and structure determination of the isolated APH(2")-Ia domain can help resolve the discrepancy in aminoglycoside binding by this domain.

3.1.5 Experimental approach

I undertook structural study of aminoglycoside binding to APH(2")-Ia using X-ray crystallography. The enzyme was crystallized in complex with a co-substrate, the phosphotransfer-resistant compound GMPPNP, and soaked with 4,6-disubstituted and 4,5-disubstituted compounds, prior to cryo-cooling, diffraction, and solution of the enzyme structure. These structures, as well as the GMPPNP-bound form, illustrate how aminoglycoside binding is facilitated in the large central cleft of the enzyme through docking into a pocket optimized for binding of 2-deoxystreptamine and the 4-linked aminohexose ring. This binding mechanism indicates that APH(2")-Ia conserves the strategy of binding the conserved neamine rings, even in the absence of a group that can be catalytically modified.

These findings allow us to suggest modifications to aminoglycosides that disrupt binding to the enzyme, and also suggest a role of 4,5-disubstituted aminoglycosides as leads in the development of anti-resistance compounds.

3.2 Methods

3.2.1 Protein production

APH(2")-Ia was produced in *Escherichia coli* BL21 (λDE3). A plasmid containing the gene coding residues 175-479 of AAC(6')-Ie/APH(2")-Ia was obtained from Dr. G. D. Wright (McMaster University, Hamilton, Ontario). Cells were transformed by heat shock and grown on ampicillin-LB agar. Single colonies were selected, and used to inoculate 2.5 mL of ZYP-0.8G media, as defined by Studier (Studier, 2005). This culture was allowed to grow overnight, at 37˚C, shaking at 300 rpm. This saturated culture was used to inoculate a new 2.5 mL culture of ZYP-0.8G with 100 µL of the overnight culture. After 1 hour of growth, this was used to inoculate 500 mL of ZYP-5052 in a 2.8 L Fernbach flask. This culture was allowed to grow at 37˚C and 300 rpm for 2.5 hours before reducing the temperature to 22˚C for overnight growth. The cells were harvested in an Avanti centrifuge in 500 mL bottles at 5000 g for 15 minutes. Cells were re-suspended in media and divided into equal portions in 15 mL conical tubes before centrifugation at maximum speed in a swinging bucket rotor for half an hour at 4˚C. The supernatant was discarded and the cells were flash-frozen in liquid nitrogen before storing at -20˚C until use. Each pellet contained approximately 1 gram of bacterial cells, and corresponded to 125 mL of culture media.

3.2.2 Protein purification

Affinity resins were screened for their efficacy in purifying the APH(2")-Ia enzyme from raw lysate. Eight affinity resins were tested: the aminoglycosides kanamycin, tobramycin, ribostamycin, and amikacin (all obtained from Sigma-Aldrich), linked to each of the two activated agarose resins Affi-Gel 10 and 15 (Bio-Rad), prepared as per manufacturer's instructions. These 8 resins were all tested for their efficacy at extracting APH(2")-Ia from lysate, with a minimum of contaminating protein. 250 µL of re-suspended beads were used to test a lysate of APH(2")-Ia in microcentrifuge spin columns. After washing and elution, kanamycin-linked Affi-gel 15 showed the largest quantity of recovered protein with the least contaminating protein as determined by SDS-PAGE. This resin was scaled up and used for future purification procedures.

Cells were re-suspended in a buffer containing 25 mM HEPES pH 7.5, 10 mM NaCl, and 2.5 mM PMSF. This suspension of cells was lysed by ultrasonification. The lysate was clarified by centrifugation at 50,000 g for 30 minutes at 4˚C. This lysate was then applied to a 5.5-12 cm high column of Kanamycin-Agarose Affi-gel 15, washed out thoroughly before eluting in a gradient from 10-500 mM NaCl.

Fractions heavily enriched in the 35 kDa band corresponding to APH(2")-Ia, as estimated by SDS-PAGE, were concentrated and loaded to a 26/60 Superdex 75 column (GE Life Sciences). The column was run with a buffer of 10 mM HEPES pH 7.5 and 5% glycerol, which became the final protein buffer that the protein was stored in and with which crystal trays were prepared. This protein eluted as a peak with some background contribution to the A260 elution profile, although SDS-PAGE indicated that the protein was quite pure. Later analysis indicated that the extra absorbance was a result of contaminating nucleic acids, but crystals were grown in the presence of these contaminants nonetheless. A typical protein purification yielded 10 mg of protein from 125 mL of bacterial culture, netting 80 mg / L of culture.

3.2.3 Crystallization

Solutions of APH(2")-Ia enzyme were screened with no ligands added, with the co-substrate GMPPNP added, with kanamycin added, and with GDP + BeF_3 + kanamycin added. Crystals grew almost immediately when the enzyme was prepared with GMPPNP and added to a condition containing magnesium formate. Other conditions with divalent cations present also grew crystals, more slowly, so the nature of the divalent salt was screened, and magnesium chloride was found to be the best salt for growing crystals of APH(2")-Ia + GMPPNP.

This initial hit was identified and optimized, reaching optimal crystal growth at 22˚C, 80-120 mM $MgCl_2$, and 10% PEG 3350. 8% glycerol was added to reduce the spontaneous nucleation rate, and crystals were streak seeded using a horse hair from previous crystals. The presence of co-purifying nucleic acid in APH(2")-Ia protein preparations necessitated pre-incubation and filtration of some protein samples to remove precipitating nucleic acids and obtain diffraction-quality protein crystals. Crystals grew to diffraction quality in 2-4 days.

3.2.4 Crystallographic soaking

Kanamycin, ribostamycin, tobramycin, neomycin, and lividomycin were all obtained from Sigma-Aldrich, and were prepared in a 100 mM stock. Gentamicin C1 was purchased from TOKU-E biosciences and also prepared at 100 mM. Aminoglycoside dilutions were prepared in 2 mM concentration in mother liquor solution. 1-2 μL of this solution was added to drops containing crystals, and allowed to equilibrate 1-3 days. Crystal growth appeared to be inhibited by the addition of aminoglycosides, so this soaking process was started once the crystals had grown large enough to obtain a complete data set at sufficient resolution.

3.2.5 Data collection

Crystals were briefly washed with mother liquor supplemented to 22-25% glycerol, before snap freezing in liquid nitrogen. The crystals were screened for diffraction on a Rigaku MicroMax 007 diffractometer with Saturn 944+ detector and annealed (Hanson et al., 2003) to improve the diffraction in cases where the initial diffraction was highly anisotropic. All data sets were collected at cryogenic temperatures. Most data sets were collected at the Canadian Light Source synchrotron, beamline 08ID-1. The second data set for ribostamycin-bound protein was collected on the Micromax 007 instrument.

Diffraction data was indexed in the $P2_1$ space group and integrated in iMosflm (Battye et al., 2011). Scaling and merging of reflections was completed using AIMLESS (Winn et al., 2011). At a data cut off of $CC_{1/2}$ of 0.5, all data sets produced diffraction of 2.45 Å or better (Table 3.1). The data collection statistics for these seven data sets are listed in Table 3.1.

	APH-GMPPNP	**APH-GMPPNP-Kanamycin A**	**APH-GMPPNP-Gentamicin C1**	**APH-GMPPNP-Tobramycin**	**APH-GMPPNP-Ribostamycin 1**	**APH-GMPPNP-Ribostamycin 2**	**APH-GMPPNP-Neomycin B**	**APH-GMPPNP-Lividomycin**
Data collection								
X-ray source	CLS Beamline 08-ID	CLS Beamline 08-ID	CLS Beamline 08-ID	CLS Beamline 08-ID	CLS Beamline 08-ID	Rigaku MicroMax 007	CLS Beamline 08-ID	CLS Beamline 08-ID
Wavelength (Å)	0.9795	0.9795	0.9795	0.9795	0.9795	1.5418	0.9795	0.9795
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
a, *b*, *c* (Å)	90.2, 100.3, 94.1	89.4, 99.2, 93.5	89.8, 98.9, 93.9	89.9, 99.3, 93.6	90.2, 99.7, 93.4	89.9, 99.8, 93.8	90.2, 100.4, 93.9	90.3, 100.2, 94.0
β (°)	105.0	105.5	105.5	105.4	104.9	105.4	105.3	105.2
Resolution (Å)	56.04-2.15 (2.19-2.15)	55.36-2.30 (2.35-2.30)	58.74-2.30 (2.35-2.30)	58.77-2.40 (2.46-2.40)	55.93-2.20 (2.24-2.20)	31.16-2.75 (2.86-2.75)	67.25-2.50 (2.57-2.50)	59.07-2.40 (2.46-2.40)
$CC_{1/2}$	0.994 (0.428)	0.996 (0.431)	0.993 (0.538)	0.992 (0.298)	0.994 (0.504)	0.991 (0.597)	0.991 (0.575)	0.988 (0.287)
R_{merge}	0.108 (1.211)	0.100 (1.307)	0.109 (0.981)	0.127 (1.463)	0.103 (1.054)	0.126 (0.691)	0.114 (1.047)	0.110 (1.345)
$I/\sigma I$	7.8 (1.4)	9.8 (1.5)	10.9 (1.6)	6.9 (1.1)	8.9 (1.7)	7.4 (1.8)	10.6 (2.0)	10.0 (1.8)
Completeness (%)	94.4 (100.0)	99.0 (98.1)	96.2 (100.0)	100.0 (100.0)	100.0 (100.0)	99.9 (100.0)	100.0 (100.0)	100.0 (100.0)
Multiplicity	4.7 (4.6)	4.3 (4.3)	4.3 (4.3)	4.2 (4.2)	4.2 (4.2)	3.7 (3.4)	4.7 (4.7)	4.2 (4.2)

Table 3.1: Data collection statistics for APH(2")-Ia datasets described in this chapter

3.2.6 Structure solution, refinement, and analysis

The structure of APH(2")-Ia bound with GMPPNP was determined by molecular replacement in Phaser (McCoy et al., 2007), using the homologous APH(2")-IIa enzyme (PDB ID 3UZR) as a search model. Non-conserved loops and side chains of the protein were removed using CHAINSAW (Stein, 2008) and manual editing. Four copies of the protein were found in the asymmetric unit of the crystal, packed as two head-to-head pairs (Figure 3.2).

Iterative rounds of refinement using REFMAC5 (Murshudov et al., 1997) and model building in Coot (Emsley and Cowtan, 2004) were used to build the model of the APH(2")-Ia enzyme. Refinement maintained torsion based NCS restraints in all stages. In later stages, TLS groups were assigned to functionally contiguous parts of the protein, which were kept identical between all structures refined. The enzyme was modelled with the co-substrate GMPPNP, two magnesium atoms, and a set of coordinating waters in the active site. This model was then used to phase and refine subsequent data sets that included soaks with aminoglycoside substrates. These structures were solved by difference Fourier synthesis directly in REFMAC. In all cases except for the lividomycin-soaked crystals, obvious and unambiguous electron density were observed for a soaked aminoglycoside compound. Aminoglycoside refinement restraints were generated with GRADE (Global Phasing, Inc.). All models were refined to convergence, with R and R_{free} statistics reflecting the top percentiles of structures at their respective crystallographic resolution. Statistics for these models are presented in Table 3.2. Structures of APH(2")-Ia were analyzed using the Normal Mode Analysis server *elNémo* at Université de Nantes (Suhre and Sanejouand, 2004) and DynDom (Hayward and Berendsen, 1998). PyMol (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) and Inkscape (https://inkscape.org/) were used to generate figures.

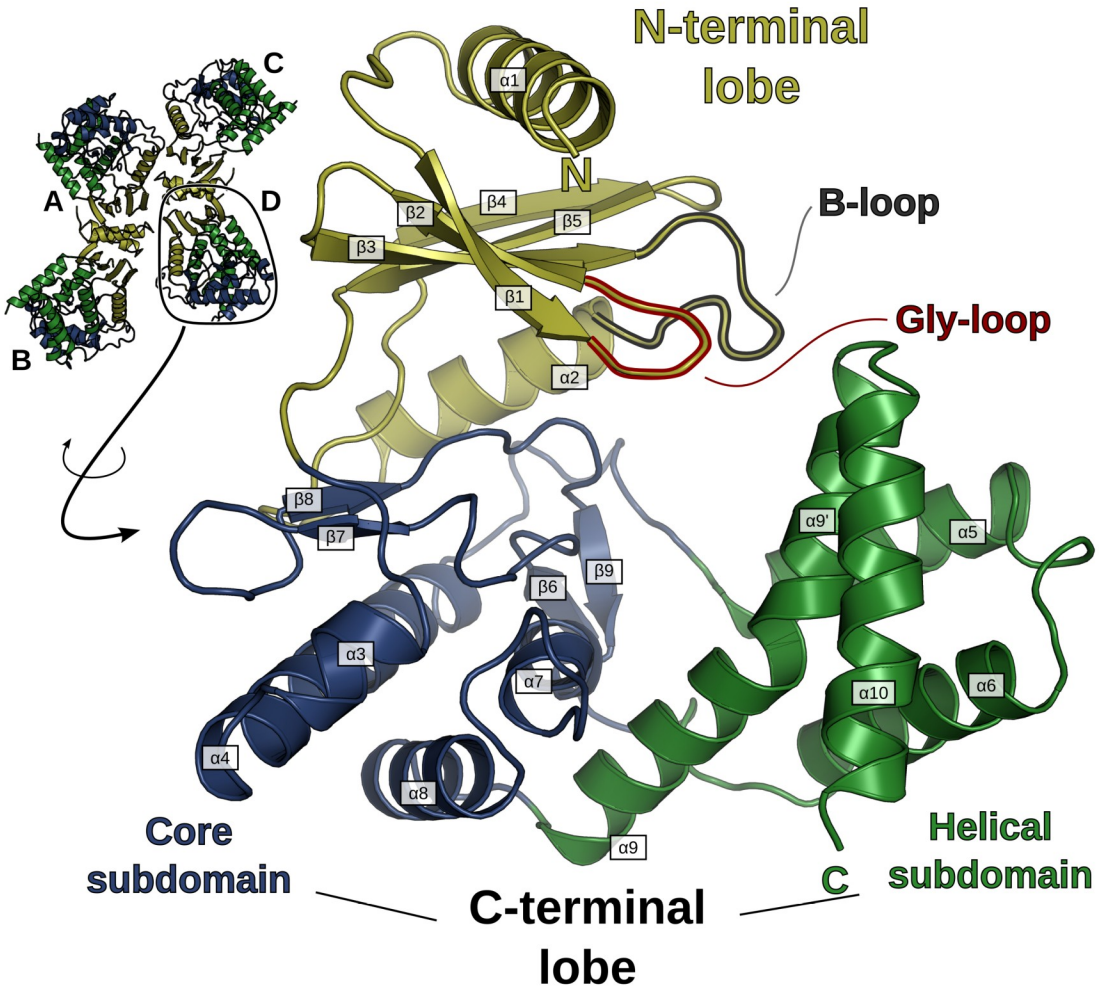

Figure 3.2: Structure of APH(2'')-Ia

Four chains (inset) are found in the asymmetric unit of crystals of APH(2")-Ia. The enzyme can be subdivided into three structural regions, comprised of the N-lobe, and the C-lobe which is further subdivided into helical and core subdomains. The nucleoside substrate binds between the N-lobe and core subdomain, while the aminoglycoside binds between the core and helical subdomains.

	APH-GMPPNP	APH-GMPPNP-Kanamycin A	APH-GMPPNP-Gentamicin C1	APH-GMPPNP-Tobramycin	APH-GMPPNP-Ribostamycin 1	APH-GMPPNP-Ribostamycin 2	APH-GMPPNP-Neomycin B	APH-GMPPNP-Lividomycin
Resolution (Å)	2.15	2.30	2.30	2.4	2.20	2.75	2.50	2.4
Wavelength (Å)								
No. unique reflections	83234	69241	67766	62095	81110	41701	55938	63273
R_{work}/ R_{free}	0.1666/0.2055	0.1664/0.2107	0.1741/0.2269	0.1889/0.2350	0.1637/0.2034	0.1805/0.2386	0.1671/0.213	0.1808/0.227
No. atoms								
Protein	9832	9624	9839	9643	9791	9791	9685	9647
Ligands	176	307	323	264	308	278	331	188
Water	721	767	996	384	1130	568	654	539
B-factors								
Protein	51.4	59.3	56.2	63.5	44.1	62.0	66.3	59.5
Ligands	45.7	51.9	54.0	53.8	42.7	80.1	93.2	45.9
Water	52.1	59.4	57.7	57.1	53.4	46.4	65.5	61.4
R.m.s deviations								
Bond lengths (Å)	0.0152	0.0140	0.0128	0.0170	0.0157	0.0145	0.0137	0.0136
Bond angles (º)	1.5710	1.5180	1.4850	1.456	1.6530	1.593	1.5420	1.506
Ramachandran								
%Favoured	97.79	97.29	96.75	96.09	97.33	96.90	96.73	97.23
%Allowed	1.70	2.62	2.56	3.39	1.72	1.90	1.98	2.25
%Outlier	0.51	0.09	0.68	0.52	0.95	1.21	1.29	0.52

Table 3.2: Structural statistics for models of APH(2")-Ia bound to GMPPNP and aminoglycoside soaks of these crystals

3.3 Results

3.3.1 Structure of the APH(2'')-Ia domain delineates a large internal cleft

The crystal structure of APH(2'')-Ia contains four enzyme molecules in the asymmetric unit (Figure 3.2). These APH(2'')-Ia molecules have the same enzyme architecture as other aminoglycoside kinases and some small-molecule kinases (Oruganty et al., 2016; Zhang et al., 2014). The N-terminal lobe of APH(2'')-Ia contains a 5-stranded beta-sheet and two alpha helices, while the C-terminal lobe contains two interleaved structural regions: a core and helical subdomain. The core subdomain is built around a hairpin structure (β7-β10) which contains catalytic and metal-binding residues held in position by the underlying domain architecture. The helical subdomain comprises a four-helix bundle, distal to the rest of the enzyme.

The core subdomain of the enzyme forms a scaffold that supports catalytic residues of the enzyme including H379, D393, and D374. H379 and D393 coordinate magnesium ions that are required for catalysis, while D374 is a critical and universally conserved catalytic aspartate residue. These residues form the bridge between the nucleoside-binding pocket and the spacious aminoglycoside-binding cleft.

The region between the core and helical subdomains delineates a large internal cleft (Figure 3.3). This cleft allows a large volume of space where an aminoglycoside can bind. Other aminoglycoside kinases like the closely related APH(2'')-IIa (Young et al., 2009) and APH(2'')-IVa (Shi et al., 2011) share this cleft. Related enzymes with a narrower range of substrates like APH(9)-Ia (Fong et al., 2010) and APH(4)-Ia (Stogios et al., 2011), have smaller and more closed clefts, while choline kinase-2 has a very shallow substrate-binding cleft that corresponds with its small substrate (Peisach et al., 2003). Despite equivalent enzyme topology, the size of cleft in these enzymes changes to correspond to the nature of the substrates the enzyme binds and acts upon.

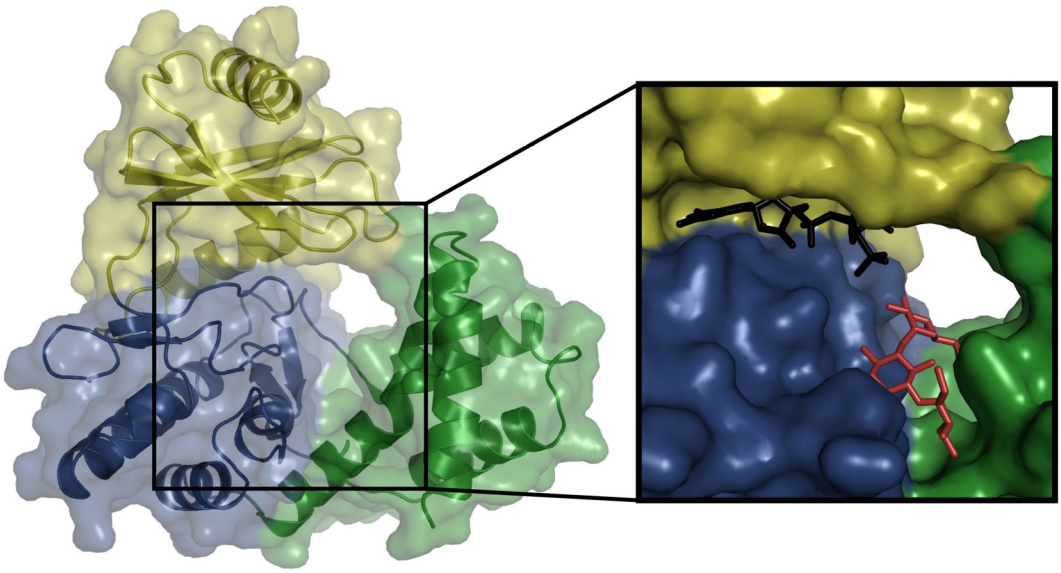

Figure 3.3: The spacious binding cleft of APH(2'')-Ia

The N-lobe (yellow) of the protein and core subdomain of the C-lobe (blue) form the cleft that binds nucleoside ligand (black) and carries out catalysis. The core and helical (green) subdomains of the C-lobe enclose a large internal cleft that is partially occupied with aminoglycoside substrate (red) but also contains a great amount of excess space.

3.3.2 APH(2'')-Ia presents a pre-organized platform that binds the neamine rings of aminoglycosides

The core subdomain of APH(2'')-Ia contains a pocket that facilitates binding of antibiotics. This pocket includes an electronegative triad formed by the residues E411, E415, and E416 which form a perfect tetrahedral site to stabilize the N3-amine of 2-deoxystreptamine (Figure 3.4). Y408 lines the bottom of this pocket, and S376 and the catalytic D374 residue complete the coordination of the aminoglycoside 2-DOS ring. Interactions with the 4-linked aminohexose ring are less substantial, but an ionic interaction between E415 and the 6' amine group also contribute to neamine binding. Two residues on the helical subdomain contact the aminoglycoside, Y448 and E445. These residues both interact with the 4-linked aminohexose, sandwiching it between the subdomains.

These 8 residues form a neamine-binding scaffold where the compound can bind the enzyme efficiently, tolerating any variation beyond the 2-deoxystreptamine and 4-linked aminohexose rings. This binding mode permits the interaction of the enzyme with a broad range of aminoglycoside antibiotics, as the 5- and 6-positions of the 2-deoxystreptamine ring remain unhindered. Groups added to either can be tolerated in the binding site of the enzyme.

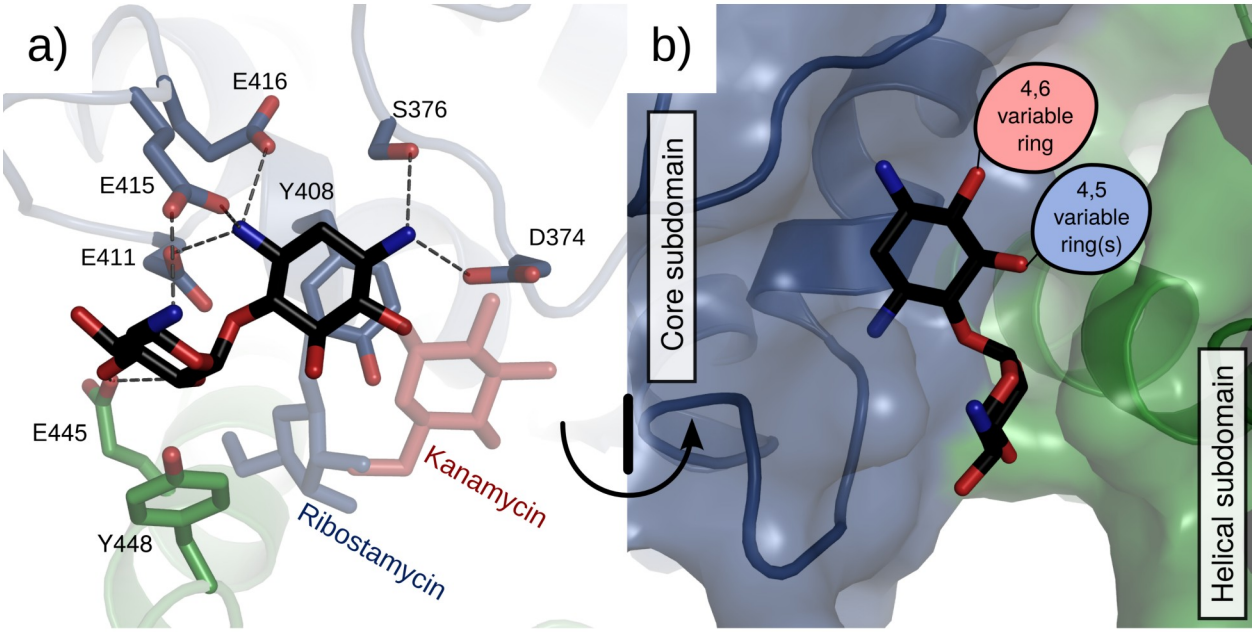

***Figure 3.4*: Residues of APH(2'')-Ia form a conserved neamine-binding platform**

a) Residues surrounding the aminoglycoside-binding pocket stabilize the binding of 2-deoxystreptamine *via* its conserved ribosome-binding elements. The tri-glutamate anionic hole binds one of the amine groups, while S376, Y408, and the catalytic D374 complete the binding pocket. Y448 and E445 from the helical subdomain contact the 4-aminohexose ring. b) rings linked to the 5- and 6-position of the compound can both be accommodated in the aminoglycoside-binding site, by protruding into the solvent-filled cleft of the enzyme.

3.3.3 APH(2'')-Ia binds the neamine rings of 2-DOS aminoglycosides in identical conformations

Kanamycin, gentamicin, tobramycin, ribostamycin, and neomycin all bind unambiguously in the active site of APH(2'')-Ia (Figure 3.5). Two independent ribostamycin-bound structures were determined, for a total of 6 structures with aminoglycoside bound. In each of four copies in the asymmetric unit of each crystal, aminoglycoside binds clearly in almost every aminoglycoside-binding site. The exceptions are a single chain of one ribostamycin-bound form, where electron density is not definitive of a single conformation and may indicate multiple weakly-bound conformations, and one chain of the neomycin-bound structure, which shows good electron density for 2-DOS, but poor definition of the additional rings. The 2-DOS ring is bound in the same position of almost every bound aminoglycoside, bound by the negatively charged glutamate residues and polar contacts of the neamine-binding platform (Figure 3.4).

Superimposition of bound aminoglycosides indicates that all of these compounds conserve binding through the neamine-binding platform between the domains. In all of these structures, the central 2-DOS and 4-linked aminohexose bind in identical positions, regardless of the identity of the antibiotic (Figure 3.6a). The remaining rings are accommodated in the spacious cleft in the centre of the enzyme.

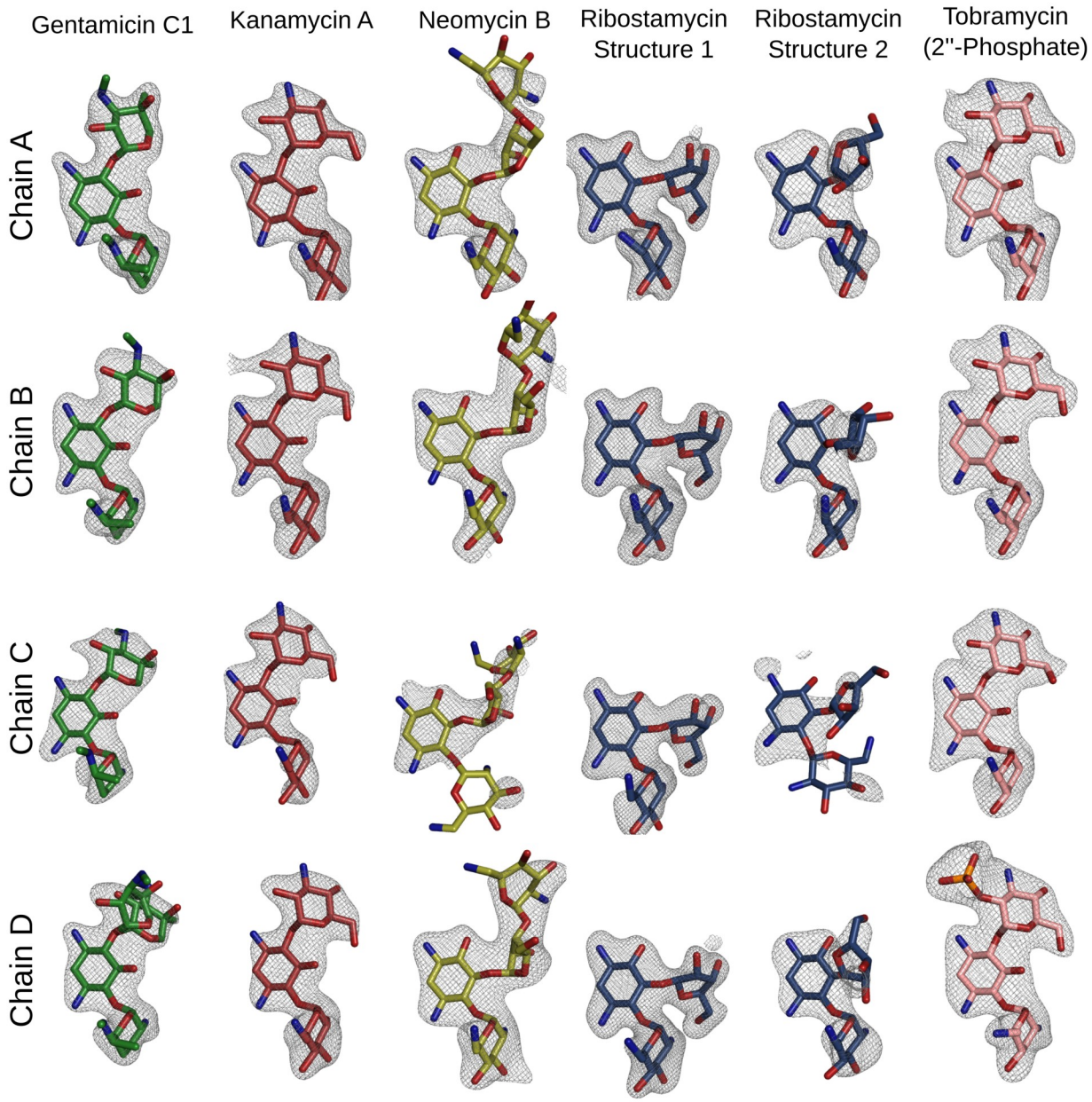

***Figure 3.5*: Aminoglycoside density binding to APH(2'')-Ia.**

Electron density and modelled aminoglycoside identity in substrates soaked into crystals of APH(2")-Ia. In four chains of the enzyme, there is some differential binding, but most binding modes conserve identical orientation of the neamine rings of the compounds. Difference maps are F_o-F_c difference maps contoured to σ = 2.8 following refinement of aminoglycoside-free structures. Chain D of the tobramycin-bound form indicated electron density for a transferred phosphate, which will be addressed in Section 3.3.5. All maps are displayed at a range of 2.25 Å from the modelled compounds.

3.3.4 Positions of of 5- and 6-linked rings of aminoglycosides in the APH(2'')-Ia binding site

The 5- and 6-linked rings of aminoglycosides are both accommodated in the cleft of APH(2'')-Ia. These rings all lie in different positions depending upon their linkage to the neamine nucleus of the antibiotic. Interestingly, in two independently determined ribostamycin-bound structures, the 5-linked ring binds in different positions. While it's not clear what differences in crystal growth or handling led to this difference in conformation, this finding illustrates an enzymatic flexibility to adopt two conformations and allows both conformations to be stabilized.

While kanamycin binds in catalytically competent fashion and gentamicin can easily access a similar conformation (Figure 3.6a), the 4,5-disubstituted neomycin and ribostamycin are precluded from binding in a way that places any modifiable groups near the catalytic centre. While these compounds are accommodated in the aminoglycoside binding site, they can not be phosphorylated by the enzyme.

Comparison of kanamycin and gentamicin show these 4,6-disubstituted compounds can still exhibit considerable flexibility of the 6-linked ring (Figure 3.6b). The 6-linked ring of gentamicin lies farther from the active site, which is possible because gentamicin lacks an equatorial group at the 5''-position, which holds kanamycin away from the helical subdomain. While gentamicin shows multiple orientations and thus flexibility of the 6-linked garosamine ring, the equivalent ring of kanamycin is more restricted, and lies in a single conformation, compatible with productive catalysis.

The fact that gentamicin naturally adopts conformations where the garosamine ring lies in sub-optimal positions for catalysis suggests that rotating toward the active site of the enzyme is slightly unfavourable. This may explain why gentamicin is modified an order of magnitude more slowly than kanamycin or tobramycin (Frase et al., 2012). The conformational changes that bring this ring of gentamicin closer to the reactive centre of the enzyme will be discussed in more detail in Chapter 4. Tobramycin showed similar conformation to kanamycin, and has even been productively modified within the crystals.

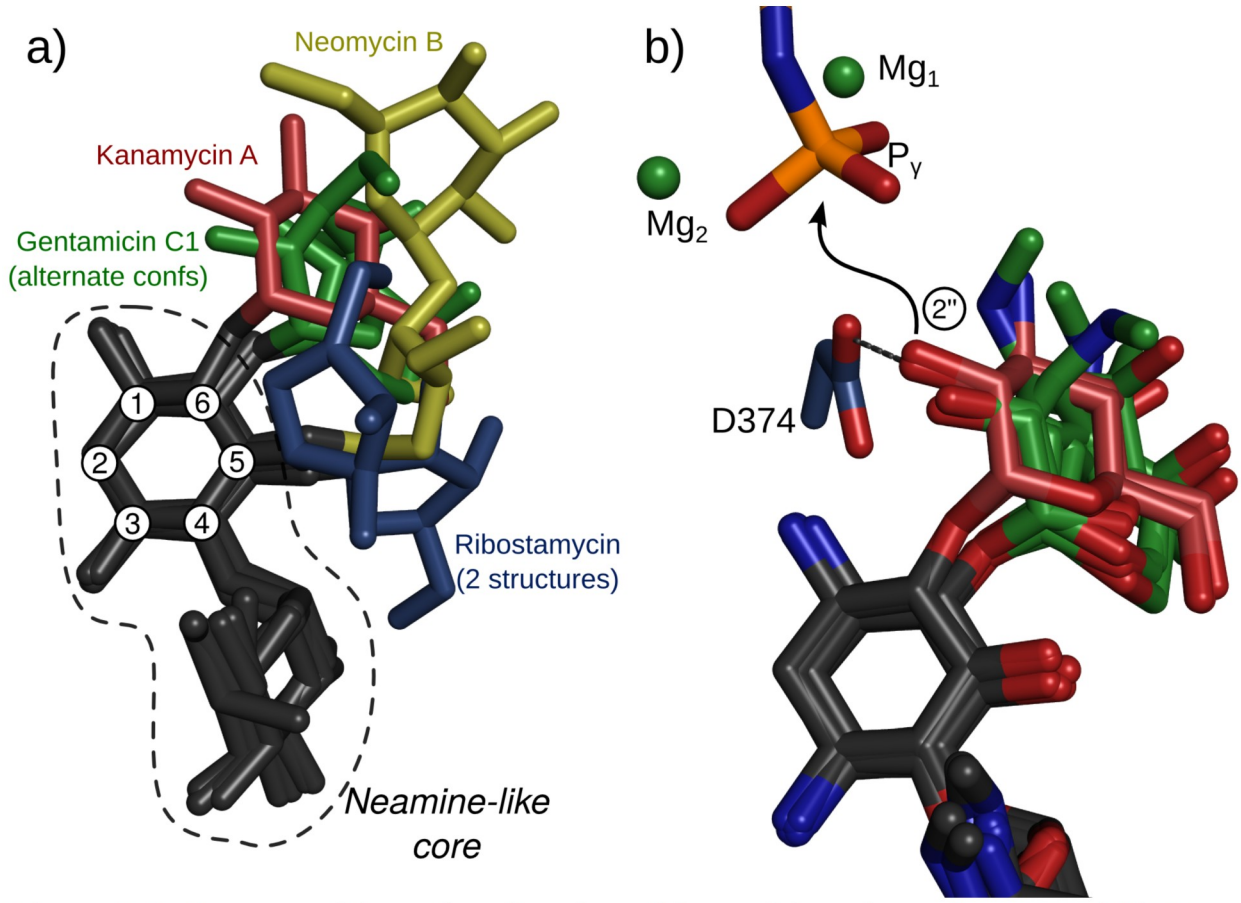

***Figure 3.6*: Superposition of aminoglycoside positions bound to APH(2'')-Ia**

a) 4,6-disubstituted aminoglycosides kanamycin (red) and gentamicin (green, modelled as a dual occupancy with two stable conformations) and 4,5-disubstituted aminoglycosides neomycin (yellow) and ribostamycin (blue, two distinct conformations observed from different crystal structures) overlay the neamine rings of these compounds in identical binding conformations. b) Kanamycin and gentamicin are both bound in a catalytically competent conformation, with the 2"-hydroxyl group in position (or easily accessible) for productive catalysis. Kanamycin binds with 6-linked aminohexose ring in the same orientation in all four chains, while gentamicin exhibits multiple different conformations.

3.3.5 Modified tobramycin indicates that aminoglycoside binding is productive, even in crystals

Tobramycin was soaked into crystals like the other aminoglycosides, and showed good electron density in all four chains (Figure 3.5). In chain D of this structure, continuous electron density extended from the 2"-oxygen of the compound toward the nucleoside-binding site. This density indicates a phosphate group linked to the 2"-position of tobramycin, illustrating that this substrate is productively modified, even in the crystal (Figure 3.7).

This was surprising as the crystals were grown using GMPPNP, an analogue of GTP with a β,γ-bridging nitrogen, which should not be active in phosphotransfer. However, studies have shown that β,γ-imidotriphosphates can still be active in enzymes (Bastidas et al., 2013), and even in APH(2")-Ia crystals, early structure determinations indicated that the γ-phosphate of the compound is labile after several days at ambient conditions.

This productive modification indicates that the mode of binding of this compound and other 4,6-disubstituted aminoglycosides is productive in the crystal form of the enzyme. The structure of 2"-phosphotobramycin in the active site illustrate that the enzyme is fully capable of carrying out this reaction with the compound bound and in the crystal.

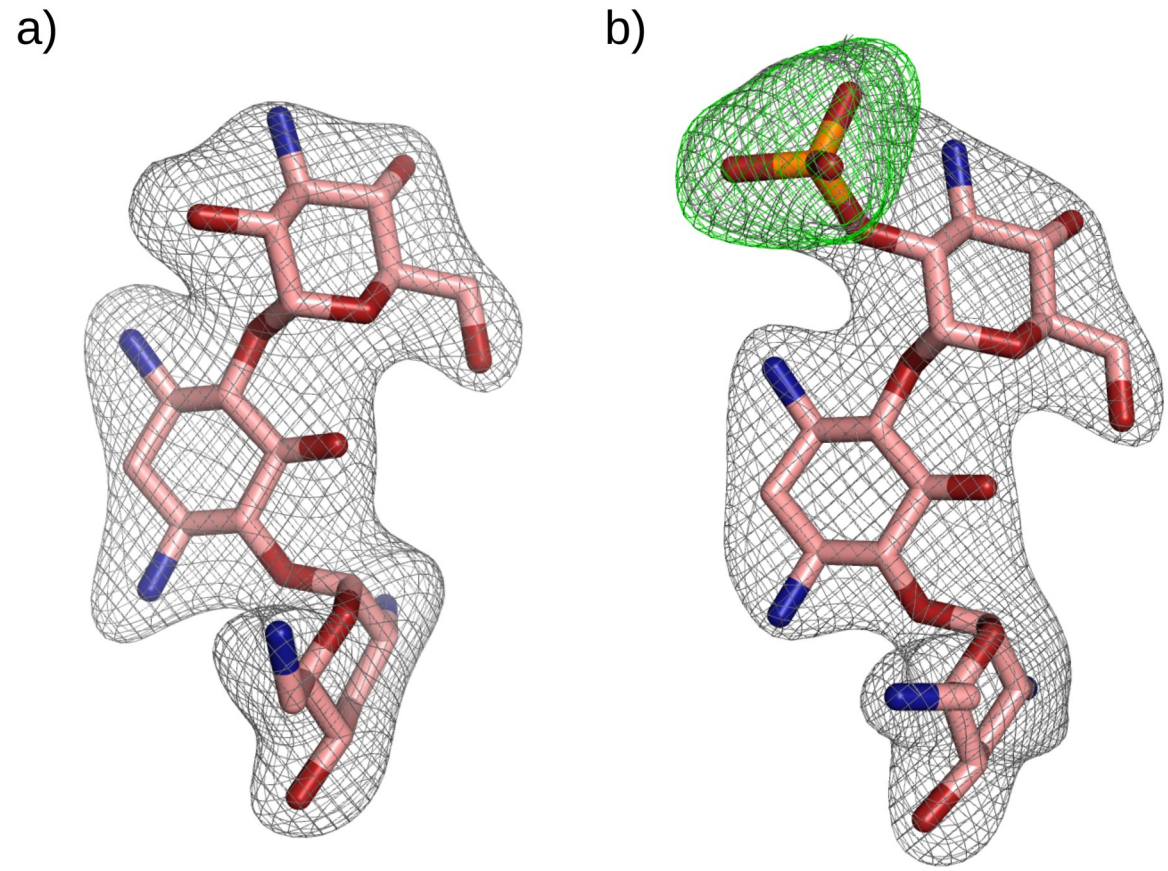

***Figure 3.7*: Tobramycin and phosphotobramycin in the APH(2'')-Ia enzyme**

a) Tobramycin in chain A of APH(2")-Ia crystal structure. b) 2"-O-phosphotobramycin in chain D of the APH(2")-Ia structure. Gray maps are F_o-F_c difference maps, calculated after removal of the ligand and REFMAC refinement, contoured at σ = 2.5. Green map is F_o-F_c difference map refined in the presence of tobramycin alone, contoured at σ = 2.5. All maps are displayed at a range of 2.5 Å from the modelled compounds.

3.3.6 Lividomycin binds weakly and suggests alternate binding modes may exist at low occupancy

Lividomycin A is the largest aminoglycoside tested in soaking experiments into crystals of APH(2")-Ia. It appears that unlike neomycin, which contains four rings and binds well to most chains (Figure 3.5), lividomycin does not easily adopt a single conformation in the aminoglycoside-binding site of APH(2")-Ia. While the electron density recovered from this crystal indicated considerable difference density indicative of a substrate, in all four chains it can not be refined to a single conformation of aminoglycoside in the enzyme binding site (Figure 3.8).

It is possible that lividomycin represents the upper limit of size at which APH(2")-Ia cannot bind aminoglycoside in a single orientation. It is also possible that the restrictions imposed by the crystal lattice preclude binding of the compound, because of the constraints imposed by crystal packing. In addition to its extra ring, lividomycin also differs from neomycin by a 6'-hydroxyl group where neomycin contains an amine, which could also be partially responsible for the low binding of lividomycin to the enzyme.

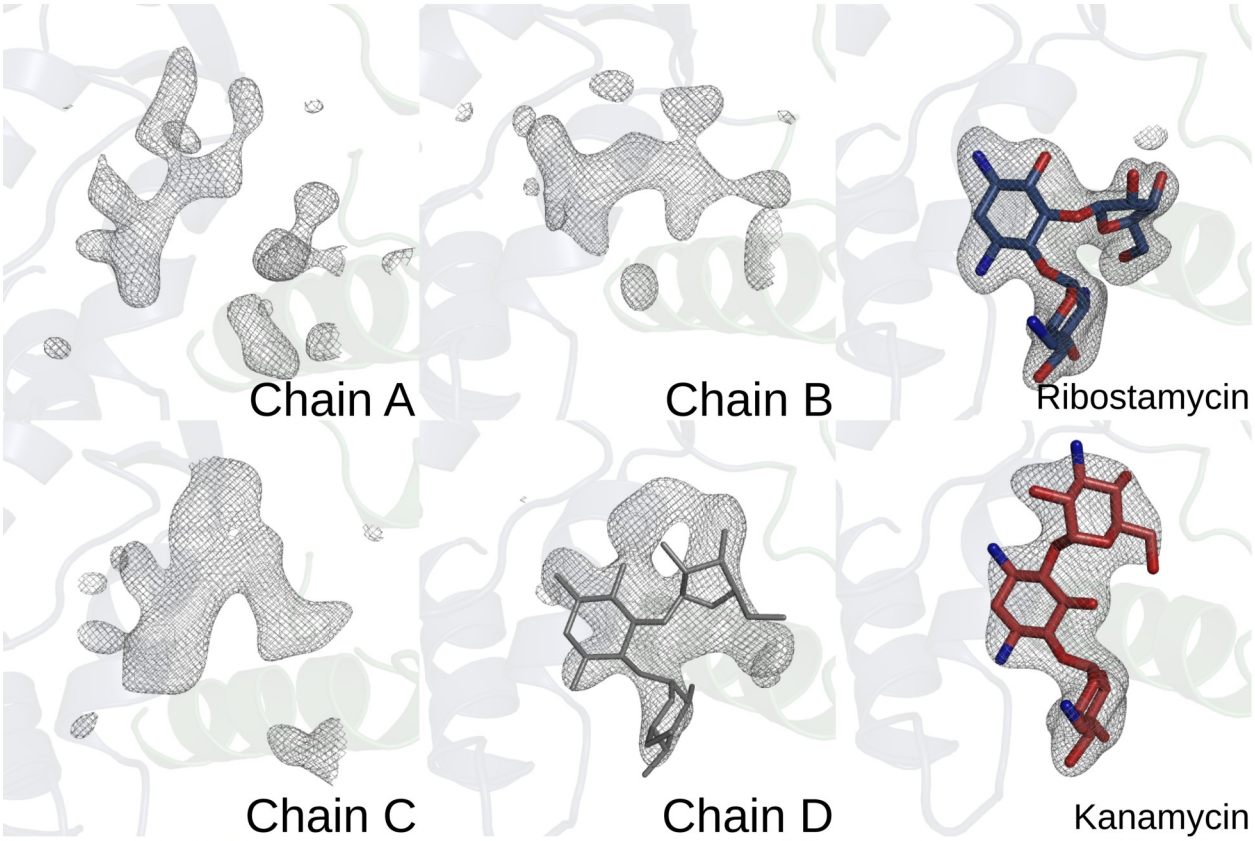

***Figure 3.8*: Difference density in the aminoglycoside-binding site of APH(2'')-Ia incubated with the antibiotic lividomycin A**

Density maps indicate electron density in aminoglycoside binding site of of APH(2")-Ia soaked with the large aminoglycoside lividomycin A. This density suggests some low-occupancy alternative binding modes are present for lividomycin in the enzyme, although none could be modelled unamiguously. In chain D, there is some indication of binding in the equivalent mode to ribostamycin and neomycin (modelled), but this mode does not fully explain the electron density in this aminoglycoside-binding site. Difference density maps calculated following refinement with the exclusion of any substrates in the aminoglycoside-binding pocket. The electron density in chain D of ribostamycin and kanamycin structures are included for comparison. All maps are F_o-F_c omit maps, σ = 2.8. All lividomycin maps are displayed at a range of 4 Å from the position of the equivalent ribostamycin molecule in the ribostamycin-bound structure.

The evidence of this interaction between lividomycin and APH(2")-Ia aligns with enzymological studies with the compound (Daigle et al., 1999a). Lividomycin has apparent K_M values 20-fold weaker than the apparent K_M of neomycin and ribostamycin. The compound was also found to be phosphorylated on the 5"-OH of the ribose ring of the compound, suggesting that it binds the enzyme in a unique manner compared to other 4,5-disubstituted compounds.

While all of the other compounds tested showed a conservation of binding to the neamine-binding platform of the enzyme, lividomycin appears to be the exception that proves that rule.

In chain D of the molecule, we can model two potential binding modes that place the 5" hydroxyl group of lividomycin in the vicinity of the reactive centre of the enzyme (Figure 3.9). One of these modes conserves the neamine-based binding to the enzyme, while the other does not and uses the 4th and 5th rings (labelled D and E) of lividomycin to bind in the place of the 2-DOS and 4-aminohexose rings instead. Either of these binding modes could be responsible for the 5"-phosphorylation of the compound. The promiscuity of aminoglycoside binding to APH(2")-Ia will be revisited in Chapter 5.

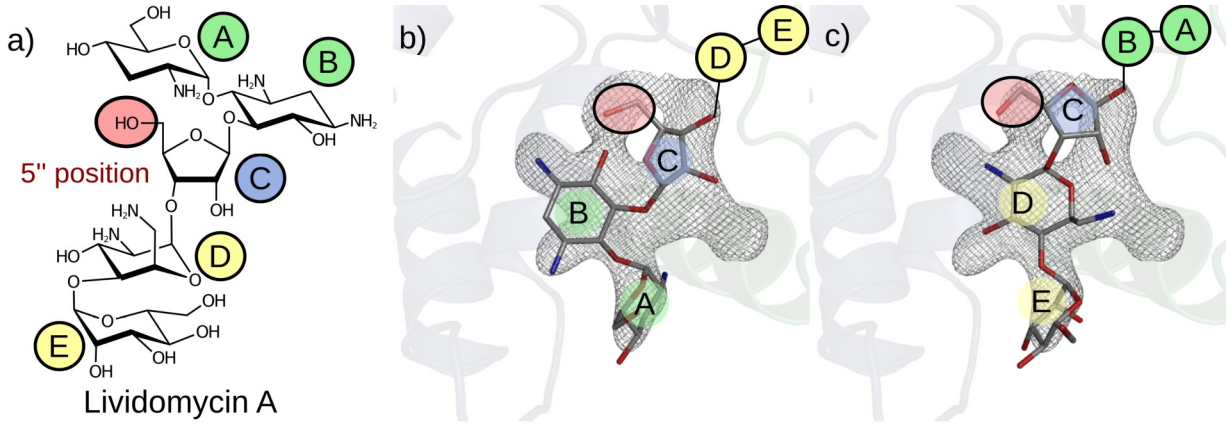

***Figure 3.9*: Alternative binding modes for lividomycin 5''-phosphorylation**

a) Lividomycin is the largest aminoglycoside, with five sugar or sugar-like rings, labelled A-E. b) In the first possible binding mode to chain D of the enzyme, lividomycin is bound in a similar fashion to other neamin-based antibiotics with the 2-DOS (B) and 4-linked aminohexose (A) rings bound to the enzyme, and the 5-linked ribose ring positioned near the reactive centre. The D and E rings are not resolved in the electron density. c) In an alternate mode of binding, the compound binds upside-down relative to other compounds, using the 4th and 5th rings of the compound (D & E) to make similar contacts to the enzyme. In this enzyme, rings A and B are not resolved.

3.4 Discussion

3.4.1 APH(2'')-Ia binds both families of neamine-based aminoglycoside by targeting the conserved rings

Soaking of multiple aminoglycosides into crystals of APH(2'')-Ia indicate that the enzyme forms a conserved platform to bind the neamine nucleus of these compounds. The additional rings on 4,5- and 4,6-disubstituted compounds are accommodated in binding to the enzyme in the large antibiotic binding site. The enzyme forms a pre-organized platform for neamine, where the central 2-DOS ring is bound most tightly in an anionic and polar pocket, while the 4-linked aminohexose forms some additional contributing interactions. The 4,5-disubstituted and 4,6-disubstituted aminoglycosides both bind to APH(2'')-Ia using these conserved rings, while the variable 5- and 6-linked rings form few interactions and are suspended in the solvent-filled cleft of the enzyme.

This finding shows that APH(2'')-Ia is similar to other broad profile resistance enzymes. Binding of both classes of compound conserves interactions with the neamine rings, while the enzymes adopt different strategies in order to accommodate the variable rings in binding. Compared with APH(3')-IIIa and AAC(6') enzymes, APH(2'')-Ia demonstrates a new means of doing so. While APH(3')-IIIa uses loop rearrangements (Fong and Berghuis, 2002) and AAC(2')-Ic ordered water molecules to compensate for alternate binding interactions (Vetting et al., 2002), APH(2'')-Ia demonstrates a third strategy. It tightly binds the neamine portion in a cleft between subdomains, while the third ring is suspended in a cavity filled with disordered solvent. This new strategy indicates that while selective forces have driven these enzymes to bind the conserved rings, the means by which they do so are not necessarily the same. APH(2'')-Ia has independently solved the problem of binding to both classes of neamine-based aminoglycoside.

3.4.2 Conservation of the neamine-binding platform

Aminoglycoside-bound structures of APH(2")-Ia illustrate a pocket of important interactions between the enzyme and neamine ring of aminoglycosides. The triad of glutamate residues, as well as interactions with S376 and D374 stabilize the neamine rings against the core subdomain. We can compare this binding platform to that of homologous enzymes to assess the importance of these interactions by conservation.

Structures of three homologous APH(2") enzymes have been determined. These homologous enzymes include three other subgroups: APH(2")-IIa, -IIIa, and IVa. Of these structures, a gentamicin-bound form of APH(2")-IIa (Young et al., 2009) (PDB ID 3HAM) and tobramycin, kanamycin, G418, and sisomicin-bound forms of APH(2")-IVa have been determined (Kaplan et al., 2016; Shi et al., 2011) (PDB ID 3SG8, 3SG9, 5C4K, 5C4L). With the exception of the sisomicin-bound form, these structures all conserve the same binding mode of aminoglycoside, which places the 2"-hydroxyl group of these 4,6-disubstituted aminoglycosides directly at the reactive active centre.

The four subgroups of APH(2") share 24-28% sequence identity, and their topology and structure are comparable, but many active site residues are exchanged between these proteins. To examine the conservation of elements of the neamine-binding platform, we can superimpose these enzymes and compare their aminoglycoside-binding sites (Figure 3.10). While circumstances of crystal packing lead to changes in the conformation of these enzymes, APH(2")-Ia, APH(2")-IIa, and APH(2")-IVa show a well-conserved aminoglycoside binding site, while APH(2")-IIIa is forced into a distorted conformation that moves some of the residues away from the aminoglycoside binding site. Nevertheless, these four homologues all show equivalent residues are still in place, and conformational changes could bring these residues into equivalent positions to the aminoglycoside-binding state of APH(2")-Ia.

Examination of the aminoglycoside-binding platform residues in these four APH(2") enzymes show that the tri-glutamate anionic hole is maintained in these enzymes, although an insertion and deletion in the APH(2")-IIIa enzyme results in a glutamate from a topologically different region filling this role. Residues S376 and D374 are universally conserved. Of the core

subdomain residues only Y408 changes appreciably, which is replaced by a serine residue in APH(2")-IVa that is buried and does not interact with the aminoglycoside. In APH(2")-IIa this residue is an electron-rich cysteine, while in APH(2")-IIIa, it remains tyrosine.

Binding of 4,5-disubstituted compounds to other enzymes in the APH(2") family has not been examined in much detail, because these enzymes did not confer high resistance toward neomycin (Chow et al., 1997; Kao et al., 2000; Tsai et al., 1998). While it would appear that 4,5-disubstituted compounds would clash with the helical subdomain (Shi et al., 2011), we have observed that the helical subdomain can move considerably and so it may still be possible for them to bind. In fact, isothermal titrations indicate that 4,5-disubstituted compounds can bind to APH(2")-IVa (Kaplan et al., 2016). Further examination of the 4,5-disubstituted aminoglycoside binding to APH(2") enzymes may reveal important interactions of these compounds, which could be important if a binding role leads to resistance through other mechanisms.

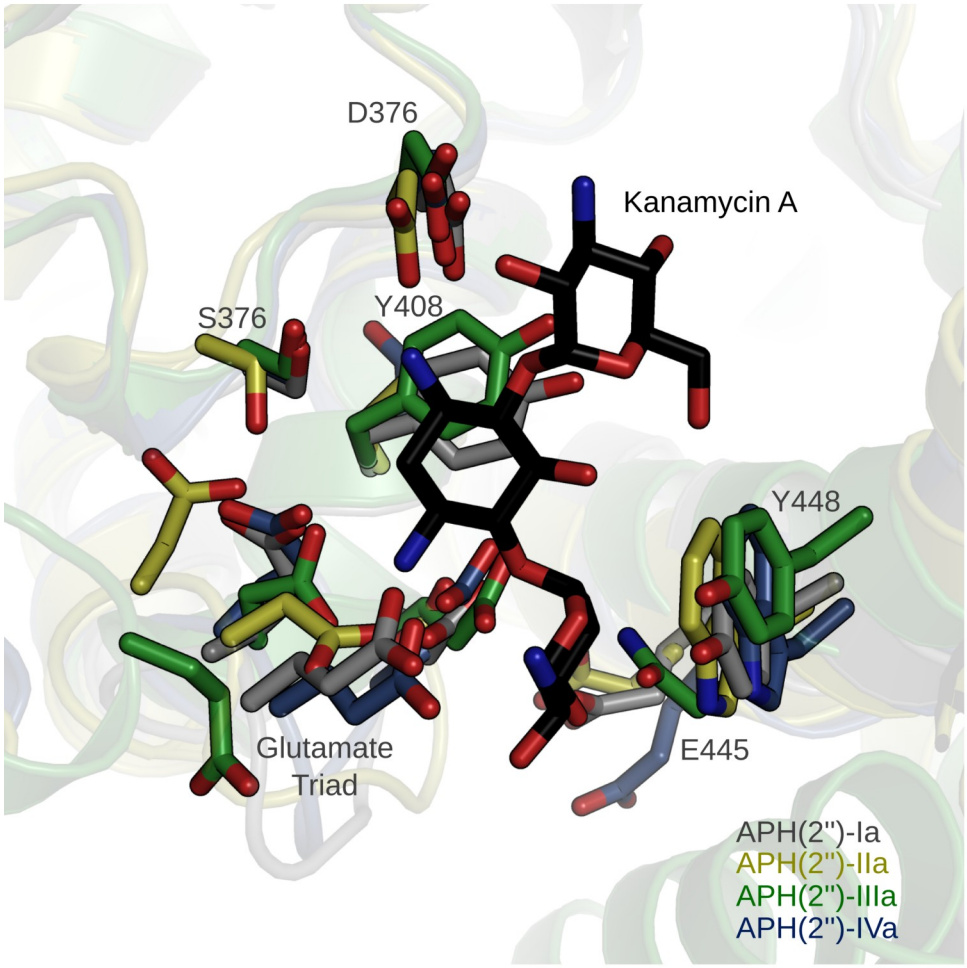

***Figure 3.10*: Conservation of the neamine-binding site in APH(2'') enzymes**

The common aminoglycoside-binding residues of APH(2")-Ia (gray), APH(2")-IIa (yellow, PDB 3HAM (Young et al., 2009)), APH(2")-IIIa (green, PDB 3TDV (Smith et al., 2012)), and APH(2")-IVa (blue, PDB 3SG8 (Shi et al., 2011)) are superimposed. S376 and D374 are universally conserved, as is an aromatic residue on the helical subdomain, Y448 in APH(2")-Ia. A conserved pocket of negative residues is present in all four enzymes, although the backbone architecture to position these residues varies and variation in crystallized conformations shows these residues have some flexibility. The residue at the bottom of the binding site, corresponding to Y408, varies the most between structures.

3.4.3 APH(2'')-Ia binds non-substrate aminoglycosides – resistance by binding alone?

The aminoglycosides kanamycin, tobramycin, and gentamicin all bind in a fashion consistent with productive modification. Ribostamycin and neomycin do not. This observation validates findings by Frase, et. al (2012) that found that while 4,5-disubstituted aminoglycosides appear to interact with the enzyme, they are not productively modified. In this case, the observed change was that upon binding aminoglycoside, the background GTP-hydrolysis rate of the enzyme increases. This effect will be further explained in the next chapter.

It has been suggested that binding alone (“apprehension”) of the antibiotic could be a major mechanism of resistance (Gates and Northrop, 1988b). There are multiple examples of antibiotic resistance by simple binding, including two cases in aminoglycoside-binding enzymes (Cox et al., 2015; Magnet et al., 2003). It is possible that binding of 4,5-disubstituted compounds to the enzyme could titrate the compound from solution and confer resistance by simple binding alone. This binding could also form a kind of short-term “holding tank” to hold aminoglycoside in place, which can then be passed to the adjacent AAC(6')-Ie domain. The enzyme could serve as a binding protein first, enzyme second. Evolution has not selected against this interaction in the enzyme, so it must not carry a heavy negative selective penalty. Possible positive benefits of binding 4,5-disubstituted compounds in APH(2")-Ia is an interesting area of future research.

3.4.4 Binding of aminoglycoside connects the core and helical subdomains

Stacking of Y448 against the 4-linked aminohexose ring of the aminoglycoside provides contact between the helical subdomain and the conserved aminoglycoside rings. This interaction can help draw this distal region closer to the rest of the protein. This tyrosine lies immediately next to the proline kink in helix α9 (Figure 3.11), which serves as a pivot point where the domain can bend toward the catalytic centre of the enzyme. E445, which also contacts the 6-linked aminohexose ring of the compound, lies just below this pivot point. These crystal structures indicate that even within the crystal, structural changes occur upon aminoglycoside binding. We

can quantify this difference using structural bioinformatics tools such as *ElNéMo* (Suhre and Sanejouand, 2004).

Comparing the aminoglycoside-free and aminoglycoside-bound forms of the enzyme, the helical subdomain shifts inward by 1.6 Å when kanamycin is introduced to the crystals. The transition is almost completely explained by a single normal mode transition in *ElNéMo*. This transition is similar to conformational changes seen in homologous APH(2")-IVa (Shi et al., 2011), although those structures were obtained from independent crystallization conditions, while the APH(2")-Ia transition occurs within the same crystal form. The transition between these forms appears to be a normal equilibrium present in the protein, biased toward a more closed conformation by the binding of aminoglycoside.

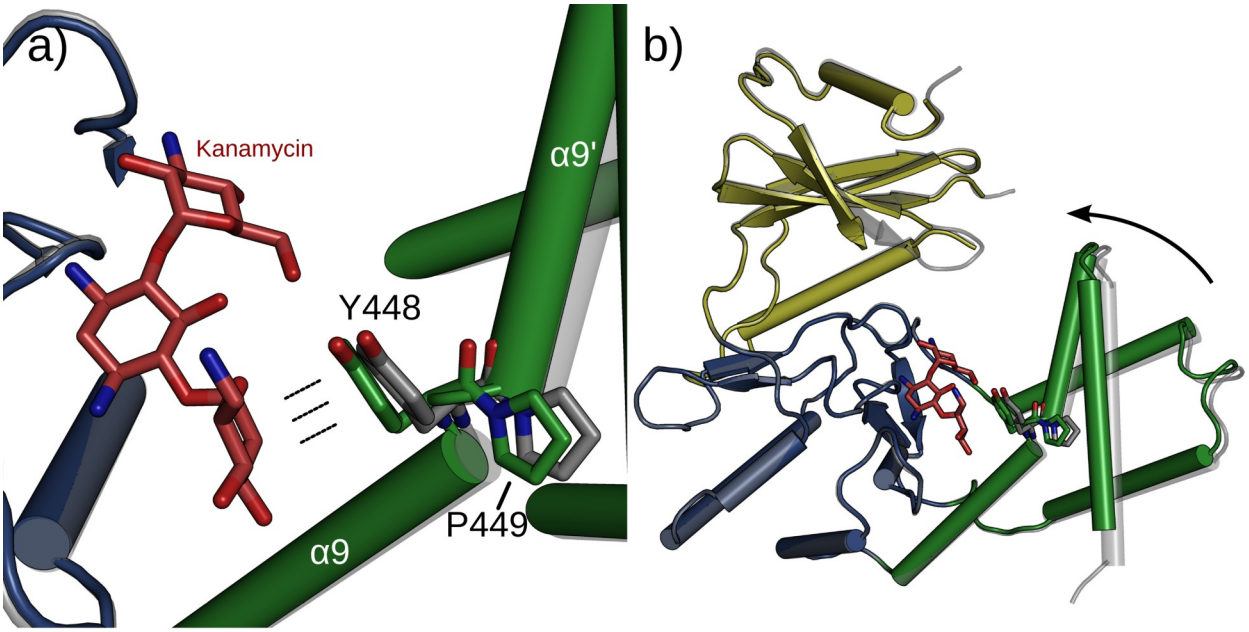

***Figure 3.11*: Aminoglycoside binding pulls the helical subdomain close**

Superimposition of chain D of GMPPNP-bound structure with no aminoglycoside (gray) and following soak with kanamycin A (coloured). a) Residue Y448 lies at a hinge point created by a discontinuity in helix α9 produced by proline 449. b) Interaction of Y448 with the aminoglycoside pulls the helical subdomain, which results in a 2 Å shift toward the rest of the protein.

3.4.5 Comparative enzyme reactivity toward different 4,6-disubstituted aminoglycosides

The 6-linked rings of kanamycin and gentamicin are built from different sugar scaffolds, corresponding to different biosynthetic pathways (Llewellyn and Spencer, 2006). The nature of this ring influences its interaction with the active site of APH(2")-Ia. While kanamycin has an aminoglucosamine ring, the garosamine ring of gentamicin rearranges the scaffold so there is no group that protrudes from the 5"-site of the ring. This allows the garosamine ring more freedom of movement in the enzyme active site, while the glucosamine ring of kanamycin and tobramycin is held closer to the active centre of the enzyme. This indeed may be why tobramycin is reactive in crystals, while gentamicin is not, and why the enzyme turns over kanamycin and tobramycin an order of magnitude more quickly (Frase et al., 2012).

For the enzyme to be fully active toward gentamicin, further structural changes are probably necessary to bring the 2"-hydroxyl group closer to the active site and for the necessary catalytic components to assemble in place. In the transition from open to closed conformation that we observe when aminoglycoside is bound, the 6-ring of gentamicin would also move toward the active site of the enzyme where it could be productively modified by triphosphate in the enzyme active site. These changes will be covered in more detail in Chapter 4.

3.4.6 4,5-disubstituted aminoglycosides provide a starting point for aminoglycoside-competitive inhibitors

The ribostamycin and other 4,5-disubstituted aminoglycosides bind in a fashion that is not catalytically competent. This suggests an avenue of inhibitor design toward APH(2")-Ia. Because this compound binds but is not chemically modified by the enzyme, ribostamycin could act as an inhibitor, competitive with respect to other aminoglycosides. Co-administration of this “dummy” substrate alongside other aminoglycosides could be a promising combination therapy for resistant infections.

An additional benefit of such compounds directed toward aminoglycoside-modifying enzymes is that they are not competitive for the nucleoside-binding pocket. Most kinase inhibitors that have been tested toward APH(2")-Ia and similar kinase enzymes bind in the nucleoside-binding pocket. A risk of targeting this binding pocket on the aminoglycoside-modifying enzyme is the chance that this inhibitor could also lead to inhibition of off-target kinase enzymes, a well-known problem in the chemotherapeutic treatment of kinase-based diseases (Bain et al., 2007; Davies et al., 2000; Shakya et al., 2011). Binding of an aminoglycoside-competitive inhibitor avoids this problem completely by not binding to the kinase pocket at all, but the antibiotic pocket instead. Unfortunately, these compounds may still be substrates for modification by the AAC(6')-Ie domain of the bifunctional protein, so this must be taken into consideration as well.

3.4.7 Implications for the development of aminoglycosides that do not bind APH(2")-Ia

The APH(2")-Ia enzyme presents an anionic neamine-binding platform that facilitates the binding of most neamine-based aminoglycosides. Disruption of the binding interface between aminoglycosides and this enzyme could then allow aminoglycosides to evade resistance by the enzyme. In fact, there are compounds that do exactly this. The N1-modified aminoglycosides like the newly developed compound plazomicin all show little modification by APH(2")-Ia (Aggen et al., 2010). This is accomplished by adding a group to the N1-amine of these aminoglycosides, which directly blocks binding to the enzyme. These compounds still act as effective antimicrobials because the group added at N1 of these compounds adds some compensatory interactions with the ribosome that offset the loss of a positively-charged N1-amine.

Unfortunately, additional modifications that interfere with binding to APH(2")-Ia will be difficult to realize and still bind the ribosome effectively. The N3-amine can not be modified without interfering with the binding of the compound to the ribosome. Modifications to the 4-linked aminohexose appear to be easily tolerated, even methylation of the 6' amine in gentamicin C1.

Outside of N1-modification the only modifications to the neamine nucleus that might be applicable in developing aminoglycosides that do not bind to APH(2")-Ia would require modification of the 2-deoxystreptamine ring itself. Modifying the 2-DOS ring by halogenation or desaturation has the potential to disrupt interactions with the neamine-binding pocket of APH(2")-Ia, while keeping important aminoglycoside-ribosome interactions intact. To the best of our knowledge, such modifications of the aminoglycoside neamine nucleus have not been attempted, and this may be a possible route of antimicrobial development.

3.5 Conclusions

The APH(2")-Ia enzyme cradles a large open cavity that allows the enzyme to accommodate binding of many different aminoglycoside antibiotics. Within this cleft, the enzyme presents a neamine-binding platform that is optimized for the 2-deoxystreptamine and 4-aminohexose rings of neamine based antibiotics. This pre-organized platform allows both 4,5-disubstituted and 4,6-disubstituted aminoglycosides to bind the enzyme using their conserved rings, while any additional rings are accommodated in the spacious cleft in the centre of the enzyme. Structural changes are induced by aminoglycoside binding that draw the distal helical subdomain closer to the reactive centre of the enzyme, helping close the active site and potentially facilitating catalysis.

While 4,6-disubstituted aminoglycosides can be productively modified as bound (and in one case is modified within crystals), the 4,5-disubstituted aminoglycosides are bound but cannot be modified. This resolves discrepancies in the field about whether these compounds are substrates or not. 4,5-disubstituted compounds are bound, but not modified by the enzyme. This binding of 4,5-disubstituted aminoglycosides suggests an additional role of the enzyme as a binding protein. APH(2")-Ia could confer low-level aminoglycoside resistance by sequestration.

The structure of this enzyme in complex with 5 different aminoglycosides gives us unambiguous structural evidence of the binding of aminoglycosides to this resistance enzyme. These models serve as the starting point for inhibitors directed at the enzyme, and for the modification of aminoglycosides that do not effectively bind to the enzyme. 4,5-disubstituted compounds could act as inhibitors for the enzyme (or as starting leads for inhibitor development). In addition, while disruption of binding to this enzyme by the addition of novel sterically blocking groups seems unlikely, modifications that alter the neamine rings themselves could reduce the binding affinity of the enzyme toward these compounds.

4 APH(2'')-Ia regulates phosphotransfer in a catalytic switch flipped by enzyme closure and the Gly-loop

4.1 Background

The APH(2'')-Ia domain of AAC(6')-Ie/APH(2'')-Ia is a kinase enzyme of the eukaryotic protein kinase-like enzyme superfamily. This lineage of enzymes emerged in ancient times, before the development of multicellular life (Scheeff and Bourne, 2005). While named for the sub-family that phosphorylates eukaryotic proteins, these enzymes are found throughout bacteria as well, and phosphorylate a broad range of substrates, not just proteins (Oruganty et al., 2016; Pereira et al., 2011). Despite the shared roots, APH(2'')-Ia and protein kinases have considerably diverged, sharing almost no identical residues, despite a shared reaction chemistry. There is a wealth of literature on the mechanisms of protein kinases, but divergence also makes it unclear how much of this functional insight is applicable to APH(2'')-Ia. Even the well-studied aminoglycoside phosphotransferases such as APH(3')-IIIa are of limited use due to a considerable divergence in sequence and structure.

Like these other aminoglycoside kinases (Hon et al., 1997), APH(2'')-Ia uses a nucleoside triphosphate, in the presence of magnesium ions, to transfer a phosphate group to the hydroxyl group of its aminoglycoside substrate. This enzyme was the first phosphotransferase to inactivate gentamicin (Le Goffic et al., 1977b), an aminoglycoside that escaped modification by other resistance enzymes. Without high-resolution structural information about the enzyme active site, a mechanistic understanding of this enzyme has been elusive. The structural details of catalysis in APH(2'')-Ia are not well understood, and we have been limited to bulk measurements of enzyme activity (Daigle et al., 1999a; Frase et al., 2012) that do not give us great mechanistic insight, and structural data from homologous enzymes (Burk et al., 2001; Shi and Berghuis, 2012). In order to study the mechanism of the enzyme, it is necessary to obtain high-resolution structural information about the APH(2'')-Ia.

4.1.1 Origins and structure of ePK enzymes

The eukaryotic protein kinase (ePK) family of enzymes is an ancient scaffold that arose in a predecessor to modern bacteria or eukaryotes (Scheeff and Bourne, 2005). The ePK enzyme framework has since been copied, recombined and elaborated in myriad combinations (Oruganty and Kannan, 2012), but maintains core features: binding of a nucleoside triphosphate and magnesium ions, binding of an acceptor substrate, stabilization of a transferred phosphate, and release of phosphorylated product and nucleoside diphosphate.

Beyond this mechanistic similarity, these enzymes have diversified into many evolutionary niches. The best studied of these niches gives the superfamily its name: the eukaryotic protein kinases that phosphorylate proteins on serine, threonine, or tyrosine residues. These enzymes play central roles in regulation of eukaryotic cells (Hanks and Hunter, 1995) and diversification into around 500 kinases in the human genome (Manning et al., 2002) produces enormous combinatorial diversity in this family of enzymes. Involved predominantly in cellular signalling, these enzymes are tuned to activate in response to a cellular stimulus and initiate and propagate signalling cascades that modulate large-scale cellular processes.

Outside of this group of canonical protein kinases, there are many other protein kinase-like and atypical kinase enzymes from this superfamily that act on non-peptide substrates. Some ePK enzymes act on lipids (Wymann and Pirola, 1998), some on small molecules (Ku et al., 2007; Peisach et al., 2003), and others have currently unknown targets. All of these enzymes conserve a catalytic architecture supported by a conserved fold and set of functional residues. This active site architecture is supported by secondary interactions with additional residues that support the catalytic elements, and these secondary elements frequently dynamically regulate the enzyme's activity (Oruganty and Kannan, 2012).

Conserved features of eukaryotic protein kinase enzymes include a cleft that binds the nucleoside triphosphate, residues that coordinate two magnesium ions, catalytic residues that activate an incoming acceptor substrate and stabilize the β-phosphate leaving group, and a flexible glycine-rich loop that caps the active site (Figure 4.1). Outside of this functional core, there are residues important for maintaining these elements in a catalytic conformation, but the

rest of the protein can diverge dramatically as selective pressures drive the non-essential parts of the protein to adopt new additional functions.

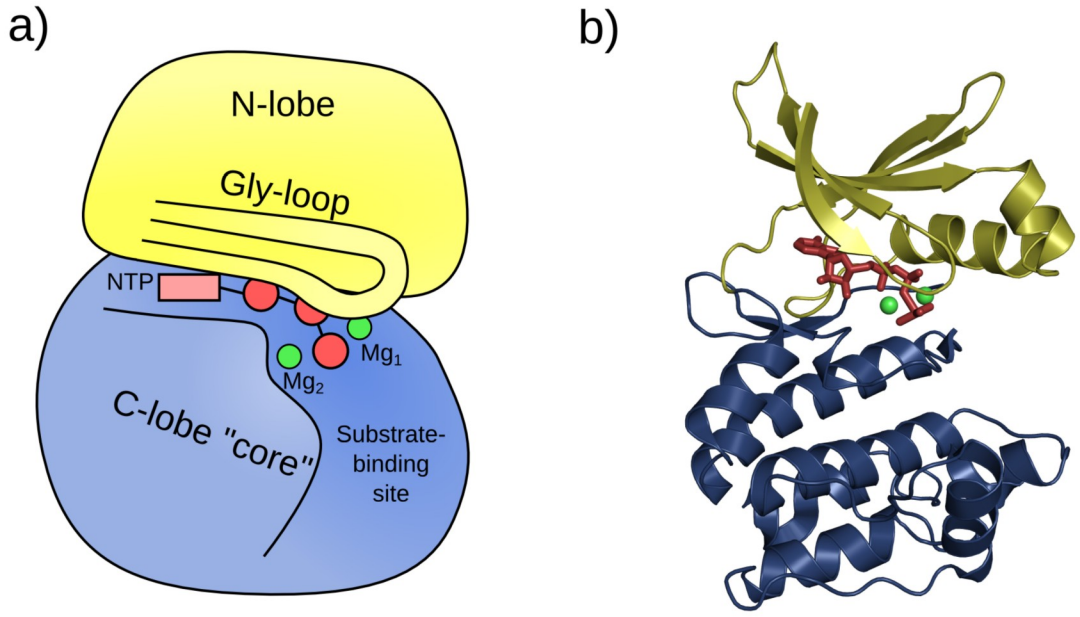

Figure 4.1: Conserved ePK kinase architecture

a) Schematic representation of the conserved ePK architecture. The N-lobe (yellow) is affixed atop the C-lobe (blue, with the nucleoside triphosphate (maroon) and magnesium ions (green) bound between. The Gly-loop of the N-lobe lies atop the triphosphate group of the nucleoside co-substrate, and stabilizes it for phosphotransfer to the substrate, primarily bound by the C-lobe. b) Crystal structure of the conserved regions of the prototypical ePK enzyme, cyclic AMP-dependent protein kinase (cAPK). This structure (PDB 1L3R) (Madhusudan et al., 2002) was determined with ADP, a triphosphate transition state mimic, and the substrate bound, and these elements represent the catalytically competent, Michaelis structure of the enzyme. Regions of the protein that are not shared with other ePK enzymes are omitted.

4.1.2 Catalytic mechanism of protein kinase-like enzymes

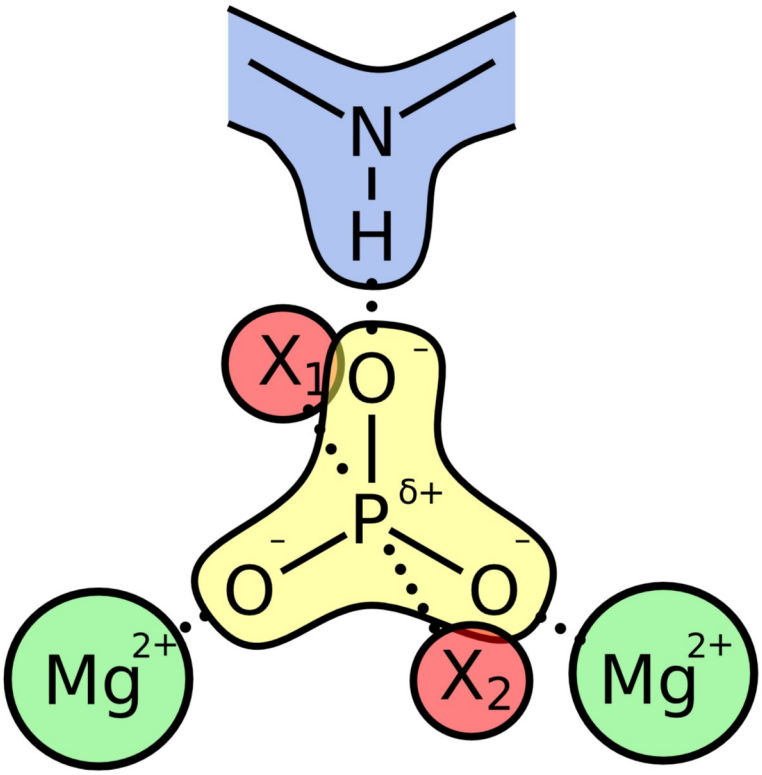

***Figure 4.2*: Stabilization of an activated phosphate group in kinase enzymes**

The phosphate group (yellow) is activated for reaction by coordination with two magnesium ions and a backbone amide of the enzyme. The incoming (X_2) and leaving (X_1) groups are collinear through the phosphate centre.

Kinases are transferase enzymes – they transfer a gamma phosphate group from a nucleoside triphosphate to an acceptor hydroxyl group using magnesium ions. This transferred phosphate needs to be stabilized for efficient transfer (Figure 4.2). The magnesium ions are used to stabilize the phosphate intermediate by drawing electron density away from the central phosphate atom, which becomes electrophilic and subject to nucleophilic attack (Valiev et al.,

2007). The third oxygen atom of the phosphate is also stabilized by a protein backbone interaction in the transition state (Madhusudan et al., 2002).

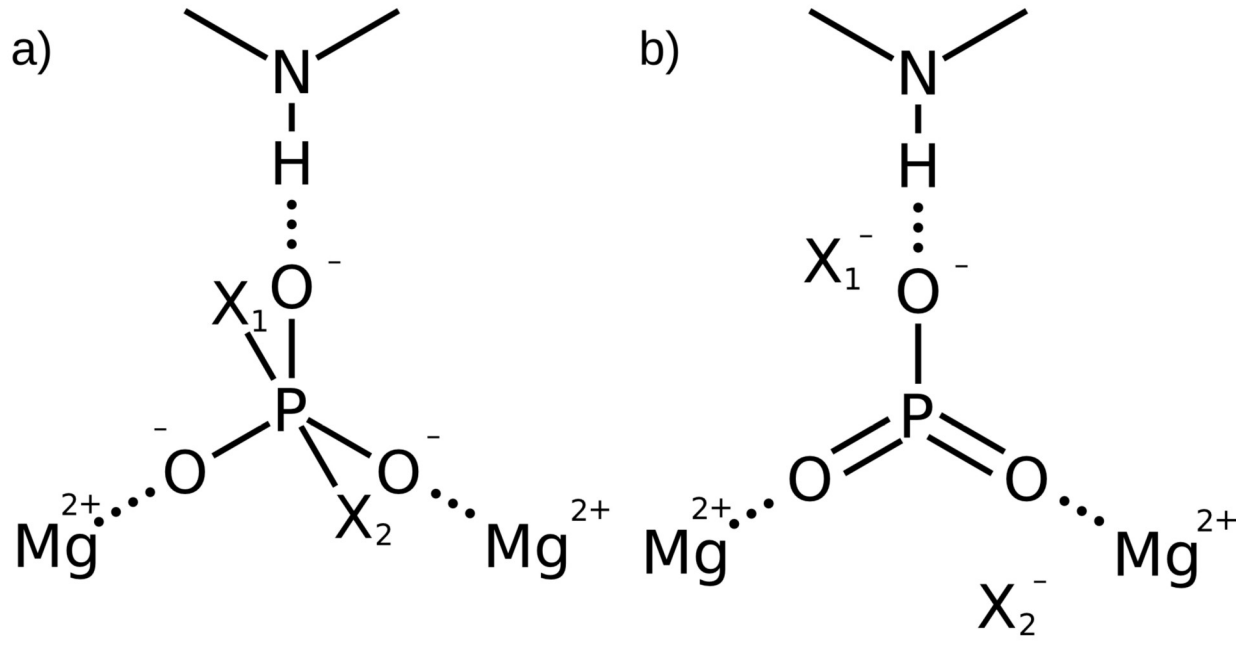

***Figure 4.3*: Mechanisms of phosphate transfer**

a) In the associative mechanism, the incoming nucleophile (X_2) associates with the phosphorus centre, creating a 5-coordinate phosphorus that then falls apart with the breakage of the bond to the leaving group (X_1) to complete the reaction. b) In the dissociative reaction, the breakage of the bond to the leaving group (X_1) leaves a metaphosphate-like intermediate that is held in the active site through coordinate interactions. The nucleophile (X_2) then reacts with the metaphosphate centre to form the phosphorylated product. The structural difference in these mechanisms is primarily the distance between leaving group and nucleophile in the transition state of the reaction.

Within this two-metal phosphate transfer framework, there is still some ambiguity in the mechanism of phosphate transfer. There are two potential mechanisms of phosphate transfer: associative and dissociative. Resembling the S_N2 and S_N1 nucleophilic substitution reactions, these mechanisms differ in the electronic configuration around the phosphorus atom, as well as the linkage of the phosphorus to other atoms during the reaction. The associative mechanism forms a penta-coordinate, phosphorane intermediate (Figure 4.3a), which has 5-coordinate trigonal bipyramidal geometry. The dissociative mechanism stabilizes a trigonal planar metaphosphate intermediate that is separated from the donor and acceptor nucleophiles (Figure

4.3b). The distance between coordinate atoms and the phosphorus centre in the dissociative mechanism are correspondingly longer, and so the distance between these atoms can been used to discern between these mechanisms. While associative and dissociative phosphate transfer mechanisms lie at the extreme, these mechanisms actually define a spectrum which can have associative-like character, or dissociative-like character. Continuous distribution of electrons means that this reaction is frequently intermediate in form between the two. Most recent evaluations of phosphate transfer in kinase enzymes find as a consensus that the reaction is primarily dissociative (Wang and Cole, 2014).

This dissociative mechanism has important consequences. A primarily associative reaction mechanism requires a tightly-coordinated acceptor substrate to facilitate the promotion of the transition state. This selects for productive substrate binding because non-optimal substrates do not easily promote the adoption of the transition state. A dissociative mechanism activates the phosphate and weakens the β-γ-phosphate linkage prior to substrate binding. This allows more flexibility in substrate binding and allows other molecules to more readily take the place of the enzyme's physiological substrate. If the enzyme activates the phosphate for transfer without the acceptor substrate present, the γ-phosphate is subject to nucleophilic attack by any appropriately-positioned nucleophile. Occasionally, this may be water, and this results in a decoupling of the reaction and a net hydrolysis of the co-substrate. As a result, many kinases have some measurable intrinsic nucleoside hydrolysis activity (Rominger et al., 2007).

4.1.3 Conserved structural motifs of protein kinase-like enzymes

Kinase enzymes are composed of two lobes, the N-lobe and C-lobe. These lobes form a cleft in which the nucleoside substrate binds. Active site residues are used to co-ordinate metal ions, usually magnesium, in the active site of the enzyme. The prototypical enzyme of this family is cyclic-AMP-dependent protein kinase (cAPK), sometimes also referred to as protein kinase A (McClendon et al., 2014). The enzyme stabilizes the transfer of phosphate from ATP to its protein substrate using two magnesium ions and conserved interactions with specific active-site residues.

One of the central motifs of the ePK fold is the β1-β2 hairpin that lies atop the nucleoside binding site. This region, known sometimes as the P-loop, G-loop, or glycine-rich loop[4], is dynamic and plays a critical role in the catalysis in transferring the γ-phosphate to its acceptor substrate. The loop is named for the three glycine residues that lie in this hairpin in cAPK, glycines 50, 52, and 55. These glycine residues are well-conserved in protein kinases, and G52 is nearly universal (Hemmer et al., 1997). These residues impose a minimal steric hindrance to movement, and maximum mobility. Correspondingly, the Gly-loop is typically one of the most mobile parts of a kinase enzyme structure.

To transfer the phosphate group, the enzyme uses two magnesium ions Mg_1 and Mg_2, which are held in place by structurally conserved residues of the protein. In cAPK, these residues are D184, which coordinates Mg_1 in a bidentate form, and N171, which binds Mg_2 together with D184 (Figure 4.4). An active-site aspartic acid residue (D166 in cAPK) is universally conserved and required to deprotonate the hydroxyl group of the acceptor substrate. The enzyme also uses a catalytic lysine residue, K72, to stabilize the negatively-charged phosphate leaving group. Completing the active site, the G52-S53 peptide of the Gly-loop coordinates the transferred phosphate. The backbone flexibility of glycine 52 is important to allow the peptide to rotate and coordinate the phosphate during transfer (Aimes et al., 2000; Hemmer et al., 1997). The near-complete conservation of this glycine residue (Kannan et al., 2007; Manning et al., 2002) indicates the importance of this flexibility.

The catalytic residues are supported by a framework that holds these residues in place, and further by a domain scaffold that stabilizes the entire protein (McClendon et al., 2014). Within the core of the enzyme, additional interactions hold these five catalytic residues in phosphotransfer-compatible conformations. A buried residue, most frequently histidine (Y164 in cAPK) supports backbones conformations of D184 and D166 that are typically be unfavourable. This residue residue forms part of an ancient “strain switch” that regulates the enzymes (Oruganty et al., 2013). A glutamate residue (E91) stabilizes the active-site lysine residue. A series of residues buried in the hydrophobic core of the enzyme form “hydrophobic spines” that stabilize the enzyme as a whole for catalysis (Taylor et al., 2012). These spines break and form in response to various regulatory mechanisms to inhibit and activate the enzyme activity. Additional

4 I will refer to this structural element as the Gly-loop for the important conserved glycine, as not all Gly-loops are “rich” in glycine residues.

residues that are less conserved that play a role in catalysis in ePK include lysine K168, which helps stabilize the transferred γ-phosphate (Szarek et al., 2008), and tyrosine Y204, which is implicated in dynamic changes in the enzyme (Yang et al., 2004a, 2005), and contacts both the substrate and K168 (Figure 4.4b).

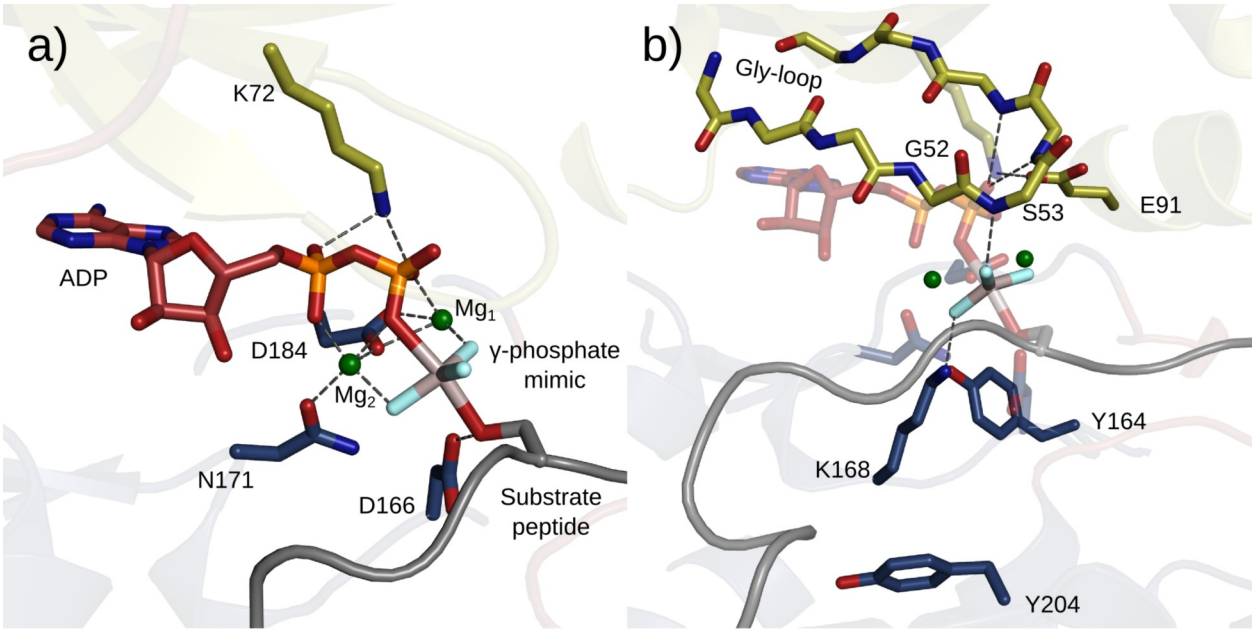

Figure 4.4: Active site architecture of a eukaryotic protein kinase

Catalytic core of a protein kinase, cyclic AMP-dependent protein kinase. This structure is the Michaelis complex mimic structure of cyclic-AMP dependent protein kinase, PDB 1L3R (Madhusudan et al., 2002). Residues in yellow are located in the N-terminal lobe, while blue residues are in the C-terminal core lobe. a) Catalytic residues. D184 and N171 coordinate magnesium, while K72 stabilizes the leaving group and D166 the acceptor for the phosphate transfer reaction. b) Secondary elements that support catalysis. The Gly-loop (side-chains omitted for clarity) directly bonds to the transition state γ-phosphate, here indicated by the mimic compound AlF_3. E91 helps stabilize the K72 residue, while Y164 supports energetically unfavourable backbone conformations of D184 and N171. K168 assists phosphotransfer by contacting the transferred phosphate as well, while Y204 has been implicated in longer-range stabilization interactions that impact catalysis.

4.1.4 Regulation and catalytic activation of protein kinases

Specific interactions that facilitate a kinase's active site chemistry are supported by an enzyme architecture that places these chemical elements in exactly the appropriate geometry for this reaction to take place. Structural changes within this architecture can in turn modulate the activity and function of the enzyme. Phosphorylation, ligand binding, protein:protein interactions and other mechanisms can influence this structural transition to an activated kinase (Kornev and Taylor, 2015).

In addition to gross changes that form and break the central spines of the enzyme or perturb the residues buried in the core, kinases also modulate the enzyme activity through interactions that constrain or release the Gly-loop. Conformational changes that force the loop into different positions can activate or inhibit the enzyme. With several glycine residues, this loop is typically mobile in the absence of stabilizing factors. Interactions that stabilize the Gly-loop allow it to, in turn, stabilize the phosphotransfer transition state (Aimes et al., 2000; Barouch-Bentov et al., 2009; McNamara et al., 2011).

The mechanisms of regulation in protein kinases are diverse, while the catalytic mechanism appears to be conserved throughout all protein kinases. It is less clear if this is the case for other enzymes in the extended ePK family, such as aminoglycoside kinases and other small-molecule kinases. These enzymes in the “understudied kinome” (Kannan et al., 2007) may share much in common with protein kinases, or they may have diverged in mechanism. To evaluate this, it is necessary to study these branches of the ePK family individually. To learn about phosphorylation in APH(2")-Ia, we need to examine it directly.

4.1.5 Aminoglycoside kinases are functionally and structurally divergent from protein kinases

The determination of the structure of the APH(3')-IIIa antibiotic resistance enzyme revealed that aminoglycoside phosphotransferase enzymes are part of the eukaryotic protein kinase-like enzyme superfamily (Hon et al., 1997). While this was initially surprising, aminoglycoside phosphotransferase enzymes are now well established non-protein kinase ePK-like enzymes.

While APH(3')-IIIa and APH(2")-Ia show some protein kinase activity (Daigle et al., 1999b), they are primarily active toward aminoglycoside substrates, phosphorylating these small molecules as a means of chemically inactivating the antibiotics.

It is tempting to consider that aminoglycoside kinases might be simpler enzymes than eukaryotic protein kinases, as they play a less nuanced role in bacterial cells, directly phosphorylating and inactivating aminoglycoside antibiotics. However, it should be remembered that these enzymes have had as much time as those protein kinases to evolve and adapt to their own evolutionary niche. Aminoglycoside phosphotransferases lack regulatory elements that are central to the activity of protein kinases like cAPK. The activation loop and N- and C-terminal regions of protein kinases are not conserved in aminoglycoside phosphotransferases. At the same time, aminoglycoside kinases contain unique elements that are not found in protein kinases, perhaps linked to APH-specific functions. These enzymes have the potential to show just as much complexity in activity as their protein-targeting counterparts.

Thorough structural analysis of APH(3')-IIIa (Burk et al., 2001; Fong and Berghuis, 2002, 2009; Hon et al., 1997) has illuminated the means by which APH(3')-IIIa carries out phosphotransfer. While APH(3')-IIIa maintains many features in common with eukaryotic protein kinases, it also diverges considerably in functional ways. The enzyme shows almost no induced movement upon substrate binding, which is the common means of helping activate eukaryotic kinases. The enzyme also lacks an activation loop, further differentiating it as a distinct kinase. These findings help us understand APH(3') enzymes, but it is unclear if the same mechanisms will apply in APH(2")-Ia and other APH(2") family kinases. Phylogenetic analysis indicates that these kinase families have independent origins (Oruganty et al., 2016), so mechanisms of catalysis in APH(3')-IIIa may not be relevant for APH(2")-Ia.

In addition to APH(3')-IIIa, multiple additional enzymes in the extended antibiotic phosphotransferase family have had structures determined. This includes the macrolide phosphotransferase enzymes MPH-I and MPH-II (Fong, *et al.*, under review), spectinomycin phosphotransferase APH(9)-Ia (Fong et al., 2010), hygromycin phosphotransferase APH(4)-Ia (Stogios et al., 2011), and aminoglycoside phosphorylating enzymes APH(3')-Ia (Cox et al., 2015), APH(3')-IIa (Nurizzo et al., 2003), APH(2")-IIa (Young et al., 2009), APH(2")-IIIa (Smith et al., 2012), and APH(2")-IVa (Shi et al., 2011). Despite this wealth of structural information

about these enzymes, the catalytic mechanism of enzymes in this group of resistance factors could not be directly studied due to a lack of structures with well-defined triphosphate substrate. Structures with triphosphate substrate are necessary to understand the nuanced role these enzymes play within bacterial cells, and the energetic consequences of catalysis.

4.1.6 Fitness cost and co-substrate breakdown in resistance enzymes

Aminoglycoside kinases are enzymes that use a metabolic co-substrate (nucleoside triphosphates) to chemically modify an antibiotic. This mechanism suffers from possible inefficiencies. Metabolic waste generated by decoupling of chemical reactions has the potential to show a considerable fitness cost for antibiotic resistance enzymes. Inefficiencies in an enzyme can lead to off-target activity, with either the wrong product produced or simply wasted donor substrate, especially if these reactions are thermodynamically favourable. If present in high enough levels, off-target enzymatic activity can impose a considerable fitness cost upon a resistance enzyme (Kim et al., 2006b). If this fitness cost is significant, evolution will select for innovations that reduce and mitigate this cost.

Abrogation of fitness cost can occur in many ways, often through active regulation. The fitness cost of aminoglycoside resistance enzyme is mitigated by making antibiotic resistance genes cryptically expressed (Magnet et al., 1999), or by placing them under antibiotic-responsive regulation (Hoffman et al., 2005; Jia et al., 2013). This molecular regulation happens on the protein synthesis or the population biology level, and is not responsive to the immediate addition of antibiotics. Because of the cascading cellular effects of aminoglycosides, minutes can matter, and so having effective proteins already synthesized can be of great benefit, but there still remains a need to reduce the fitness cost of these already existing aminoglycoside resistance factors. It is possible that resistance factors like APH(2")-Ia carry intrinsic means of reducing their fitness cost – structural study can probe these mechanisms.

4.1.7 Experimental approach

The mechanism of action of APH(2")-Ia is not easily inferred from structures of homologous enzymes. To examine the structural transitions in the active site of APH(2")-Ia, the enzyme was crystallized with the nucleoside GMPPNP (as described in Section 3.2). This structure revealed a structural transition between two triphosphate conformations that necessitated the determination of multiple additional crystal structures to study the mechanistic details of phosphate transfer in this enzyme. The wildtype enzyme was also crystallized with GTP, GTP-γ-S, GDP, and GMPPCP. Crystals prepared with GTP were also soaked with gentamicin to generate a dead-end complex with GDP and gentamicin bound to the enzyme.

In addition to these structures determined with the wildtype enzyme, two mutants were generated to probe interactions of residues with the active site of the enzyme. S214A and Y237F mutants were generated using site-directed mutagenesis, and purified using the same protocol as the wildtype enzyme. These mutants were crystallized in under the same conditions, which allows us to directly probe the impact of these residues on the catalytic centre of the enzyme, and this also allows us to probe the phosphate hydrolysis activity of these mutants to track their impact on the activation of the enzyme's GTP co-substrate. Collectively, these structures, along with the aminoglycoside-bound structures first presented in Chapter 3, permit us to propose a mechanism for activation of the APH(2")-Ia enzyme that mitigates the fitness cost of the resistance factor, and indicates that APH enzymes can show complicated modulation of activity through novel means independent from that of distantly related protein kinases.

4.2 Methods

4.2.1 Site-directed mutagenesis

Primers for site-directed mutagenesis were purchased from BioCorp, Inc (Montreal). Sequences for these primers are provided in Table 4.1. These primers were prepared in molecular biology grade water at a concentration of 0.1 ng/μL. Diluted primers were used to prepare an amplification reaction in 50 μL with 0.05 ng/uL template DNA – the pET-22b-APH(2")-Ia expression plasmid. This reaction was prepared with 100 μM respective dNTPs and the PfuX7 enzyme (Nørholm, 2010). This reaction was run for 30 cycles of alternating heat in a thermocycler, starting with 2 minutes denaturation at 95˚C, and 15 minutes annealing and extension at 72˚C. At completion of this cycle amplification product was easily visible on an agarose gel, and was transformed in to competent *E. coli* DH5α cells and grown on ampicillin-agar. Single colonies were grown in Luria-Bertani Broth and the plasmids were extracted and purified by the alkaline lysis. Plasmids were sequenced at the Genome Québec Innovation Centre to confirm the successful generation of mutant plasmids.

Primer	**Sequence**
S214A forward	5'-GTATTGAAATAATCGGTAGTGGTTATGATGCTGTGGCATATTTAGTTAATAATGAATAC
S214A reverse	5'-GTATTCATTATTAACTAAATATGCCACAGCATCATAACCACTACCGATTATTTCAATAC
Y237F forward	5'-CAAAATTTAGTACTAATAAGAAAAAAGGTTTTGCAAAAGAAAAAGCAATATATAATTTTTTAAATAC
Y237F reverse	5'-GTATTTAAAAAATTATATATTGCTTTTTCTTTTGCAAAACCTTTTTTCTTATTAGTACTAAATTTTG

Table 4.1: Sequence of primers used for site-directed mutagenesis studies of APH(2")-Ia

4.2.2 Protein production and purification

Wildtype APH(2")-Ia protein was produced and purified as described in Sections 3.2.1-3.2.2. Plasmids for the Y237F and S214A mutant enzymes were transformed into BL21(λDE3) cells, and the protein was produced in the same fashion as the wildtype protein. The Y237F mutant enzyme and additional wildtype protein were subjected to a modified purification protocol that greatly improved purity by eliminating nucleic acid contamination that had been present in previous wildtype protein purifications. In this purification method, cells were lysed

by ultrasonication and clarified by ultracentrifugation, in the same manner as previous preparations. The lysate from these cell pellets was run over a kanamycin-agarose column and eluted in a gradient from 25-500 mM NaCl. Elution fractions containing the enzyme were concentrated and diluted in buffer with 25 mM NaCl to bring the salt concentration below 40 mM. This exchanged elution was then loaded to a 6 mL ReSource Q column (GE Biosciences) and eluted with a gradient up to 1M NaCl. The fractions containing APH(2")-Ia were then loaded to a Superdex 75 26/60 column (GE Biosciences) as before, with a running buffer of 10mM HEPES pH 7.5, 5% glycerol. This protein was concentrated to ~12 mg/mL and snap frozen in aliquots in liquid nitrogen, stored at -80˚C.

4.2.3 Crystallization

Crystallization of APH(2")-Ia was carried out in a similar fashion as described for most crystals in Section 3.2.3. All compounds were obtained from Sigma-Aldrich unless otherwise indicated. Reservoir solution was prepared that was 10% polyethylene glycol 3350, 8% glycerol (Fisher Scientific), 100mM HEPES pH 7.5, and 100mM $MgCl_2$. Concentrated protein at 10-15 mg/mL was prepared with 1-2 mM GDP, GMPPNP, GMPPCP, GTP, or GTP-γ-S and 2-4 mM $MgCl_2$ and equilibrated 2:1 with reservoir solution, prior to spin filtration and crystallization drop setup using a 2:1 ratio of this clarified protein solution and reservoir solution, and streak-seeded using a horse-hair fibre.

WT and Y237F protein that was more pure following a modified purification protocol did not require a pre-incubation step, and instead was prepared as a simple saturated protein solution in hanging-drop crystal trays (VDXm model, Hampton Research). In this case, the protein was prepared with 1-2 mM GMPPNP or GDP, 2-4 mM magnesium chloride, and directly mixed with equal volume of reservoir solution, and streak seeded from previous experiments using a horse hair.

4.2.4 Structure solution, refinement, and analysis

In addition to the APH-GMPPNP structure and aminoglycoside soaked-structures described in Chapter 3, structures of the wildtype enzyme with GMPPCP and GDP bound were also determined. The GTP-γ-S and GTP crystals showed no density for a γ-phosphate group, so these structures represented the GDP-bound form, and the highest resolution of these datasets, that

determined from co-crystallization with GTP-γ-S was taken as the GDP-bound model (Table 4.2). Similarly, the best data set for a GDP-gentamicin bound complex was determined from crystals prepared with GTP, but by the time the crystals were subjected to diffraction, there was no observable electron density for the γ-phosphate of the compound.

In addition to structures of the wildtype enzyme, the structure of the S214A and Y237F mutants were determined with bound GMPPNP, while an additional structure of Y237F with bound GDP was also determined. All of these structures were solved by the following methods.

Structures were determined by difference Fourier synthesis using the crystal structures determined of APH-GMPPNP (Section 3.2.6) as starting model. A model without non-protein atoms, with flexible loops and termini excluded, was used to calculate approximate phases and begin refinement of the structure. Rigid-body refinement was followed by NCS-restrained refinement in REFMAC5 (Murshudov et al., 1997). Flexible loops were modelled in manually, where possible, followed by placement of water molecules and ligands, where justified by Fo-Fc difference map density.

Models determined and described in Chapter 3 will also be described in this chapter. The conformation of the bound nucleoside changes in these aminoglycoside-bound structures. The conformational changes of the enzyme (mentioned in section 3.4.4) was observed and compared to aminoglycoside-free forms. The triphosphate in the enzyme active sites was carefully modeled in all cases.

The occupancy of two triphosphate conformations of models was determined by finding optimal stabilized and activated conformations from structures with only one of these conformations visible, and merging these into a single ligand with 50% occupancy of each, including water molecules displaced by this transition. This occupancy was refined in REFMAC5, although in most cases manual intervention was also required to converge upon the occupancy that best reflected the visible electron density. Occupancies lower than 20% were ignored, and otherwise occupancies were rounded to the nearest 5%.

4.2.5 Phosphate release assay

An assay was developed for tracking released phosphate from GTP catalysed by APH(2")-Ia. This assay tracks free released inorganic phosphate produced by hydrolysis of the co-substrate, using a Malachite Green based colourimetric reagent, purchased from BioAssay Systems. Incubation of APH(2")-Ia or mutant with GTP at room temperature was used to liberate phosphate, following which the reaction was quenched and developed for 30 minutes before measuring absorbance of the solution at 630 nm. A standard curve of inorganic phosphate from 1.25-40 μM phosphate was used to calibrate the assay, and a control of GTP in the absence of enzyme was measured to control for background hydrolysis of the co-substrate.

The enzyme was incubated for variable time periods with GTP in the presence of 10 mM $MgCl_2$ and 50 mM HEPES pH 7.5. Following quenching, development and measurement at 630 nm in a microplate reader, the concentration of liberated phosphate was determined and used to infer the rate of phosphate release per enzyme active site per minute. This assay was used to determine comparative rates of hydrolysis of the S214A and Y237F mutants versus the wildtype enzyme, and to track changes to the rate of phosphate release upon addition of aminoglycoside to the enzyme.

	APH-GMPPCP	APH-GTPγS (GDP)	APH-GTP (GDP)-Gentamicin	APH S214A-GMPPNP	APH Y237F-GMPPNP	APH Y237F-GDP
Data collection						
X-ray source	CLS Beamline 08-ID	Rigaku MicroMax 007	CLS Beamline 08-ID	CLS Beamline 08-ID	Rigaku MicroMax 007	Rigaku MicroMax 007
Wavelength (Å)	0.9795	1.5418	0.9795	0.9795	1.5418	1.5418
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
a, *b*, *c* (Å)	90.3, 99.9, 93.6	90.5, 99.1, 92.3	90.3, 98.5, 93.0	90.2, 99.7, 93.3	90.2, 99.7, 93.0	89.9, 99.7, 92.9
β (°)	105.9	105.2	105.3	105.3	105.1	104.9
Resolution (Å)	58.92-2.15 (2.19-2.15)	33.19-2.35 (2.41-2.35)	55.61-2.50 (2.57-2.50)	58.84-2.25 (2.30-2.25)	33.60-2.15 (2.19-2.15)	33.35-2.25 (2.30-2.25)
$CC_{1/2}$	0.993 (0.718)	0.997 (0.511)	0.997 (0.466)	0.985 (0.571)	0.996 (0.479)	0.997 (0.514)
R_{merge}	0.091 (0.621)	0.139 (1.211)	0.096 (1.091)	0.074 (0.477)	0.082 (0.623)	0.134 (1.219)
$I/\sigma I$	12.3 (2.6)	10.9 (1.7)	9.0 (1.6)	10.6 (2.4)	8.4 (1.6)	9.1 (1.5)
Completeness (%)	95.0 (65.6)	100.0 (100.0)	100.0 (99.9)	96.6 (98.3)	99.8 (97.2)	100.0 (100.0)
Multiplicity	3.9 (3.8)	7.2 (6.9)	4.2 (4.2)	2.0 (1.9)	3.4 (3.0)	7.2 (7.0)

Table 4.2: Data collection statistics for APH(2")-Ia datasets described in this chapter

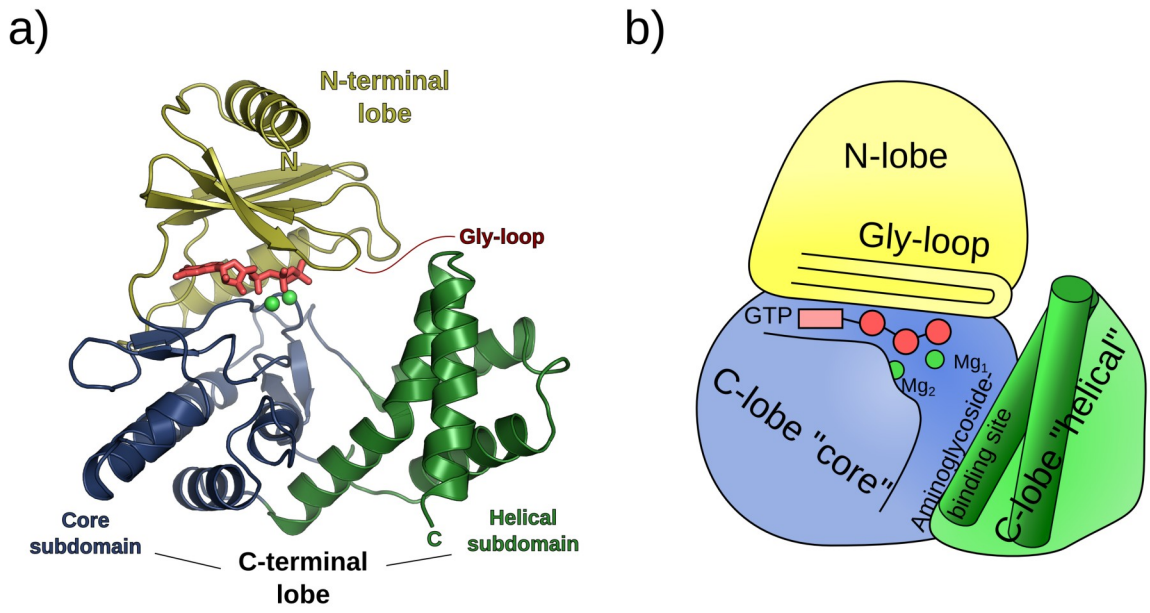

Figure 4.5: Summary of features of APH(2'')-Ia

a) Crystal structure of APH(2")-Ia domain in complex with GMPPNP and magnesium. b) Schematic of the APH(2")-Ia domain including the N-lobe (yellow) core subdomain of the C-lobe (blue) and helical subdomain of the C-lobe (green), with the C-terminal helices of the enzyme indicated. Comparable regions to cAPK (Figure 4.1) are labelled.

	APH-GMPPCP	APH-GTPγS (GDP)	APH-GTP (GDP)-Gentamicin	APH S214A-GMPPNP	APH Y237F-GMPPNP	APH Y237F-GDP
Resolution	2.15	2.35	2.5	2.25	2.15	2.25
No. unique reflections	83033	65574	54412	72914	86206	75134
R_{work}/ R_{free}	0.1559/0.1983	0.1795/0.229	0.1850/0.2298	0.1572/0.1987	0.1635/0.2101	0.1720/0.2227
No. atoms						
Protein	9827	9934	9758	9646	9785	9854
Ligands	194	141	261	164	145	141
Water	1059	925	466	1074	1660	1469
B-factors						
Protein	48.2	50.2	62.8	49.2	47.4	53.6
Ligands	47.3	43.5	62.6	46.5	43.5	47.9
Water	54.3	52.2	57.5	55.7	58.7	60.1
R.m.s deviations						
Bond lengths (Å)	0.0159	0.0130	0.0136	0.0152	0.0151	0.0139
Bond angles (°)	1.5820	1.5800	1.6250	1.5360	1.6460	1.6260
Ramachandran						
%Favoured	97.57	97.22	96.60	97.03	97.10	96.60
%Allowed	2.00	1.93	2.98	2.45	1.96	2.89
%Outlier	0.43	0.84	0.43	0.52	0.94	0.51

Table 4.3: Structural statistics for models of APH(2")-Ia bound to GTP analogues, GDP, and mutants bound with GMPPNP and GDP, as well as a gentamicin soak of the GDP-bound form

4.3 Results

4.3.1 APH(2'')-Ia conserves important catalytic features with eukaryotic protein kinases

APH(2'')-Ia shows structural hallmarks of the ePK superfamily of enzymes, conserving important elements with these proteins from their ancient ancestors. As discussed in Chapter 3, the enzyme can be subdivided into three sections: the N-terminal lobe, core subdomain of the C-terminal lobe, and helical subdomain of the C-terminal lobe (Figure 4.5). The catalytic elements of the enzyme are contained within the cleft between N-lobe and core subdomain, where the enzyme coordinates the nucleoside triphosphate ligand using catalytic magnesium ions. The binding site for these compounds lies adjacent to the aminoglycoside-binding site, where these compounds can bind and be modified.

Examination of the structure and comparison to eukaryotic protein kinases indicates that the elements of catalysis of the protein kinase enzymes are conserved in the same positions in APH(2'')-Ia. This is especially clear in comparisons of chain D of the GDP + gentamicin-bound structure of APH(2'')-Ia with the transition state mimic-bound structure of cAPK (PDB 1L3R, Figure 4.6). The catalytic residues of APH(2'')-Ia lie in equivalent positions to the analogous residues of cAPK, keeping the phosphates of the nucleoside and magnesium ions in the identical position. D393 coordinates Mg_1, while Mg_2 is coordinated between D393 and H379. These magnesium ions are present in every structure of the enzyme, aided by the saturating (100 mM) concentration of magnesium present in the crystallization solution.

A notable observation in the study of structures of APH(2'')-Ia is the mechanism of change in magnesium coordination, which can be linked to catalysis in these enzymes. From the activated triphosphate to the product GDP, Mg_2 moves from a trigonal bipyramidal coordination to an octahedral coordination. The oxygen atom bridging between beta and gamma phosphates coordinates to this magnesium ion, and during transfer this magnesium ion's transition likely offsets some energetic costs of the reaction. The coordination change of the magnesium may provide a path by which the energy barrier for transfer of the phosphate to acceptor is lowered, facilitating the reaction through Lewis Acid attack (Valiev et al., 2007). The change in active site

magnesium ion coordination of APH(2")-Ia also reflects a mechanistic similarity conserved with protein kinases, which also undergo changes in the coordination of magnesium ions during catalysis.

In APH(2")-Ia, lysine 226 interacts with the β-phosphate of the analogue, and glutamate 240 stabilizes that lysine. In contrast to ePK enzymes where these residues can move to activate or deactivate the enzyme, these residues remain in the same conformation, fixed in the “active” state in APH(2")-Ia in every structure determined. Aspartate 374, the universally conserved catalytic base, lies in position to abstract a proton from the substrate hydroxyl group, and also shows no change in any structures. The backbone interaction that stabilizes the transferred phosphate, from the G52-S53 peptide of cAPK, is fulfilled by the G211-Y212 peptide of APH(2")-Ia, where it adopts the exactly identical conformation in the APH(2")-Ia superimposed with cAPK (Figure 4.6b). While I could not determine a structure with a phosphate mimic bound in this position, the loop still adopts the position where it can move and transfer phosphate to the aminoglycoside substrate. The product-bound form of the enzyme with tobramycin-2"-phosphate also exhibits this loop conformation (Figure 4.7).

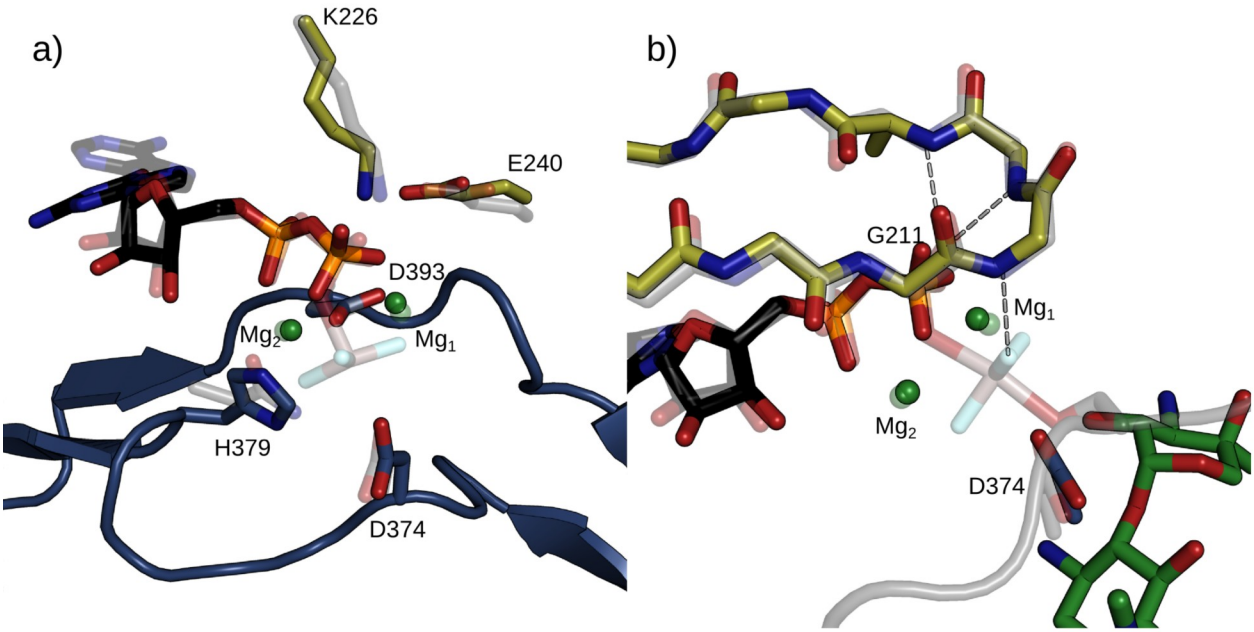

***Figure 4.6*: Comparison of active site of APH(2'')-Ia to a catalytically active protein kinase structure**

a) ADP-AlF_3-substrate-bound cAPK (transparent) and GDP- and gentamicin-bound APH(2")-Ia structures superimposed. The catalytic residues, including magnesium-coordinating residues and leaving-group stabilizing lysine residues superimpose in the same catalytic framework. b) Superimposition of the same structures to illustrate the Gly-loop conformation that is positioned identically to transfer the γ-phosphate group to the acceptor substrate. Structure of cAMP is from PDB 1L3R (Madhusudan et al., 2002).

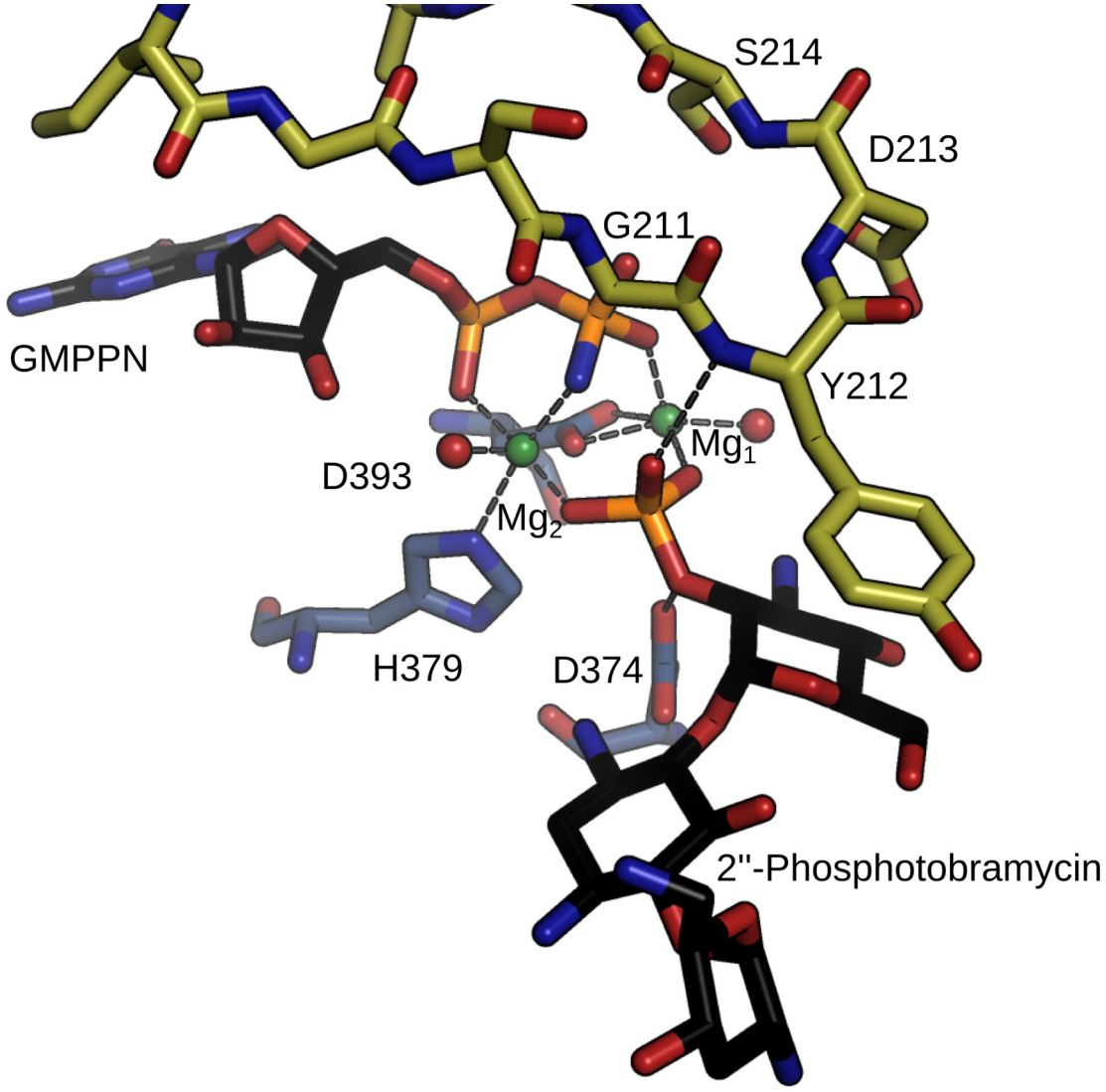

***Figure 4.7*: 2''-Phosphotobramycin coordination in the active site of APH(2'')-Ia**

The Gly-loop (yellow) forms a stabilizing interaction with the transferred phosphate group, consistent with mechanistic studies that implicate this residue in the productive transfer of phosphate from donor to acceptor substrate.

4.3.2 Structural elements that regulate protein kinases are altered in APH(2")-Ia

While the conserved core and catalytic residues of APH(2")-Ia lie in the same position as those of protein kinases, the enzyme also shows deviations in topology, sequence, and tertiary structure. The most obvious difference is the large helical subdomain inserted in the C-terminal lobe of APH(2")-Ia. This region of the protein forms very few interactions with the rest of the enzyme, and even aminoglycoside binding interactions only make use of Y448 and E445 in this section (Section 3.3.2). This large structural element of the enzyme takes the place of the activation loop in protein kinases, which is not present in APH(2")-Ia.

In protein kinases, phosphorylation of the activation loop leads to activation in two ways. First, it removes occlusion of the substrate binding site, allowing peptides to bind. In addition, the phosphate of this group forms a salt bridge with an arginine residue, which forms a a conserved “strain switch” (Oruganty et al., 2013) with the N-terminal aromatic residue and C-terminal aspartic acid, which together form the HRD motif of the enzyme. In APH(2")-Ia, this residue is replaced with a glycine, which results in the adjacent histidine 374 and aspartate 376 residues always remaining in the activated position. The strain switch of APH(2")-Ia is permanently set to “on”.

Like other features of protein kinases that are missing in APH(2")-Ia, a tyrosine residue that plays a critical role in the dynamics of the protein kinases is missing in APH(2")-Ia. In cAPK tyrosine 204 is implicated in dynamic transitions, and it stabilizes catalysis by interacting with residues adjacent to the active site (Yang et al., 2005). This residue is missing in APH(2")-Ia, but the location of Y204 in cAPK is instead filled by the aminoglycoside binding site in APH(2")-Ia. In this way, it is possible that bound aminoglycosides fill the equivalent role and help stabilize the active site to help drive the phosphotransfer reaction.

Another notable difference between APH(2")-Ia and protein kinases is a residue on the Gly-loop of the enzyme. Serine 214 of APH(2")-Ia takes the place of a glycine residue in most protein kinases. Of the three conserved glycine residues of the Gly-loop, this glycine residue is the least conserved, and can be replaced with the minimal negative effect on enzyme catalysis (Hemmer

et al., 1997). In contrast to protein kinases, where a serine in this position appears to play little functional role, there are structural changes that appear to have functional importance. This serine residue changes in response to larger conformational changes in the APH(2")-Ia enzyme.

4.3.3 APH(2")-Ia opens and closes in response to substrate binding

Despite the crystal packing environment, structural changes can be observed in crystal structures of APH(2")-Ia. These changes occur both within and between crystal forms consistent with ligand-induced conformational changes. Four protein chains exist in the asymmetric unit of these structures, and chain D shows more mobility than the other chains, a result of reduced crystal packing upon this chain and increased freedom of motion. This allows us to observe structural changes that indicate the behaviour of the “free” form of the protein, outside of a crystal lattice. The enzyme transitions between conformations that can be classified as open, closed, and intermediate.

In the open conformation of the enzyme, the helical subdomain does not form any contacts with the rest of the enzyme. To move to intermediate and closed conformations, this subdomain hinges toward the rest of the protein, closing the large central cleft, where the aminoglycoside binds at the hinge point of this movement. In addition to this rigid-body movement of the helical subdomain, the B-loop and Gly-loop rearrange to condense over the active site (Figure 4.8a,b).

The open and closed conformation occur as a result of different bound nucleoside, where chain D of GMPPNP and GMPPCP-bound structures adopt open conformations, while this chain of the GDP-bound structure is fully closed. This difference indicates that the transition between these conformations responds to the bound nucleoside.

This change is also influenced by aminoglycoside binding. In structures soaked with aminoglycoside, the helical subdomain closes 1-2 Å and brings the helical subdomain closer to the rest of the enzyme, as previously described in Section 3.4.4. This change happens with kanamycin, gentamicin, and neomycin bound and in one of the ribostamycin-bound structures, although the second structure with ribostamycin bound does not show extensive closure (Figure 4.8c, d).

The link between the active site nucleoside and the helical subdomain lies on the Gly-loop. Closure of the helical subdomain brings it into contact with the Gly-loop, resulting in steric clashes and a displacement of the Gly-loop, indicated by poorer electron density for this loop in intermediate conformations. In the fully-closed conformations, the Gly-loop adopts a new conformation condensed over the active site with a hydrogen bond to tyrosine 455 of the helical subdomain (Figure 4.8b). The structure determined with the active-site Y237F mutant recapitulates this finding. When crystallized with GDP, the enzyme does not close, as an important interaction between Y237 and the Gly-loop cannot form (examined in further detail in section 4.3.5). As a result, the APH(2'')-Ia enzyme in the Y237F-GDP structure remains in the open conformation.

The importance of structural transitions of the Gly-loop is evident when we consider interactions of this loop with the nucleoside triphosphate substrate of the enzyme, which adopts a novel conformation in the structures.

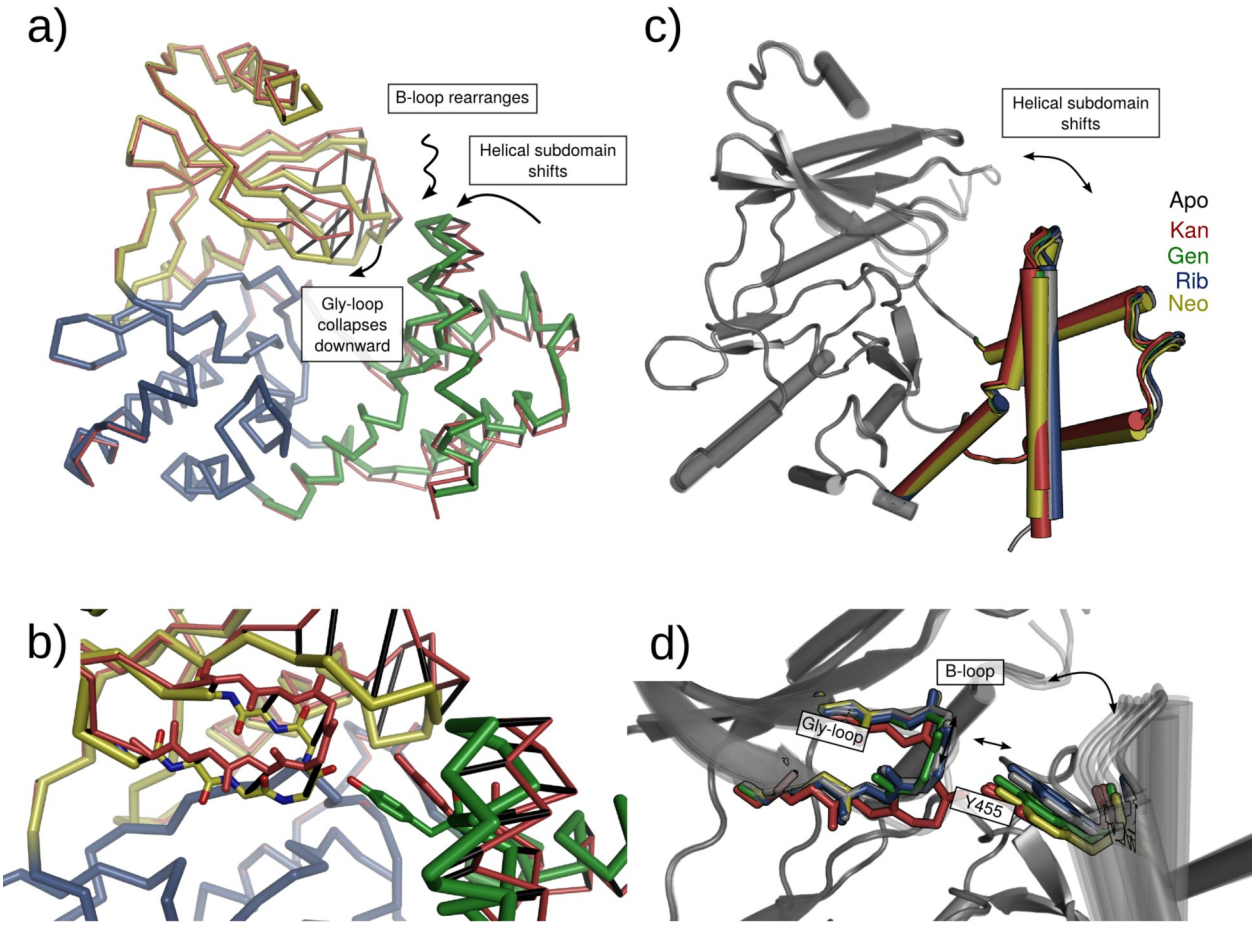

Figure 4.8: Conformational changes of APH(2'')-Ia

a) Open conformation (Chain A, GMPPNP-bound structure) and closed conformation (Chain D, GDP-bound structure) show displacements of the helical subdomain, Gly-loop and B-loop. b) Active site view of these structures indicate that this transition brings Y455 of the helical subdomain in position to stabilize the new conformation of the Gly-loop. c) Soak structures of the APH-GMPPNP complex show changes in chain D of the structures. 1-2 Å shifts are seen when gentamicin, kanamycin, and neomycin are soaked into the crystal. The shift occurs for one bound ribostamycin structure (not pictured), but not the other (blue). d) Active site view of these structure again indicate that this shift brings the helical subdomain and loops into contact.

4.3.4 APH(2'')-Ia binds triphosphate in two conformations

In structures of APH(2'')-Ia in complex with GMPPNP, there are two clearly defined conformations of the triphosphate group. In both cases, two magnesium ions are present in the active site, coordinating the phosphate groups. The base, ribose ring, and α-phosphate lie in identical positions, but those of the β- and γ-phosphate are distinct between these conformations.

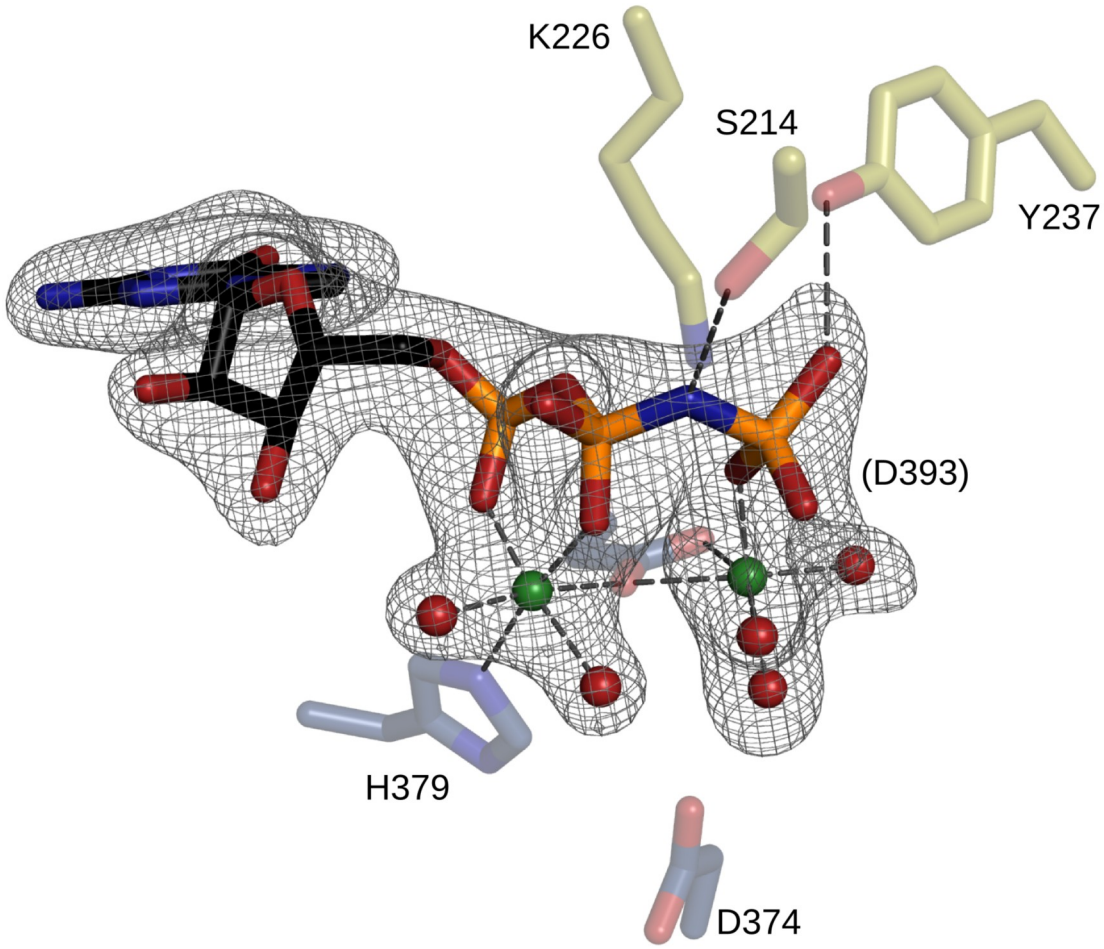

***Figure 4.9*: GMPPNP in the stabilized conformation in APH(2'')-Ia active site with magnesium ions and coordinating waters**

GMPPNP bound in chain A of the APH-GMPPNP structure, with co-ordinating magnesium ions and waters excluded from model for map determination. Map is a F_o-F_c omit map with GMPPNP, Mg and associated waters excluded prior to refinement. Map is displayed at σ = 3.5, illustrated within 2 Å of the omitted atoms.

The conformation that predominates in structures determined with GMPPNP is a stabilized triphosphate conformation in which the γ-phosphate is placed in a different location than that typically observed in kinase enzymes. Instead of being positioned between catalytic magnesium ions, the γ-phosphate is directed toward a cleft in the back of the protein, where it is sequestered from the catalytic base and site of substrate binding(Figure 4.9).

This stabilized conformation is not consistent with what is known about the activation of kinases from structural and mechanistic studies, as outlined in Figure 4.2. However, the activated conformation is also visible in structures of APH(2")-Ia. The activated triphosphate conformation becomes visible when accounting for un-modelled electron density in some chains (Figure 4.10). These partial occupancies of activated and stabilized conformations reflect the equilibrium between states of the enzyme, and changes depending on which substrates are bound to the enzyme (Figure 4.11). This activated conformation is fully compatible with the active conformation crystallized in many protein kinases, and the aminoglycoside kinase APH(3')-IIIa (Burk et al., 2001).

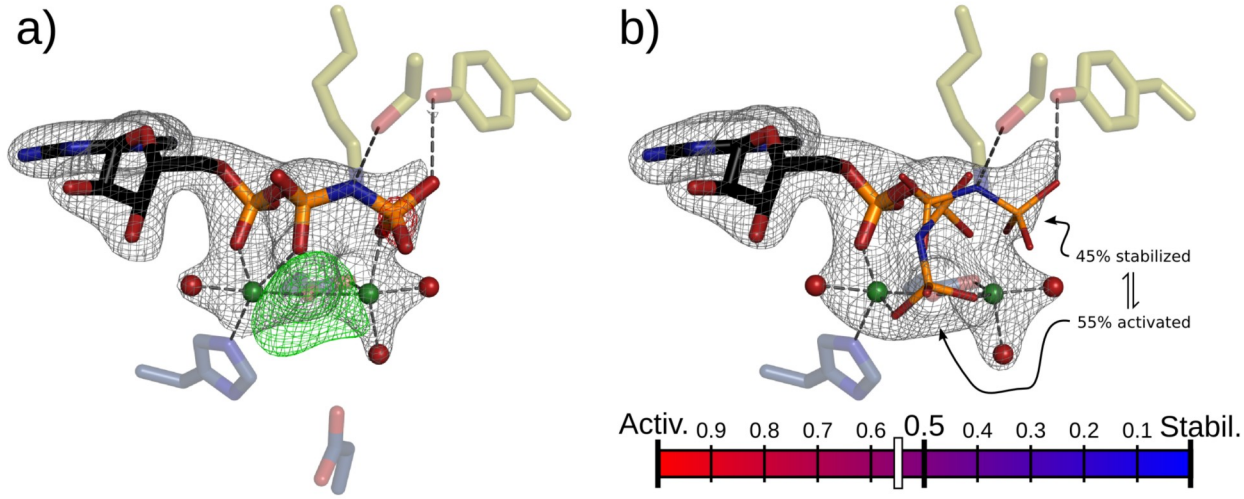

***Figure 4.10*: Difference map of stabilized-activated triphosphate equilibrium**

a) GMPPNP ligand in active site of chain D of APH-GMPPNP structure, modelled completely as the stabilized conformation. $2F_o$-F_c map displayed at 1.5σ in grey, while difference map following refinement with stabilized GMPPNP displayed at +3.5σ (green) and -3.5σ (red). Maps are displayed with 1.5-1.6 Å cutoff from ligand. b) Model reflecting near-equal occupancies of a stabilized and activated GMPPNP conformation in the active site. Refined F_o-F_c difference map displayed at σ = 3.0, at a cutoff of 1.6 Å from omitted atoms. Sliding scale represents the fraction of the nucleoside bound in the activated conformation.

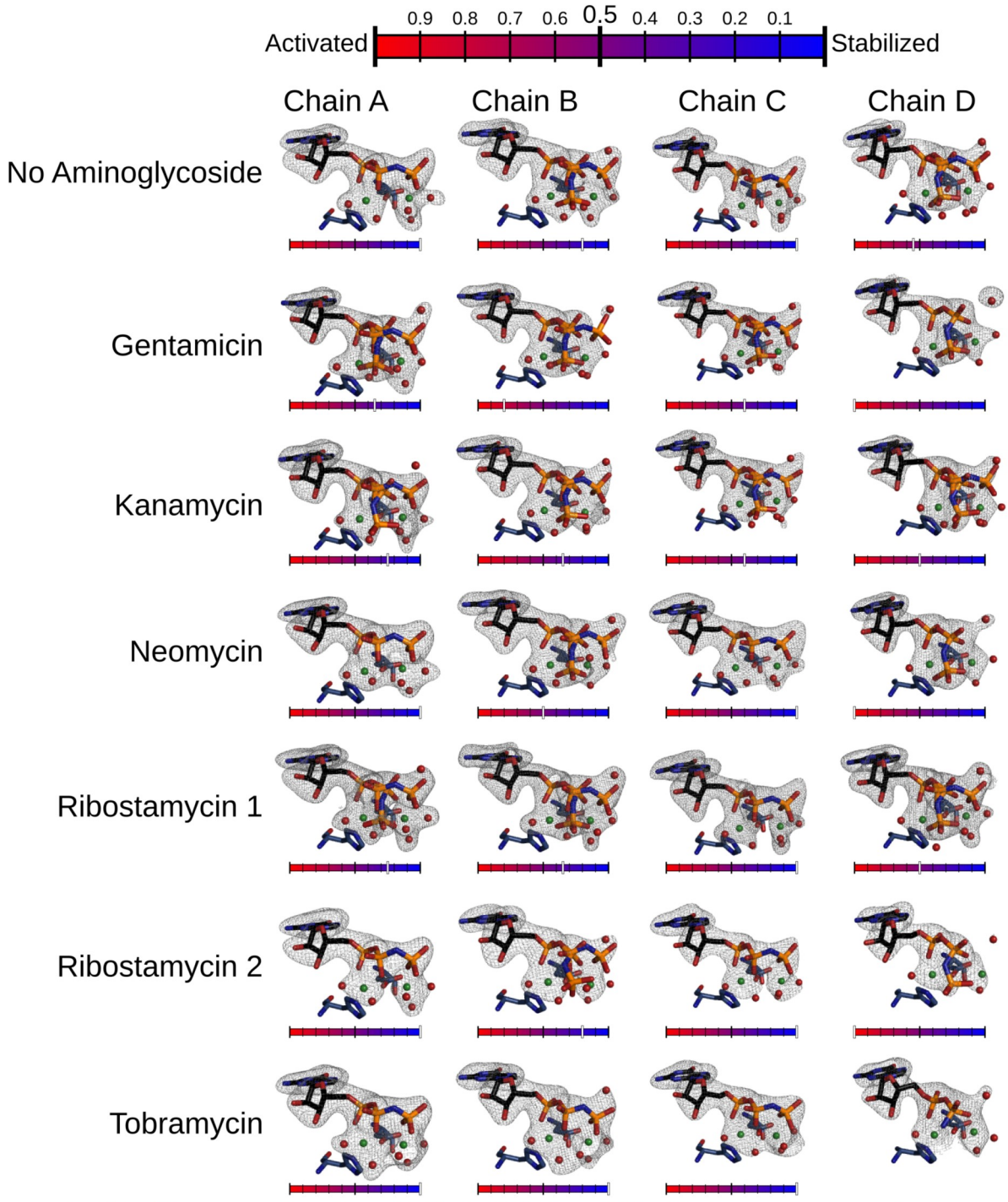

***Figure 4.11*: Difference density of APH(2'')-Ia GMPPNP co-substrate in structure determined with GMPPNP alone, and following soaking with aminoglycoside antibiotics**

Difference electron density refined in the absence of nucleoside, magnesium, or coordinating waters. Maps are all F_o-F_c refined maps, σ = 3.5, illustrated within 2 Å of the omitted atoms. Proportion of triphosphate modelled in activated or stabilized conformation is indicated using the sliding indicator.

In the stabilized conformation, the Gly-loop makes a single contact with the β-γ bridging atom of GMPPNP. A hydrogen bond between S214 on the Gly-loop and this bridging atom appears to stabilize this γ-phosphate conformation of the co-substrate. In the structure determined with GMPPCP bound, and in the mutant S214A enzyme crystallized with GMPPNP, the activated conformation forms instead (Figure 4.12b, c), and so the S214-GMPPNP hydrogen bond appears to be critical to the adoption of the stabilized conformation.

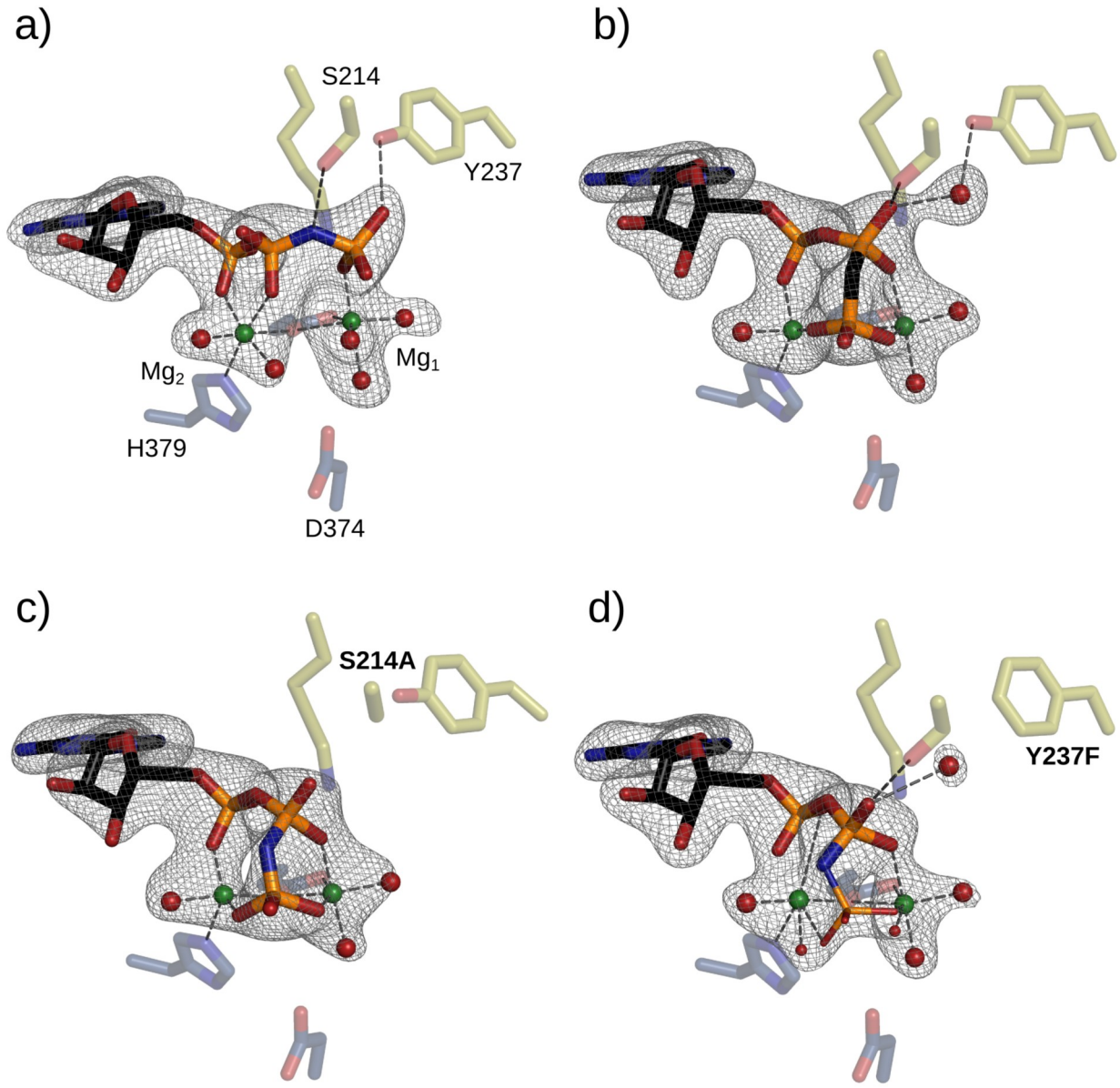

Figure 4.12: Electron density for selected triphosphate configurations

a) Stabilized conformation observed in chain C of the APH-GMPPNP structure. In this case, the phosphate is observed in a completely stabilized form. b) Activated triphosphate in chain B of the APH-GMPPCP structure. c) Activated triphosphate in chain B of the S214A structure. d) Activated triphosphate modelled in chain B of the Y237F structure, modelled with 50% occupancy of γ-phosphate atoms, to correspond to the reduced electron density for this part of the ligand in this structure. All maps are refined F_o-F_c difference maps, contoured to σ = 3.5, illustrated within 2 Å of the omitted atoms.

Removal of the second contact with the triphosphate group, a hydrogen bond between Y237 and the γ-phosphate also showed that the stabilized conformation is destabilized, although the GMPPNP in the active site of this mutant exhibited greatly reduced electron density, suggesting either disorder or breakdown of the co-substrate within the crystals. A dataset from a fresh crystal of wildtype APH-GMPPNP complex made with the same reagents was collected to confirm that the bound GMPPNP remained intact in wildtype crystals. This crystal was incubated as long as the Y237F crystal and showed full occupancy of the γ-phosphate, indicating that the changes in the nucleotide observed for Y237F are indeed due to the mutation. The hydrogen bonds to S214 and Y237 appear to hold the γ-phosphate in an catalytic trap, in a conformation distinct from the catalytic activated conformation.

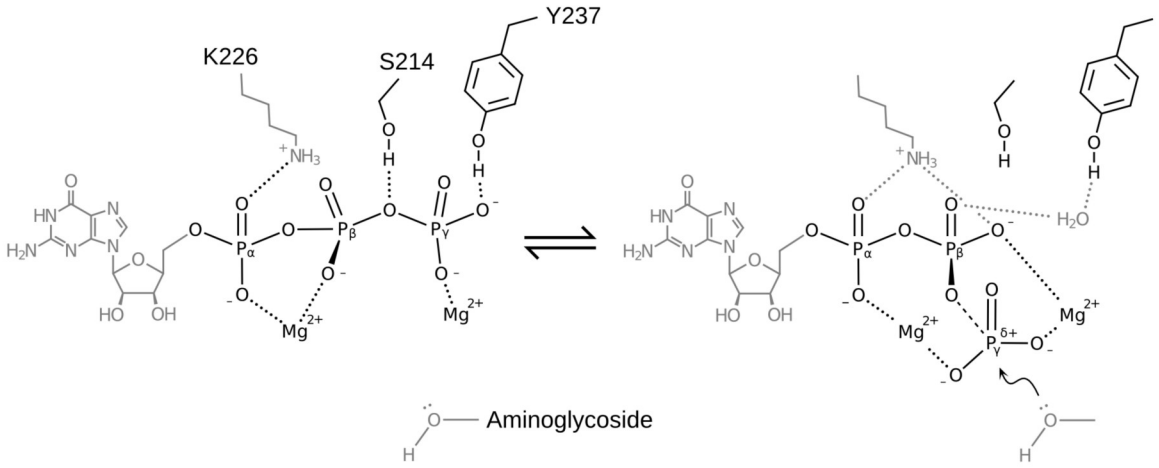

***Figure 4.13*: Schematic indicating equilibrium between activated and stabilized triphosphate conformations**

These findings indicate that APH(2")-Ia contains two conformations of nucleoside triphosphate, which are in equilibrium subject to conformation of the enzyme (Figure 4.13). This equilibrium could constitute a catalytic switch from inactive to active form of the enzyme. In order for this activated conformation to facilitate productive catalysis, additional changes need to occur to transfer the phosphate. Stabilizing interactions for the transferred phosphate need to take place and for this to happen, the enzyme needs to move the Gly-loop.

4.3.5 The Gly-loop governs the triphosphate conformation and connects enzyme closure to ligand activation

Changes from the open to closed conformation of APH(2")-Ia appear to centre around one structural element: the Gly-loop. This loop contacts the substrate triphosphate, the adjacent B-loop, and helical subdomain of the enzyme upon closure. These interactions in turn place the Gly-loop in position to productively transfer the γ-phosphate from the donor GTP to acceptor aminoglycoside substrate.

In fully open conformations, the Gly-loop sits above the triphosphate of GMPPNP and residues S214 and Y237 hold the triphosphate in the stabilized position (Figure 4.14a). On partial enzyme closure, the helical subdomain shifts inward, which brings it into contact with the open conformation of the Gly-loop. In intermediate conformations, the loop is disordered, releasing the triphosphate to adopt the activated conformation (Figure 4.14b).

In the fully closed conformation, Y455 from the helical subdomain contacts the peptide backbone at the tip of the Gly-loop, while N459 of the helical subdomain and T231 on the B-loop form hydrogen bonds that hold these structural elements together and condense this loop atop the Gly-loop using residue F229 (Figure 4.14c).

In the closed conformation, S214 and Y237 now form hydrogen bond to each other, closing the hole vacated by the nucleoside γ-phosphate. These residues switch between supporting the stabilized triphosphate and helping the Gly-loop reach a catalytic conformation. These conformations appear to be central to catalysis in the enzyme. In the GDP-bound complex of the Y237F mutant enzyme, the S214-Y237 hydrogen-bond cannot form, so the Gly-loop is not brought into this catalytic position, and the enzyme remains open when compared to the wildtype enzyme-GDP complex.

The activated triphosphate is more subject to phosphotransfer and hydrolysis than the stabilized form, because it contains the structural elements necessary for productive catalysis such as K229 and D374 in the appropriate positions. To test the interactions of the Gly-loop that drive transfer of phosphate, the rate of hydrolysis was measured in these mutants relative to the wildtype enzyme.

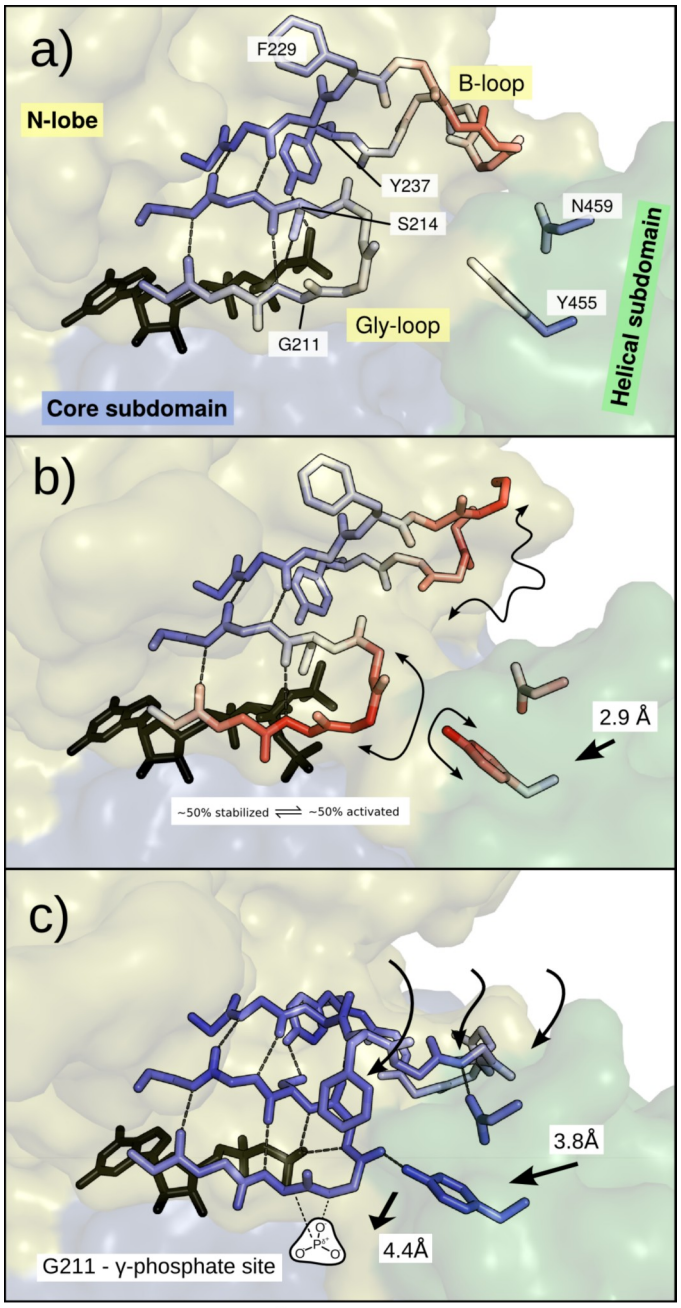

***Figure 4.14*: Conformations and transitions of Gly-loop in APH(2'')-Ia**

4.3.6 Decreased intrinsic GTP hydrolysis in mutant APH(2'')-Ia enzymes

The release of phosphate from GTP was tested for the S214A, Y237F, and wildtype APH(2'')-Ia enzyme at ~0.4 μM enzyme. The corrected phosphate released per enzyme active site could be measured and tracked as a linear reaction with time. At equivalent enzyme concentrations and 10 μM GTP, APH(2'')-Ia released 0.262 phosphate per enzyme per minute from the wildtype enzyme, 0.122 from the Y237F mutant, and 0.056 per minute from the S214A mutant (Figure 4.15). This indicates that the rate of background hydrolysis of APH(2'')-Ia depends upon the action of these residues. Loss of Y237 reduces the catalytic rate by more than half, while loss of S214 drops the rate 5-fold.

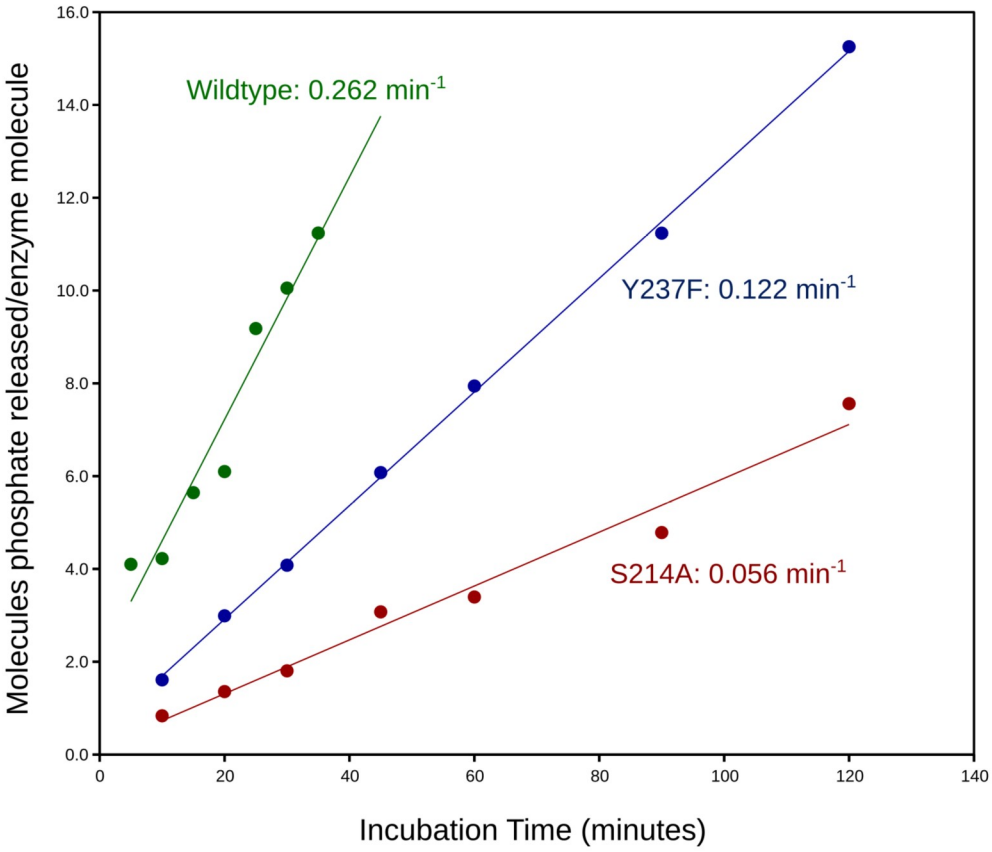

***Figure 4.15*: Hydrolysis of GTP induced by the APH(2'')-Ia enzyme and mutants**

Malachite green-based tracking of phosphate release from enzyme to establish linearity and compare mutant enzymes. Phosphate is normalized to the concentration of enzyme, so rates reflect the turnover of phosphate release by APH(2'')-Ia.

This change in reactivity indicates that the loss of Gly-loop contacts in the active site decreases the ability of the enzyme to facilitate effective catalysis. The other change we can use to probe the enzyme is the effect of aminoglycoside binding, as we have observed in structures that the addition of aminoglycoside also drives the enzyme toward the activated state.

4.3.7 Aminoglycoside binding accelerates phosphate hydrolysis in APH(2'')-Ia

With a linear rate of hydrolysis established for the wildtype APH(2'')-Ia, it was also possible to measure change in rate of hydrolysis upon addition of substrates to the enzyme. 4,5-disubstituted compounds are not substrates for the enzyme, and so the change in rate of hydrolysis in the enzyme can be measured by adding neomycin and ribostamycin as allosteric effectors of hydrolysis. Addition of neomycin raised the intrinsic rate of hydrolysis in the enzyme by ~4-fold, while ribostamycin raised it ~8-fold (Figure 4.16). These reactions showed an inflection in the low millimolar range, reflecting a saturable interaction with the enzyme at these concentrations. The addition of these compounds leads to closure of the enzyme and catalytic activation even in the absence of acceptor substrate, indicating that the enzyme closure seen by comparing structures reflects real enzymatic activation as part of APH(2'')-Ia catalytic cycle.

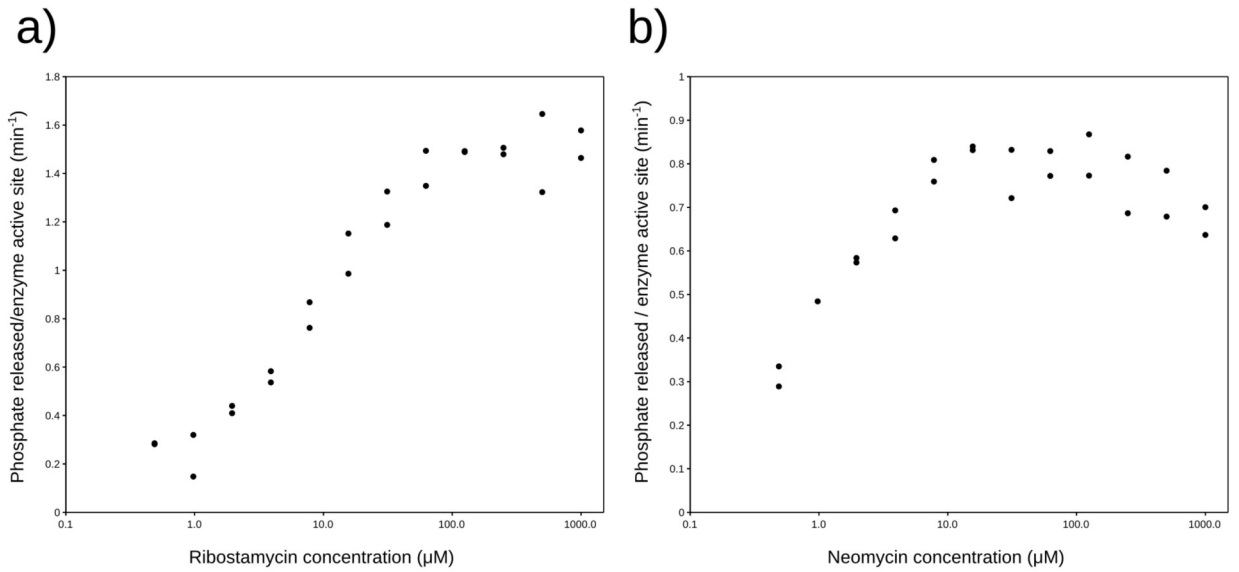

***Figure 4.16*: Aminoglycoside induction of hydrolysis in APH(2'')-Ia**

a) Increasing concentrations of ribostamycin drive an increase in phosphate release by APH(2'')-Ia. The rate increase appears saturable, indicating specific binding of ribostamycin drives this effect. An inflection ~10 μM suggests binding in this range. b) Equivalent measure using neomycin addition to the enzyme. The acceleration of hydrolysis by this compound is less pronounced than ribostamycin, but occurs at lower antibiotic concentrations. Lower concentrations of antibiotic could not be measured because the enzyme concentration began to exceed that of the antibiotic. One point was omitted due to a mechanical fault in the apparatus.

4.4 Discussion

4.4.1 APH(2'')-Ia active site maintains ancient catalytic motifs shared with all kinases

The active site of APH(2'')-Ia conserves residues in the same spatial arrangement as other enzymes in the eukaryotic protein kinase-like superfamily. This finding confirms that despite a long divergence from these enzymes this aminoglycoside kinase conserves a catalytic architecture from its ancient roots with eukaryotic protein kinases. This occurs despite otherwise diverging enormously in structure and regulation. APH(3')-IIIa, the other well-studied aminoglycoside kinase, contains an insertion in the Gly-loop, which shifts the geometry of the active site somewhat (Burk et al., 2001; Thompson et al., 2002). The divergence between APH(2'') and APH(3') enzymes is not as surprising as we might expect – they cluster as two distinct lineages with independent roots in the greater ePK superfamily (Oruganty et al., 2016). APH(2'') and APH(3') enzymes appear to have arrived upon aminoglycoside modification twice independently and the structures of these enzymes active sites reflect this independence.

The catalytic residues of APH(2'')-Ia are almost perfectly co-incident with cAPK, while immediately supporting residues are also maintained in structurally equivalent positions. Structural features of protein kinases that support the catalytic architecture (McClendon et al., 2014) are also shared with APH(2'')-Ia. Two stacks of hydrophobic residues that form catalytic and regulatory spines are maintained in APH(2'')-Ia (Figure 4.17), only varying at the bottom of the regulatory spine, farthest from the active site. Moving further outward from the catalytic centre, structural similarity is progressively less conserved, and protein motifs that are mechanistically important in the regulation of protein kinases have no equivalent in APH(2'')-Ia (Zhang et al., 2014). Thus, known regulatory mechanisms from protein kinases can not exist in APH(2'')-Ia.

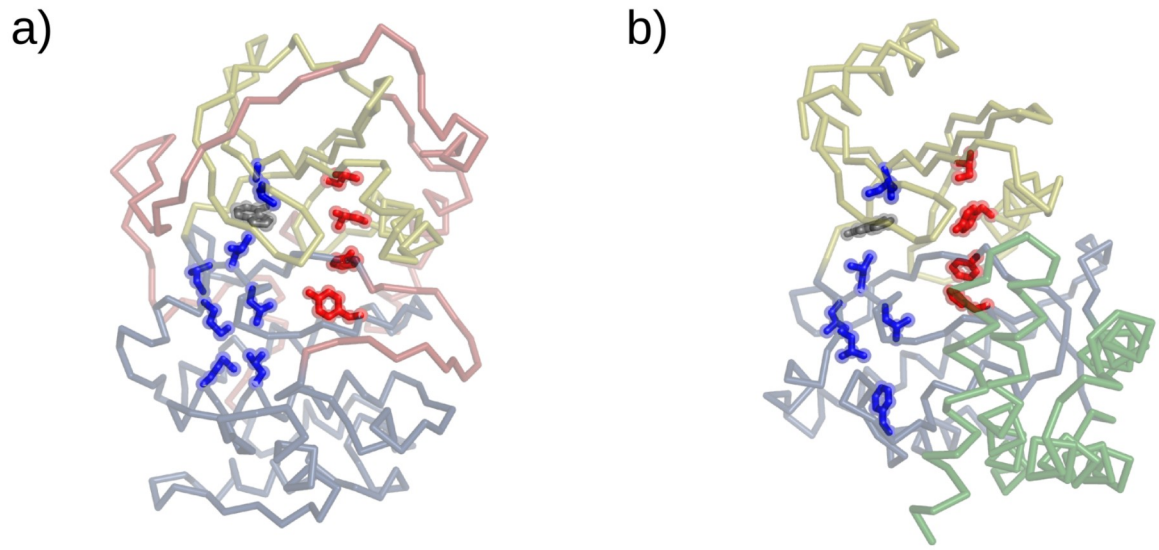

***Figure 4.17*: Kinase core spines in cAPK and APH(2'')-Ia**

a) The regulatory (blue) and catalytic (red) spines of cAPK. b) The equivalent spines in APH(2")-Ia. Despite dramatic changes in enzyme architecture, these spines are largely conserved between these divergent kinases.

Despite these large differences in topology and conservation, APH(2")-Ia does exhibit structural flexibility that may accomplish similar functions to the regulatory mechanisms of protein kinases. In both cases, conformational changes move parts of the enzyme together, and the Gly-loop is locked in place by a convergence of features from around the protein (Masterson et al., 2010). In the structure determined with GDP and gentamicin bound, the backbone conformation of the Gly-loop of APH(2")-Ia becomes identical to the equivalent loop of protein kinases. This convergence reflects a thermodynamically optimized strategy: there is one optimally active enzyme architecture, while distortions in this architecture decrease or destroy catalysis.

4.4.2 APH(2'')-Ia binds a stabilized triphosphate conformation that is incompatible with productive catalysis

The structure of APH(2'')-Ia in complex with GMPPNP reveals a novel, stabilized triphosphate conformation. In this conformation, two magnesium ions are bound to the enzyme and the γ-phosphate is directed away from the aminoglycoside-binding site, toward the solvent-filled cleft and poorly-ordered B-loop. The active state of the enzyme, as described in Section 4.1.2, can not be achieved.

This stabilized conformation moves the β-phosphate away from contact with the active site lysine, and the γ-phosphate away from the catalytic aspartic acid. Without these interactions, the incoming nucleophile and β-phosphate leaving group are not stable, leaving the triphosphate group inert. The magnesium coordination of the triphosphate also changes in this state, fixing this co-substrate in an inactive conformation.

This conformation appears to be a novel form of triphosphate binding to a kinase enzyme. There are no comparable examples of this conformation in kinases in the protein databank. A couple of superficially similar conformations are found in some kinases (Lisa et al., 2015; Tereshko et al., 2001; Wu et al., 2008; Young et al., 2009), but none are well-defined with productive hydrogen bonds and the equivalent co-ordinations to magnesium.

In the stabilized conformation, two residues contact the nucleoside phosphates directly. Serine 214 on the Gly-loop hydrogen-bonds to the bridging atom of the beta-gamma linkage. Tyrosine 237, which lies where the B-loop joins the N-terminal end of helix α2, also forms a hydrogen-bond with the gamma-phosphate of the group. These contacts seem to be critical to the adoption of the stabilized triphosphate conformation.

4.4.3 Breaking the stabilized conformation favours the activated triphosphate

The S214 and Y237 contacts appear to hold the stabilized conformation of the triphosphate in place. Removal of either of these contacts results in a loss of the stabilized conformation. In the crystal of the S214A mutant of APH(2")-Ia in complex with GMPPNP, the co-substrate moves toward the activated conformation. The same effect was true when GMPPNP was replaced by GMPPCP, which can no longer interact with S214 because its β,γ-bridging methylene group can not interact with S214. In both of these cases, the activated form of the triphosphate is favoured, which indicate that S214 helps hold the triphosphate group in the stabilized conformation.

Structures of GMPPNP bound to the Y237F show possible degradation of the GMPPNP co-substrate, or alternatively just a large increase in disorder. To rule out degradation of the reagents prior to crystallization, a new data set of the wildtype protein prepared with the same reservoir solution and GMPPNP stock were prepared, which showed no breakdown of the GMPPNP co-substrate. The structure of the Y237F mutant determined with GDP bound confirmed that there is still residual density that remains for the γ-phosphate in the GMPPNP-bound form, by comparison. There is precedent for kinase enzymes acting upon β,γ-imido compounds within crystals (Bastidas et al., 2013), so despite GMPPNP being resistant to breakage of the linkage, it is possible this has occurred to the GMPPNP in the Y237F structure.

The switch in triphosphate conformations is close to equilibrium in our crystals, and in some cases both conformations are observed within a single active site. Under these conditions, even subtle changes have the potential to push the equilibrium toward the activated state. In structures determined with aminoglycoside bound to the enzyme, there is a shift toward the activated form of the triphosphate compared to the GMPPNP-bound form alone (Figure 4.11). This occurs despite the fact that the aminoglyosides bind too far away from the stabilized triphosphate to exert any direct influence. The shift of the helical subdomain inwards upon aminoglycoside binding puts this region of protein into contact with the Gly-loop and nearby B-loop, which in turn affect the disposition of the triphosphate. Through these elements, aminoglycoside binding drives enzyme closure and switch to the activated triphosphate. Echoing

mechanisms of convergent catalysis in protein kinases, the central linchpin in this interaction is the Gly-loop.

4.4.4 Activation of catalysis through stabilization of the Gly-loop

The switch between activated and stabilized triphosphate conformations is linked to a switch of two residues in the enzyme active site – S214 and Y237. These residues hold the triphosphate group in a catalytically inactive form when the enzyme is open, but upon closing, conformational changes to the enzyme release these connections, allowing the triphosphate to adopt the activated conformation. Together, S214 and Y237 form a bi-stable arrangement where they fix both the open, triphosphate-stabilized conformation, and the closed, triphosphate-activated conformation.

S214 and Y237 play a second role in stabilizing the Gly-loop for productive catalysis. In fully-closed conformations with GDP, a change in conformation allows these residues to move closer to each other and close the gap opened by displacement of the now-activated γ-phosphate. In this closed conformation, these residues now H-bond to each other, and fix the loop in place. Comparison of the wildtype and Y237F mutant in complex with GDP shows that this interaction is important for the closure of the enzyme – in Y237F, the loop does not adopt this form and as a result the enzyme does not remain closed (Figure 4.18).

The Gly-loop also cannot reach the closed conformation with the stabilized triphosphate bound – the triphosphate sterically blocks access to the Y237 side chain. As a result, the structural changes to the triphosphate group and Gly-loop are interdependent and change in concert to activate the enzyme upon closure. This makes the Gly-loop the central link in connecting gross enzyme closure to catalytic activation of the donor triphosphate group.

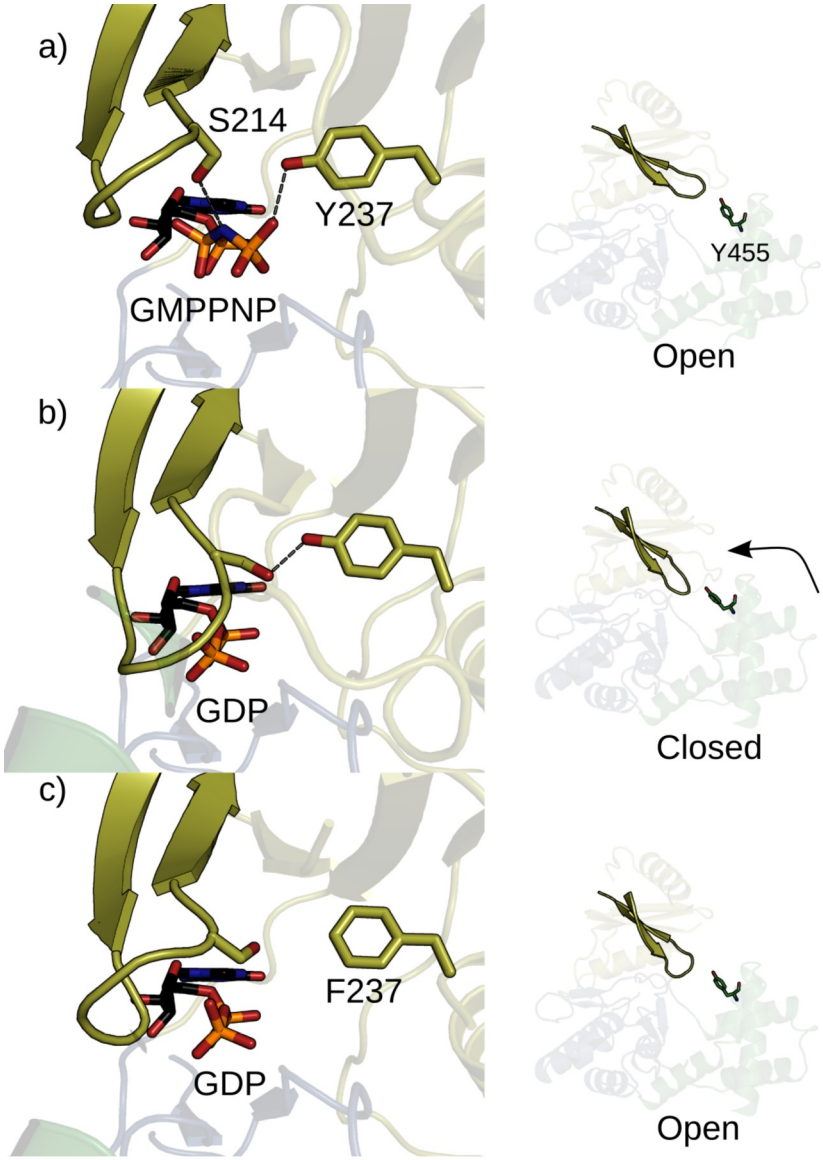

Figure 4.18: S214 and Y237 are involved in stabilization of triphosphate and enzyme closure

a) In the open enzyme, stabilized triphosphate form, S214 and Y237 both contact the triphosphate of the co-substrate. b) in the GDP-bound fully closed wildtype enzyme, S214 and Y237 form hydrogen bond to each other, holding the loop in place. c) In the Y237F mutant with bound GDP, this interaction does not form, the loop remains poorly-ordered, and contacts with the distant helical subdomain do not form, precluding closure of the enzyme.

4.4.5 A conformational mechanism for APH(2'')-Ia activation

Our structures allow us to propose a mechanism for activation of the APH(2'')-Ia enzyme to form a catalytically competent state (Figure 4.19). In the GTP-bound form, the Gly-loop fixes the GTP triphosphate in the stabilized, unreactive form. The aminoglycoside-binding cleft remains unoccupied and the helical subdomain is flexible and can accommodate binding of substrates as necessary. When an aminoglycoside is bound, the helical subdomain closes inward.

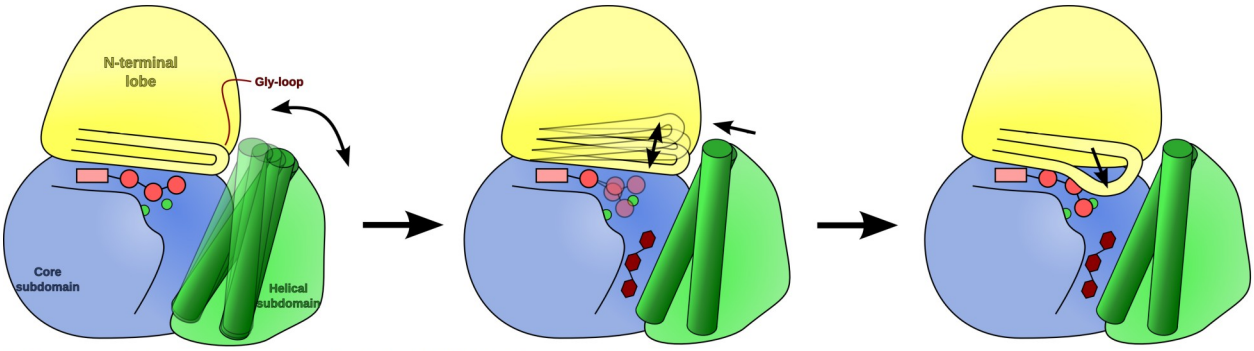

***Figure 4.19*: Model of APH(2'')-Ia activation**

The N-lobe (yellow) and core subdomain of APH(2'')-Ia (blue) remain mostly fixed in respect to each other throughout the conformational transitions. The helical subdomain (green) has mobility in the unbound form. Binding of aminoglycoside antibiotic fixes the helical subdomain in a position closer to the N-lobe and nucleoside, where it clashes with the Gly-loop. The Gly-loop becomes mobile, adopting many conformations before it becomes stabilized through new interactions with the helical subdomain. The triphosphate (pink) is liberated in this state, and switches between stabilized and activated states. In this new conformation, the loop can facilitate productive phosphotransfer to the aminoglycoside antibiotic.

The inward position of the helical subdomain is incompatible with the initial, open conformation of the Gly-loop. Once the helical domain shifts inward, the Gly-loop is destabilized, and disorder of this loop is accompanied by a release of the stabilized triphosphate. Without stabilizing interactions with the Gly-loop, the triphosphate can adopt either of two conformations, with the activated conformation favoured when there is no loop present.

In addition to positioning the triphosphate, the Gly-loop must also reach a position to facilitate effective catalysis. Displacement of the loop over the triphosphate occurs through conformational sampling and is fixed in place by a new hydrogen-bond to the buried Y237, which is accessible once the triphosphate moves from the stabilized conformation. A new hydrogen bond with Y455 of the helical subdomain, and packing interactions with F229 of the adjacent B-loop all combine to support the Gly-loop in a catalytic position. This convergence of supporting interactions on the Gly-loop echoes similar mechanisms in protein kinases that drive catalysis.

This mechanism of activation is different from protein kinases which distort the catalytic core of the enzyme to regulate the enzyme activity. In this enzyme, we observe a new innovation: a stabilized triphosphate conformation, held in place by the same structural elements that also facilitate activation switching of the Gly-loop. APH(2")-Ia has developed a novel means of regulation distinct from its distant protein kinase relatives. This mechanism appears to be a convenient way of mitigating off-target substrate hydrolysis, which would impose a fitness cost on this antibiotic resistance factor.

4.4.6 Aminoglycoside-induced catalytic activation – a mechanism to mitigate the fitness cost of resistance in APH(2'')-Ia

Due to a dissociative mechanism, the activated triphosphate of APH(2'')-Ia and other kinase enzymes is considerably weakened through binding to the enzyme. In this state, the nearest available nucleophilic atom may react with the activated phosphate and form a new bond, completing the reaction. If an aminoglycoside is bound in this position, then catalysis proceeds normally. If there is no aminoglycoside present, it is possible that a water molecule fills this role instead. This results in a net loss of the co-substrate and no productive modification of substrate – an undesirable outcome for the enzyme that increases its fitness cost.

This form of enzymatic decoupling induces a considerable fitness cost, which can be high enough to drive selection against the resistance enzyme (Kim et al., 2006b). Reduction of this fitness cost could confer great selective advantage to APH(2'')-Ia. Any mechanism of mitigating this wasteful hydrolysis provides a selective benefit.

The aminoglycoside-induced activation of catalysis in APH(2'')-Ia requires the helical subdomain and active site loops to converge together to facilitate catalysis. This results in measurable increases in enzymatic activity on aminoglycoside binding, which reflect a conformational transition in the enzyme that drives catalysis.

4.4.7 APH(2'')-Ia activation in the context of the full-length AAC(6')-Ie/APH(2'')-Ia enzyme

The mechanism of activation of APH(2'')-Ia should be considered in the context of the full-length AAC(6')-Ie/APH(2'')-Ia enzyme. The Gly-loop undergoes a transition between the open, stabilized state, and the closed activated state of the enzyme. Interestingly, the N-terminus of the domain lies near this loop. Our previous SAXS-based modelling of the full-length AAC(6')-Ie/APH(2'')-Ia protein (Chapter 2) has shown that the AAC(6')-Ie domain of the enzyme packs immediately next to this N-terminus.

This is especially important when we look to enzyme kinetics studies conducted on the APH(2")-Ia domain in comparison with the full-length AAC(6')-Ie/APH(2")-Ia protein. The K_M for GTP is higher in the APH(2")-Ia fragment than the full-length protein (Boehr et al., 2004), indicating that the addition of the nearby acetyltransferase domain can impact the affinity of the APH(2")-Ia domain for GTP.

The rate-limiting step in catalysis by ePK enzymes is release of the product diphosphate (McKay and Wright, 1996; Zhou and Adams, 1997). This is facilitated by the movement of the Gly-loop out of the way to allow the release of the GDP or ADP. Obstruction of movement of this loop will lead to a slower transition between bound and unbound, and can thus modulate the enzyme. It is conceivable that changes in the AAC(6')-Ie domain can exert influence on the APH(2")-Ia domain through interactions with the Gly-loop. If there is interaction between these domains, this provides a mechanism of potential allosteric action between these two domains, which could contribute to the selective advantage of the bifunctional particle.

4.5 Conclusions

Despite a long divergence in evolutionary history, the APH(2")-Ia enzyme conserves the active site architecture of protein kinases and other ePK enzymes. These enzymes have developed independently for a long time and evolved independent means of regulation, but the core functional unit of the APH(2")-Ia remains conserved with the distantly related PKs.

APH(2")-Ia stabilizes a novel conformation of its catalytic triphosphate substrate. This conformation is incompatible with productive hydrolysis. Breaking this conformation allows the substrate to adopt the conventional, activated conformation in the active site. Subsequent changes to conformation of the Gly-loop that lock it into position further facilitate catalysis, which is driven by aminoglycoside-induced enzyme closure.

Aminoglycoside binding drives the enzyme to release its sequestered triphosphate substrate from an inert conformation, revealing a new mechanism of catalytic activation of a kinase, unique to this family of enzymes. This activation is confirmed by the observation that binding of non-substrate aminoglycosides greatly elevates the intrinsic hydrolysis rate of the enzyme.

A reduced rate of hydrolysis in the absence of aminoglycoside substrates indicates that the enzyme has developed novel means of reducing its fitness cost in the absence of substrate. This reduced fitness cost could lead to better fitness and evolutionary success of this widespread antibiotic resistance factor.

5 Structural studies on binding of N1-substituted aminoglycosides to wildtype and S376N mutant APH(2'')-Ia

5.1 Background

The AAC(6')-Ie/APH(2'')-Ia bifunctional enzyme inactivates almost every aminoglycoside antibiotic efficiently, through the action of one enzymatic domain or the other. The exception to this trend is the group of semisynthetic N1-substituted aminoglycosides, including amikacin, arbekacin, netilmicin, and plazomicin, which are all modified at very low levels or not at all by AAC(6')-Ie/APH(2'')-Ia. As a result, N1-substituted aminoglycosides remain effective against strains of bacteria expressing AAC(6')-Ie/APH(2'')-Ia.

Several studies find a low level of resistance conferred by the wildtype enzyme (Daigle et al., 1999a; Frase et al., 2012), although this mechanism is not efficient enough to be clinically problematic. However, the ability of this enzyme to inactivate N1-substituted aminoglycosides is important to observe and track because changes to the enzyme that improve the efficiency of this interaction have the potential to render these antibiotics also inactive toward bacteria expressing mutant AAC(6')-Ie/APH(2'')-Ia.

5.1.1 N1-substituted aminoglycosides are second generation aminoglycosides designed to resist resistance

N1-substituted aminoglycosides are semisynthetic compounds that generate novel properties in comparison to their natural counterparts. They are inspired by the natural aminoglycoside butirosin, first identified in 1972 (Howells et al., 1972). This aminoglycoside, from a fermentation culture of *Bacillus circulans*, is a 4,5-disubstituted aminoglycoside similar to ribostamycin, that carries a novel modification. Butirosin is acylated with an (*S*)-2-hydroxy-4-aminobutyrate group (AHB) at the N1 position of the 2-deoxystreptamine ring. The N1-linkage in this compound inspired a new generation of aminoglycosides, through the semisynthetic alteration of existing antibiotics with this acyl group (Kondo and Hotta, 1999). Compounds

carrying this modification act as effective antibiotics, even in the presence of some resistance factors (Price et al., 1976).

Amikacin (Kawaguchi et al., 1972) and arbekacin (Kondo et al., 1973) are the most prominent N1-AHB aminoglycosides in current clinical use. These compounds are N1-modified kanamycin A and dibekacin, respectively (Figure 5.1). The N1-AHB group of these compounds is tolerated at the site of action of the antibiotic (Kondo et al., 2006), but interfere with binding of these compounds to many resistance enzymes. As a result, N1-substituted aminoglycosides are effective as antibiotics against many aminoglycoside-resistant microbial strains.

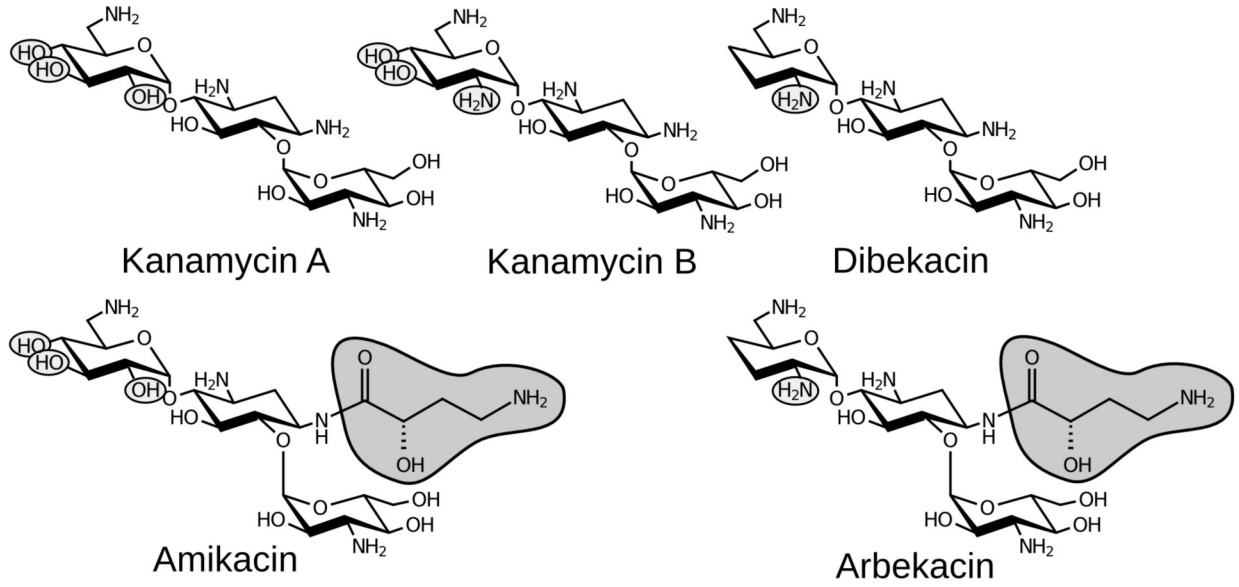

Figure 5.1: Semisynthetic aminoglycosides

Amikacin and arbekacin are N1-modified kanamycin A and dibekacin, respectively. The N1-AHB group is indicated in dark grey, while the divergent groups on the 4-linked aminohexose are indicated in light grey.

In addition to the AHB group addition, dibekacin is also a semi-synthetic compound created by removing the 3' and 4' hydroxyl groups from kanamycin B (Umezawa et al., 1971). This alteration confers protection against resistance enzymes that modify the 3' or 4' hydroxyl groups, which further improves the activity of this compound and its derivative, arbekacin, toward resistant strains. The different groups on the 4-linked aminohexose of arbekacin and dibekacin

relative to amikacin and kanamycin have produced some puzzling results when it comes to binding to macromolecules – arbekacin retains activity despite acetylation at two sites, while the same is not true of amikacin (Hotta et al., 1996, 1998). Dibekacin, which lacks a 4' group, is modified by the ANT(4')-Ia enzyme, by adopting a novel binding mode that facilitates modification at the 4" site (Carlier and Courvalin, 1990). Because of these peculiar findings, it is important to test both sets of compounds in their interactions with macromolecules before generalizing from one set to the other.

5.1.2 Resistance to N1-substituted aminoglycosides

There are very few aminoglycoside-modifying enzymes that naturally confer resistance to the N1-substituted aminoglycosides amikacin and arbekacin. This might be anticipated as these semi-synthetic aminoglycosides are not present in the environment, where aminoglycoside-modifying enzymes developed. As a result, these antibiotics are some of the most effective compounds currently used in treating infectious disease.

While still somewhat uncommon, amikacin resistance has been identified in strains expressing AAC enzymes (Meyer et al., 1983) and through mutation of the ribosomal binding site (Alangaden et al., 1998). Some enzymes can inactivate this compound by nucleotidylation (Jacoby et al., 1990) and phosphorylation (Fong and Berghuis, 2009), but so far this antibiotic has remained effective against strains of bacteria expressing AAC(6')-Ie/APH(2")-Ia and similar resistance factors.

5.1.3 Structural interaction of N1-substituted aminoglycosides with APH(2")-Ia

Our understanding of aminoglycoside binding to APH(2")-Ia (Chapter 3) indicates that the enzyme binds to neamine-based aminoglycosides using the conserved neamine-based rings. While it binds and inactivates many compounds, semisynthetic aminoglycosides with N1 modifications are not modified at high rates by the enzyme. The introduction of a bulky acyl group at the N1 position likely disrupts binding to the enzyme and keeps the compound from being productively modified.

While established and catalytically optimized antibiotic resistance to amikacin and arbekacin is not conferred by AAC(6')-Ie/APH(2")-Ia, *de novo* mutations can expand the substrate specificity of existing enzymes to act on these compounds. Mutations that introduce new features to a resistance enzyme are a well established phenomenon in enzymes that confer resistance to β-lactams (Knox, 1995), but not common in aminoglycoside resistance enzymes, even less in APH enzymes as most identified AMEs are acetyltransferases (Lambert et al., 1994; Robicsek et al., 2006).

Mutations to APH(2") enzymes that increase resistance to N1-substituted aminoglycosides have been generated in laboratory settings (Lee et al., 2002; Toth et al., 2010), but only one clinical mutant has been identified that increases resistance to N1-substituted aminoglycosides by APH(2")-Ia.

Resistance to N1-substituted aminoglycosides in APH(2")-Ia is particularly important in the consideration of a new aminoglycoside, plazomicin, which carries both the N1-modification and an N6'-modification (Aggen et al., 2010). Structures I have determined with bound aminoglycosides (section 3.3.2) indicate that modifications at the N6' position should not impact the binding of any compounds to APH(2")-Ia, and so changes that impact amikacin and arbekacin binding will also influence the binding of this newly-developed, resistance-resistant compound.

5.1.4 Modification of N1-substituted compounds by APH(2")-Ia

AAC(6')-Ie/APH(2")-Ia has not typically been resistant to amikacin and arbekacin at clinically important levels. This gives clinicians confidence that an N1-substituted aminoglycoside will be effective toward resistant bacteria, including those that express this protein. Thus, the emergence of AAC(6')-Ie/APH(2")-Ia-based resistance to these compounds is of clinical importance. The enzyme does appear to show weak activity toward these compounds (Daigle et al., 1999a; Frase et al., 2012; Yuan et al., 2011), although different methodologies measure K_M values that vary considerably, but always with a considerably higher K_M for the N1-substituted compound. Studies of intermediate-resistance strains indicate that the enzyme does 2"-*O*-phosphorylate and 6'-*N*-acetylate the antibiotic (Kondo et al., 1993), so the underlying

catalytic activity appears to exhibit the same regiospecificity on the 2"- and 6'-sites of these compounds as the enzyme does toward aminoglycosides without the N1 substitutution.

Prediction of the binding and specificity of aminoglycoside-modifying enzymes is challenging, even with available crystal structures. The structures of four APH(2") enzymes have been determined, yet it is still necessary to experimentally test the substrate-binding profiles for individual enzymes – structures alone are not predictive. There are discrepancies within the APH(2") enzymes where they confer differing amounts of resistance to different compounds, but all confer very little resistance to N1-substituted amikacin and arbekacin (Toth et al., 2009).

Three strains of pathogenic bacteria carrying AAC(6')-Ie/APH(2")-Ia have been identified that carry genetic changes which confer increased resistance to arbekacin. In one case, a promoter mutation leads to overproduction of the enzyme without any change to the coding sequence of the gene (Matsuo et al., 2003). This amplification of the weak arbekacin-modifying activity seems to be sufficient to confer resistance. In another case, a D80G mutation in the AAC(6')-Ie domain leads to 4'"-acetylation of arbekacin, on the N1-acyl group itself (Fujimura et al., 2000). In this enzyme, this change appears to give the enzyme flexibility that permits an alternate binding mode of arbekacin to the enzyme. This results in modification of the alkyl tail of the N1-group.

The third arbekacin-resistant strain is of interest given our earlier studies on the aminoglycoside-binding specificity of APH(2")-Ia (Chapter 3). In this case, mutation of aminoglycoside-binding serine 376 to asparagine is associated with arbekacin resistance (Ishino et al., 2004). The S376 residue is involved in coordination of N1 of the aminoglycoside, but this amine group is modified in N1-substituted semisynthetic aminoglycosides. There is no obvious explanation why this structural change might alter binding of N1-substituted compounds. Both the N1-linked AHB group and mutant S376N asparagine residues introduce changes that disrupt the normal binding of neamine-based aminoglycosides to the enzyme (Figure 5.2a). The N1-groups of substituted aminoglycosides create clashes between the aminoglycoside and enzyme (Figure 5.2b), while the S376N mutation removes a conserved aminoglycoside-binding interaction and introduces clashes that preclude the same binding mode of aminoglycosides (Figure 5.2c).

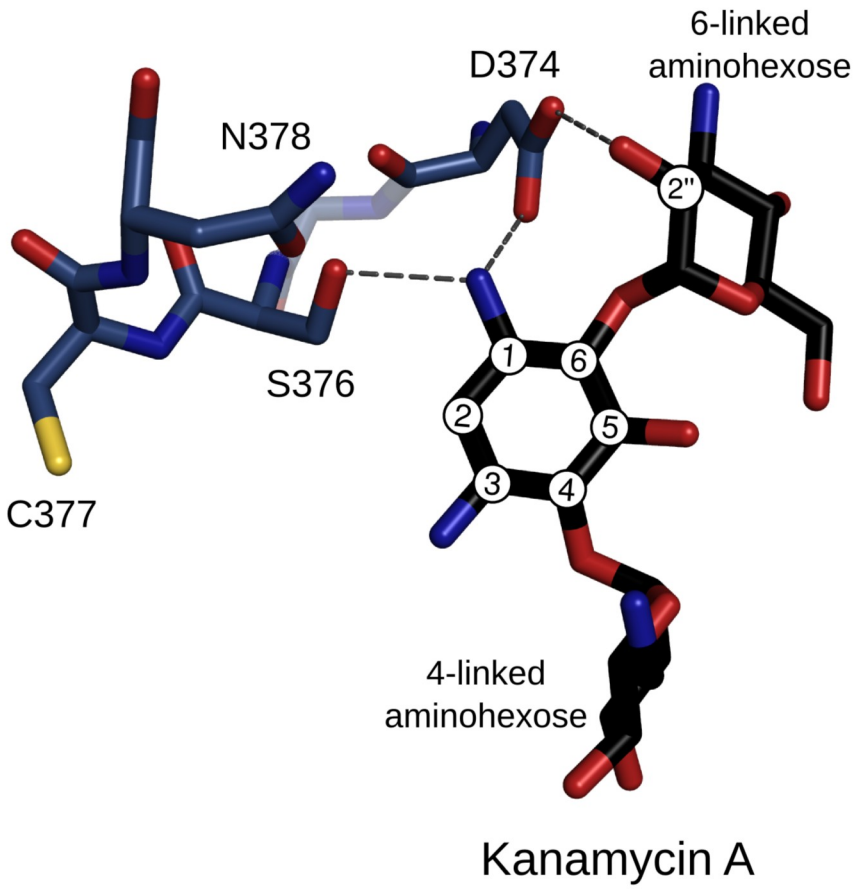

***Figure 5.2*: Binding of an N1-unsubstituted aminoglycoside to APH(2'')-Ia**

Kanamycin, bound to the APH(2")-Ia enzyme forms hydrogen bonds between the N1 group and residue S376 and the catalytic base D374. These and other interactions hold the compound in a position where the 2"-hydroxyl group of the 6-linked aminohexose ring can contact the catalytic base and react with the triphosphate (not pictured).

The S376N mutation of APH(2")-Ia and its activity toward arbekacin is the first reported instance of a natural mutation that enhances the activity of APH(2")-Ia toward N1-substituted aminoglycosides. The S376N mutant is more active toward arbekacin than amikacin, but the mechanism of this activity is not clear. Modification still occurs at the 2"-hydroxyl function of the antibiotic (Ishino et al., 2004), which is equivalent between these compounds. A structural

approach will help evaluate the interactions that occur in this enzyme and the wildtype enzyme that help facilitate phosphorylation of N1-substituted aminoglycosides.

5.1.5 Experimental approach

The wildtype APH(2")-Ia enzyme was purified and crystallized as previously described. Soaks with the semisynthetic aminoglycosides amikacin, dibekacin, and arbekacin were used to test for APH(2")-Ia binding to these compounds. Co-crystals could be grown with APH(2")-Ia, GMPPNP, and amikacin, and within these crystals aminoglycoside binding could be observed and the mode of binding modelled. This is the first instance where diffraction-quality crystals of APH(2")-Ia could be grown in the presence of an aminoglycoside compound.

The S376N mutant of APH(2")-Ia was produced by site-directed mutagenesis. The enzyme was purified and crystallized in the same means as the wildtype enzyme. The structure of this mutant was determined, and examined to track changes to the binding site of this enzyme upon mutation. Introduction of kanamycin, amikacin, arbekacin, and dibekacin by soaking was used to test for aminoglycoside binding to the resistance enzyme.

5.2 Methods

5.2.1 Protein production and purification

Site-directed mutagenesis of APH(2")-Ia was conducted using the same protocol as described in Chapter 4. The sequences of primers used to generate the S376N mutant are provided in Table 5.2.

Primer	Sequence
S376N forward	5'-GTGTTTATGCCATAATGATTTTAaTTGTAATCATCTATTGTTAGATGGC
S376N reverse	5'-GCCATCTAACAATAGATGATTACAAtTAAAATCATTATGGCATAAACAC

Table 5.1: Sequences of primers used to generate the S376N mutant of APH(2")-Ia

The S376N mutant enzyme was prepared and purified by the same protocol used for WT and other mutant proteins. While the protein purified using the same affinity resin and buffers, the yield was considerably lower than the wildtype protein and other mutants, which is likely linked to the active site mutation that altered the binding site of the enzyme, which is likely involved in binding to the first, aminoglycoside-affinity step on a kanamycin-linked agarose resin. In addition, the protein was less stable, degrading over time at room temperature, where other variants of the enzyme appeared stable.

5.2.2 Crystallization

The S376N enzyme was crystallized under similar conditions to the wildtype enzyme, but showed a higher degree of precipitation, making crystal growth harder to control. Protein at 10-15 mg/mL was combined with 1-2 mM GMPPNP and 2-4 mM $MgCl_2$ and incubated at room temperature for 30 minutes prior to setting drops in hanging-drop vapour diffusion crystal trays. These drops were streak seeded with protein crystals at initial set-up, but did not grow crystals until the drops were opened, the precipitated material removed, and the drops seeded again.

5.2.3 Aminoglycoside soaking and preparation of APH(2'')-Ia-GMPPNP-Amikacin co-crystals

Arbekacin and dibekacin were obtained from Meiji Seika co., Japan. Kanamycin and amikacin were purchased from Sigma-Aldrich. These compounds were all prepared at 2 mM in the reservoir solution for crystals (10% PEG 3350, 8% glycerol, 100 mM $MgCl_2$, 100 mM HEPES pH 7.5). 1 µL of this solution was mixed added to drops containing wildtype or S376N mutant enzyme, and allowed to incubate for 24 hours prior to crystallographic screening.

In soaking experiments with amikacin, it was observed that crystal growth did not cease following the addition of this compound, as it does following the addition of aminoglycosides without an N1-substitution. In this case, it was possible to set crystal growth experiments in the presence of amikacin. The protein was mixed with GMPPNP and $MgCl_2$ as with other experiments, but amikacin was also included at 1mM in the crystal growth conditions. These crystals grew in a manner similar to those pre-incubated without aminoglycoside, and could be cryo-protected and subjected to diffraction in the same manner as APH(2'')-Ia-nucleoside crystals.

5.2.4 Diffraction, model building and analysis

Crystals were screened on a Rigaku MicroMax 007 with Saturn 944^+ detector, and those which exhibited good diffraction were subjected to X-ray diffraction analysis. Data sets for these crystals were collected either on this instrument or at the 08-ID beamline of the Canadian Light Source Synchrotron (Saskatoon, SK). Images were integrated using iMosflm, and scaled in AIMLESS.

Crystals soaked with N1-substituted and N1-unsubstituted aminoglycosides were determined by the same methods and the F_o-F_o difference maps between the aminoglycoside-free and aminoglycoside-soaked structures were determined using the CAD, SCALEIT, and FFT packages of CCP4.

All models were phased using the final APH(2'')-Ia-GMPPNP structure, subjected to a round of refinement in REFMAC5, and the active site was inspected and corrected in Coot. pK_a of active site residues was calculated using the PropKa server.

	APH-S376N-GMPPNP	**APH-S376N-GMPPNP (Amk soak)**	**APH-S376N-GMPPNP (Kan soak)**	**APH-S376N-GMPPNP (Abk soak)**	**APH-S376N-GMPPNP (Dbk soak)**	**WT APH-GMPPNP (Abk soak)**	**WT APH-GMPPNP (Dbk soak)**	**WT APH-GMPPNP (Amk soak)**	**WT APH-GMPPNP (Amk co crystal)**
Data collection									
X-ray source	CLS Beamline 08-ID	CLS Beamline 08-ID	CLS Beamline 08-ID	Rigaku MicroMax 007	Rigaku MicroMax 007	Rigaku MicroMax 007	Rigaku MicroMax 007	CLS Beamline 08-ID	CLS Beamline 08-ID
Wavelength (Å)	0.9795	0.9795	0.9795	1.5418	1.5418	1.5418	1.5418	0.9795	0.9795
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
a, *b*, *c* (Å)	89.9, 99.7, 92.9	90.7, 99.7, 93.6	90.9, 99.3, 93.4	90.2, 99.7, 93.9	90.3, 99.8, 93.7	90.1, 100.5, 94.2	89.7, 98.4, 93.2	89.3, 100.1, 93.8	90.1, 100.2, 94.0
β (°)	104.9	105.2	105.3	104.8	105.0	105.1	105.4	105.0	105.1
Resolution (Å)	33.35-2.25 (2.30-2.25)	55.94-2.65 (2.75-2.65)	55.88-2.65 (2.74-2.65)	35.82-2.70 (2.80-2.70)	36.50-2.55 (2.63-2.55)	34.35-2.55 (2.63-2.55)	33.23-3.05 (3.24-3.05)	55.71-2.35 (2.41-2.35)	50.11-2.20 (2.24-2.20)
$CC_{1/2}$	0.997 (0.514)	0.993 (0.546)	0.993 (0.568)	0.991 (0.511)	0.997 (0.587)	0.986 (0.410)	0.965 (0.513)	0.991 (0.451)	0.991 (0.557)
R_{merge}	0.134 (1.219)	0.125 (1.047)	0.095 (0.755)	0.201 (1.099)	0.087 (0.676)	0.157 (0.906)	0.200 (0.615)	0.124 (1.077)	0.115 (1.022)
$I/\sigma I$	9.1 (1.5)	7.6 (1.9)	7.7 (1.6)	9.2 (1.8)	11.4 (1.7)	6.7 (1.4)	6.0 (2.0)	9.5 (1.6)	10.5 (1.8)
Completeness (%)	100.0 (100.0)	94.1 (77.5)	100.0 (100.0)	100.0 (100.0)	99.7 (99.1)	99.8 (99.5)	99.8 (99.9)	100.0 (100.0)	100.0 (100.0)
Multiplicity	7.2 (7.0)	4.3 (4.3)	3.7 (3.7)	7.1 (7.0)	3.6 (3.5)	3.5 (3.3)	3.4 (3.4)	4.2 (4.2)	4.3 (4.3)

Table 5.2: Data collection statistics for APH(2")-Ia and APH(2")-Ia S376N datasets described in this chapter

	APH-S376N-GMPPNP	WT APH-GMPPNP (Dibekacin soak)	WT APH-GMPPNP (Amikacin co-crystal)
Resolution	2.25	3.05	2.20
No. unique reflections	75134	28436	77748
R_{work}/ R_{free}	0.1720/0.2227	0.2332/0.2825	0.1797/0.2198
No. atoms			
Protein	9854	9837	9750
Ligands	141	263	237
Water	1469	368	639
Mean B-factors			
Protein	53.6	48.3	50.5
Ligands	47.9	41.2	47.3
Water	60.1	23.5	50.1
R.m.s deviations			
Bond lengths (Å)	0.0139	0.0094	0.1460
Bond angles (º)	1.6260	1.344	1.5480
Ramachandran			
%Favoured	96.60	94.49	97.24
%Allowed	2.89	4.83	2.07
%Outlier	0.51	0.68	0.69

Table 5.3: Structural statistics for models of APH(2")-Ia and APH(2")-Ia S376N

5.3 Results

5.3.1 N1-substituted aminoglycosides block binding to APH(2")-Ia in the crystal form

Crystals of wildtype APH(2")-Ia prepared with GMPPNP were soaked with arbekacin, dibekacin, and amikacin. The structure determined by soaking of kanamycin reported in Chapter 3 was also included in this analysis. Kanamycin binds to the conserved neamine-binding platform and places the 2" hydroxyl group in position for phosphotransfer from the GTP substrate, as discussed in Chapter 4. Dibekacin adopts the same conformation, despite changes in the 4-linked aminohexose in this compound. Both of these compounds exhibit clear difference density in F_o-F_o difference maps, indicating unambiguous binding in the aminoglycoside-binding site when corrected relative to the aminoglycoside-free structure (Figure 5.3a, b).

Soaking of amikacin and arbekacin into these crystals indicates that introduction of the N1 group indeed blocks the binding of compounds in these crystals, as made clear by the lack of difference density between crystals (Figure 5.3c, d). There is no evidence for any alternative binding modes occupied by these compounds, so the addition of an N1-linked AHB group to kanamycin and dibekacin completely abrogates binding to the APH(2")-Ia enzyme, at least in the enzyme's pre-crystallized form.

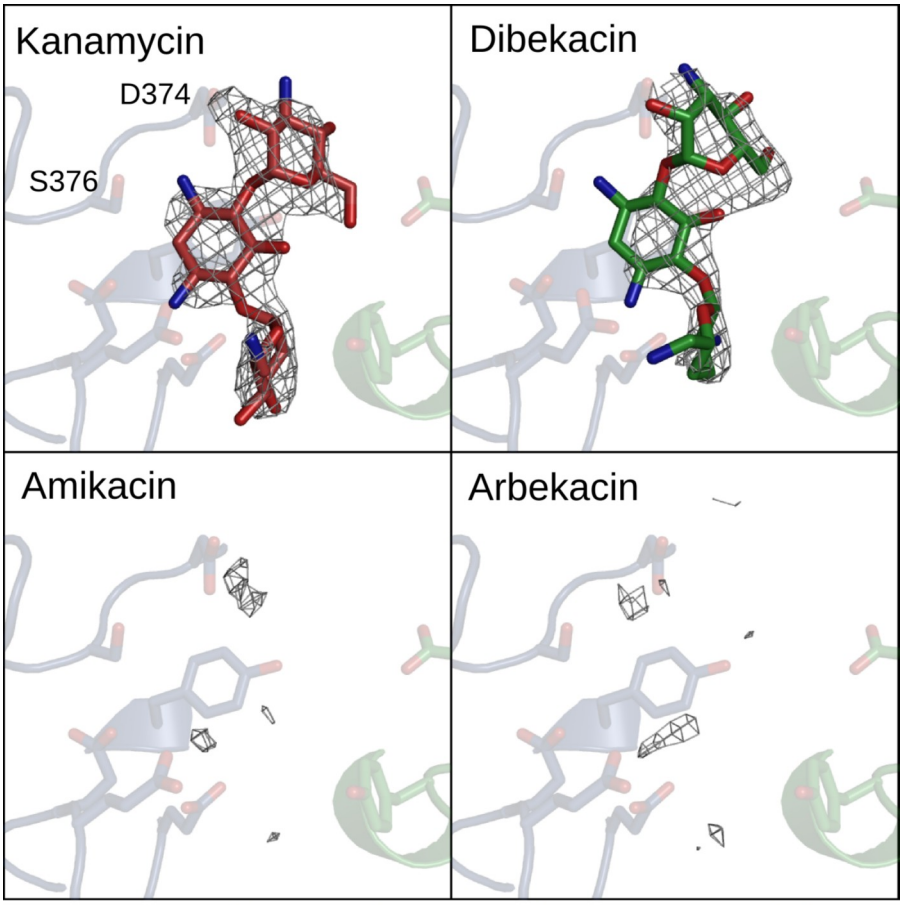

Figure 5.3: **Difference density in the active site of the APH(2'')-Ia enzyme soaked with unsubstituted and N1-substituted aminoglycoside antibiotics**

F_o-F_o difference electron density for crystals of APH(2'')-Ia soaked with N1-unsubstituted (kanamycin, dibekacin) and N1-substituted (amikacin, arbekacin) aminoglycoside antibiotics. Difference maps are calculated as the difference of the aminoglycoside-soaked and APH-GMPPNP data sets (Chapter 3), using the phases from the unsoaked structure. All images represent chain D of the respective structure. Maps are carved at 2 Å around the position of the kanamycin in the kanamycin-bound structure, displayed at σ = 3.0, within 2 Å of the equivalent molecule of kanamycin in the kanamycin-bound structure.

The equivalent binding modes observed for kanamycin and dibekacin indicate that despite their alterations on the 3' and 4' sites, there is little difference that can be distinguished between these compounds binding to APH(2'')-Ia. Analysis is limited by the low resolution of this data set but dibekacin appears to show some variability in the position of its 6-linked aminohexose ring

(Figure 5.4), similar to gentamicin (Section 3.3.3). Dibekacin conserves the neamine-based interactions with the enzyme and doesn't exhibit any additional interactions facilitated by the altered 4-aminohexose ring of dibekacin. The deoxygenation of dibekacin and arbekacin do not appear to play a role in the differential activity of the enzyme toward these compounds.

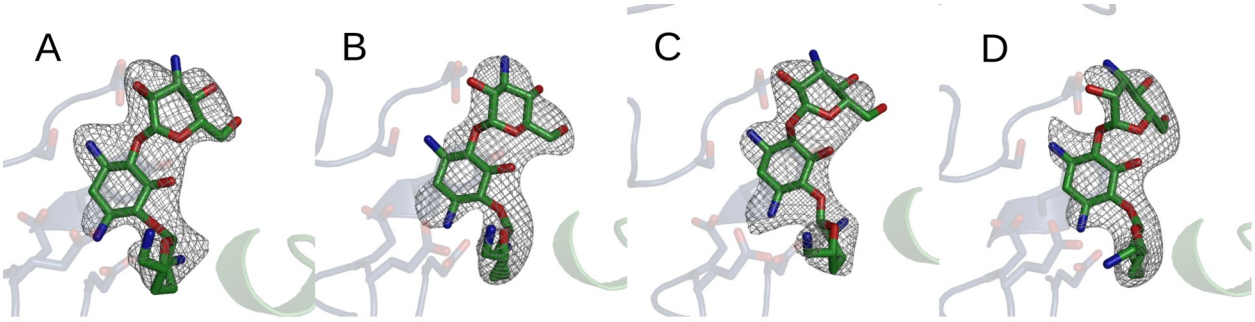

***Figure 5.4*: Difference density for dibekacin soaked into crystals of wildtype APH(2'')-Ia**

F_o-F_c refined difference density at σ = 2.8 for chains A-D of APH-GMPPNP crystals soaked with the aminoglycoside dibekacin, displayed within 2 Å of the modelled aminoglycoside. Despite limited resolution, the electron density in these structures indicates binding of this compound consistent with other 4,6-disubstituted aminoglycosides.

5.3.2 Co-crystals of amikacin and wildtype APH(2'')-Ia indicate two weak binding modes

In previous soaking experiments with aminoglycosides in APH(2")-Ia, introduction of the aminoglycoside substrate typically halted crystal growth. Upon observation that crystal growth continued upon soaking with amikacin, co-crystallization trials were carried out with APH(2")-Ia, GMPPNP, magnesium, and amikacin. Crystals were obtained under these conditions and the structure of these crystals was solved. Upon examination of the aminoglycoside binding sites in this structure, it was found that two of the four protein chains exhibited evidence of a bound aminoglycoside (Figure 5.5). In both cases, the electron density is comparatively weak when compared to N1-unsubstituted compounds, but models of these two binding modes could be built.

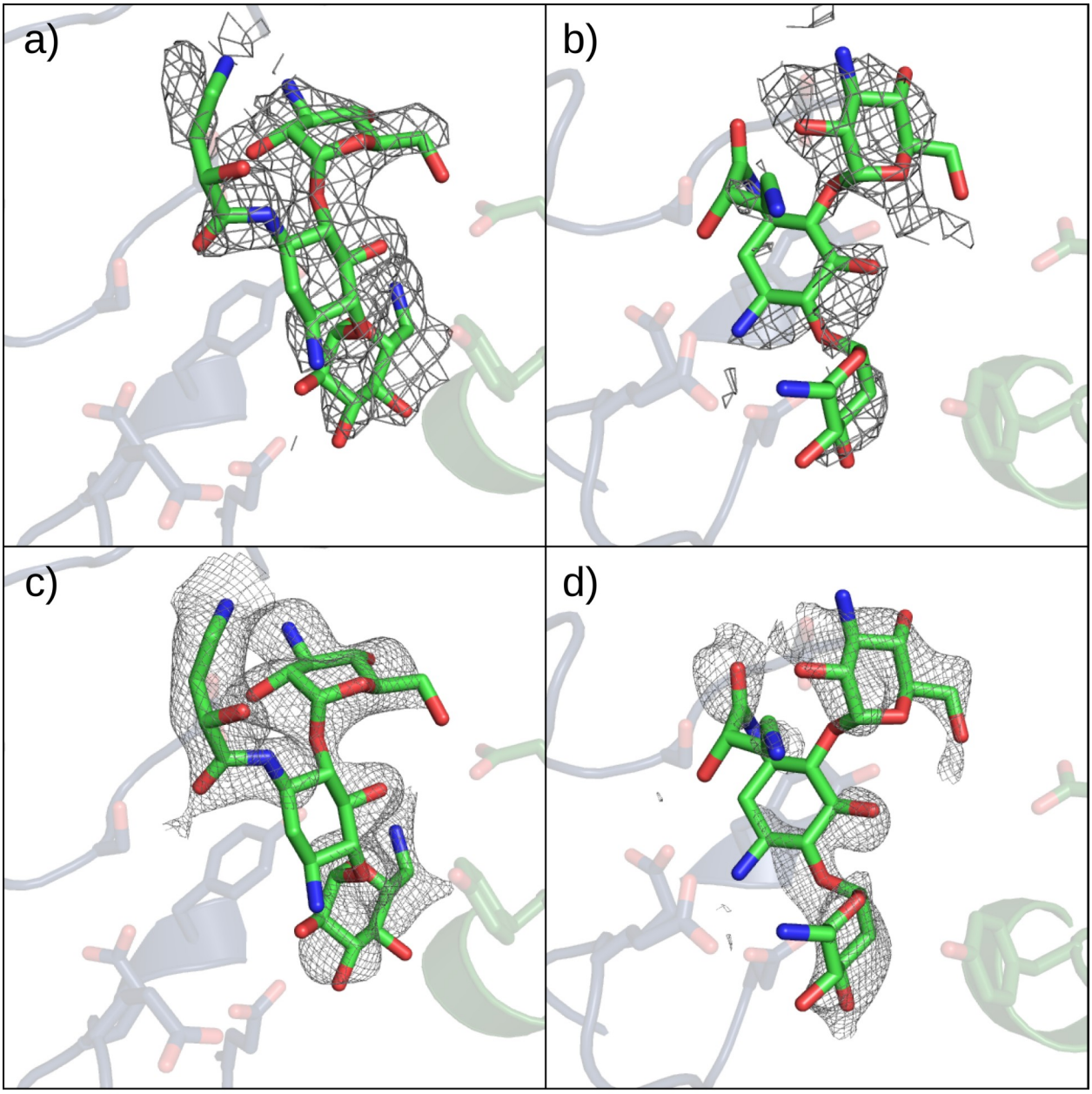

Figure 5.5: **Electron density in the active site of APH(2'')-Ia co-crystallized with GMPPNP, Magnesium, and amikacin**

a) F_o-F_o map density in the aminoglycoside-binding site of chain A of the APH-GMPPNP-amikacin co-crystal structure. Contoured at σ = 2.5 and displayed within 2 Å of the modelled aminoglycoside. b) The equivalent map, calculted in chain D of the same structure. Contoured at σ = 2.5 and displayed within 2 Å of the modelled aminoglycoside. c) F_o-F_c refined omit difference map following refinement of the APH(2")-Ia-GMPPNP-Amikacin structure, chain A. Contoured at σ = 2.0 and displayed within 1.75 Å of the modelled aminoglycoside. d) The equivalent map calculated in the active site of chain D. Contoured at σ = 2.0 and displayed within 1.75 Å of the modelled aminoglycoside.

These conformations are still poorly defined, likely due to high mobility in the active site of the enzyme. Nevertheless, it's possible to model approximate conformations for these co-crystallized aminoglycosides. In chain A of this structure, the aminoglycoside adopts a conformation that is unique when compared to that previously observed for neamine based compounds. The 6-linked aminohexose ring and the AHB group are confined and bound near the closed Gly-loop in this domain, although the rest of the compound is less well defined, refined at half-occupancy. Electron density for the 4-linked aminohexose ring places it in contact with some of the same residues that bind the 6-linked ring of other aminoglycosides, but it binds in an orientation upside-down relative to those compounds. It also completely avoids forming contacts between the central ring and the neamine-binding site of the protein.

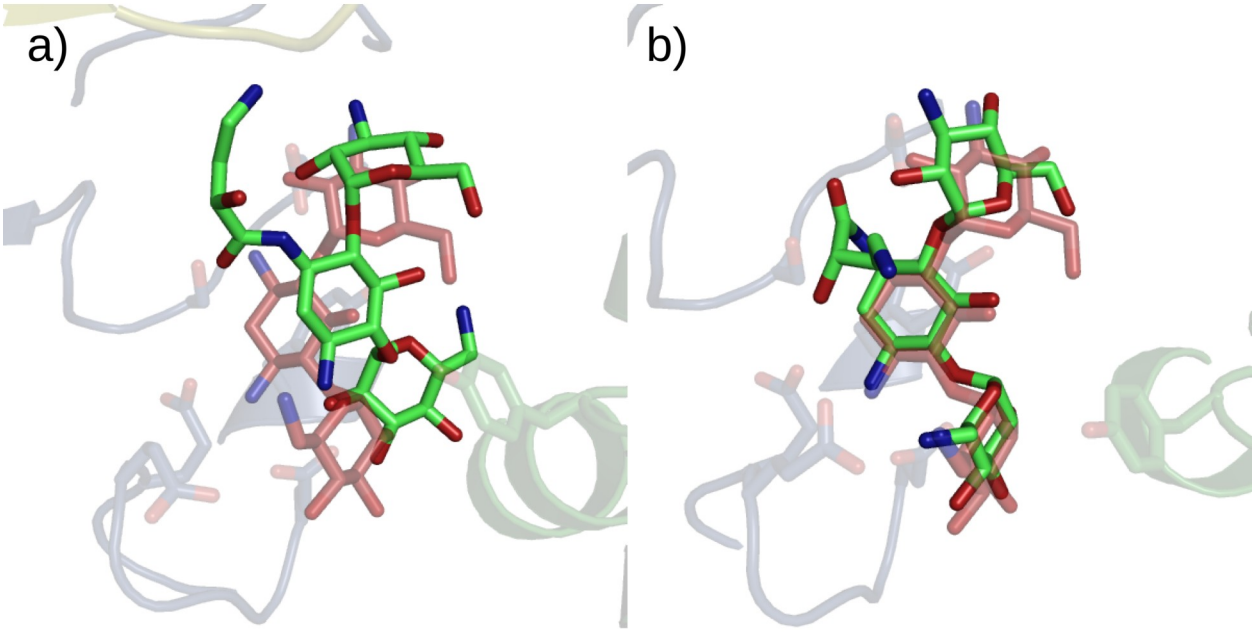

***Figure 5.6*: Superimposition of kanamycin binding to wildtype APH(2'')-Ia and newly observed amikacin binding modes**

a) In chain A of the amikacin co-crystal structure, amikacin (green) adopts a new binding interaction with the enzyme when compared to kanamycin bound to the wildtype enzyme (maroon). The 4-linked aminohexose lies in a different position in this structure and binds in an inverse orientation relative to kanamycin. b) In chain D of the same structure, the weak binding mode is similar to that of kanamycin, superimposing well and adopting an unfavourable conformation of the amide group in order to bind.

In chain D of this structure, amikacin binds in a similar manner to its non-substituted equivalent kanamycin (Figure 5.6b), but only at ~60% occupancy. In this case, the N1 AHB group refines as an unfavourable *cis*-amide to fit in the binding site, but this awkward conformation permits the compound to bind in a conformation otherwise compatible with the binding mode of unsubstituted aminoglycosides like kanamycin and gentamicin.

One or both of these interactions may contribute to the weak 2"-O-phosphorylation activity of the enzyme toward amikacin and arbekacin. In both of these cases, there is a 2" hydroxyl group placed in contact with the catalytic aspartic acid residue, but neither is placed in a good geometric orientation for a productive reaction. As discussed in Chapter 4, induced changes to the catalytic machinery of the enzyme are necessary to activate the enzyme, and changes to the conformation of these aminoglycosides must also be necessary to allow the reaction to take place. Some of these changes may not be accessible in crystals of APH(2")-Ia, so these binding interactions may indicate the structural changes that are necessary to occur in solution for the enzyme to be active toward these N1-substituted aminoglycosides. The high K_M values determined for interaction of these compounds with APH(2")-Ia indicate that these interactions are weak, but we can infer some of their character from this binding interaction with the enzyme.

5.3.3 Crystal structure of the APH(2")-Ia S376N mutant

The structure of APH(2")-Ia S376N was determined by Fourier synthesis of the wildtype structure in complex with GMPPNP. Crystals of this mutant took longer to grow than wildtype, and correspondingly, there was degradation of the triphosphate co-substrate in these crystals. Inspection of this structure revealed that the S376N mutation is accommodated in the aminoglycoside-binding site with very little change. The only visible changes to the enzyme structure are adjustments to the nearby C377 and N378 residues that adapt to the introduction of the larger asparagine side chain in the mutant (Figure 5.7).

Substitution of N for S376 pushes N378 into a new conformation, rotated away from the catalytic site of the enzyme. The equivalent residue in homologous APH(2")-IIa was found to be involve in increased resistance to N1-substituted aminoglycosides (Toth et al., 2010), so the structural change to this residue may lead to improved binding and modification of N1-substituted aminoglycosides in APH(2")-Ia as well.

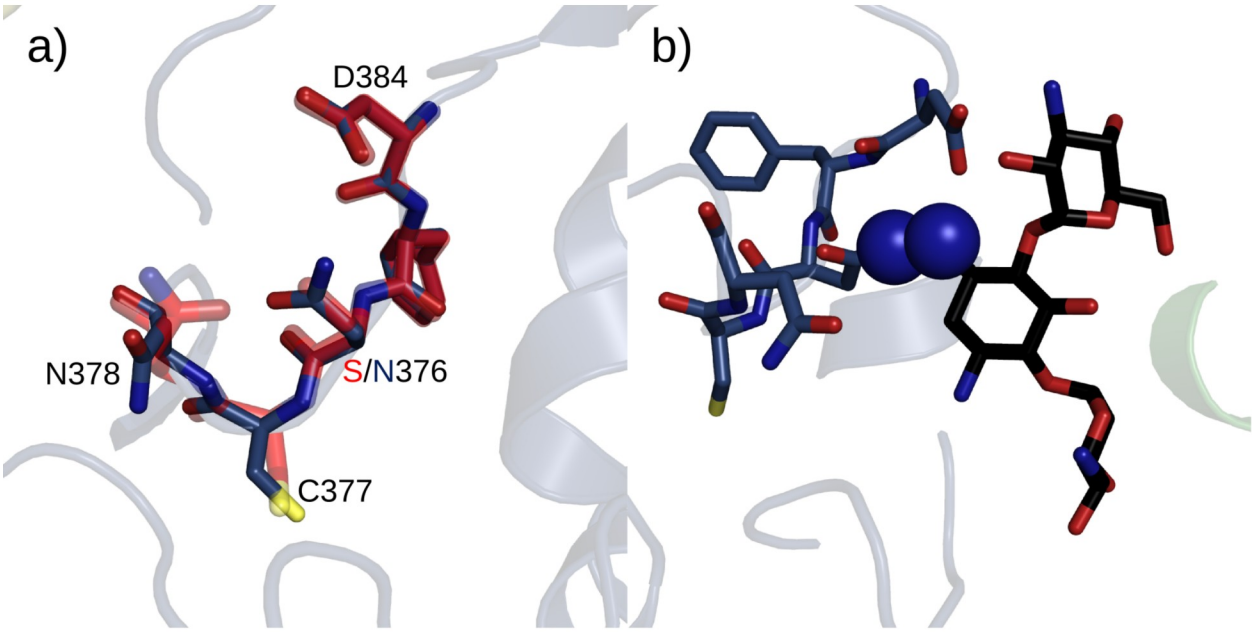

***Figure 5.7*: The active site of wildtype APH(2'')-Ia in comparison with the S376N mutant**

a) Superimposition of wildtype (red) and N376 mutant APH(2")-Ia. Subtle shifts to adjacent C377 and N378 residues are observed, while the catalytic D384 residue is not affected. b) Superimposition of S376N mutant with kanamycin bound in soaks of wildtype crystals. Clash of atoms is indicated by overlapping spheres of the N1 nitrogen of kanamycin and the amide nitrogen of asparagine 376.

The structure of S376N reaffirms the observation that gentamicin is no longer modified by the enzyme (Ishino et al., 2004). As there is almost no change to the backbone of the protein in the active site, the asparagine group disrupts the binding platform where gentamicin, kanamycin, and other neamine-based aminoglycosides bind. However, with no rearrangement to this active site, there is also no clear means by which an N1-substituted aminoglycoside could bind to the protein in the same fashion as unsubstituted compounds bind (Section 3.3.3). The S376N mutant does not alter the enzyme in a way that facilitates improved N1-substituted aminoglycoside binding.

Without obvious explanation of the binding of N1-substituted compounds to this marginally changed active site, I turned to crystallographic soaking experiments to look for interactions between aminoglycosides and the S376N mutant enzyme.

5.3.4 Soaks of compounds into S376N mutant indicate a lack of additional contacts that stabilize aminoglycosides to this enzyme

Amikacin, dibekacin, arbekacin, and kanamycin were all introduced into crystals of APH(2")-Ia S376N by crystallographic soaking, with the aim of identifying an alternative binding mode for N1-substituted aminoglycosides facilitated by the S376N substitution. Unfortunately, in these structures, there is very little difference electron density that would indicate the presence of these soaked aminoglycoside compounds (Figure 5.8). The introduction of the S376N mutation or the accompanying shifts in the aminoglycoside-binding site do not appear to make any new contacts that facilitate strong aminoglycoside binding as visible by this technique.

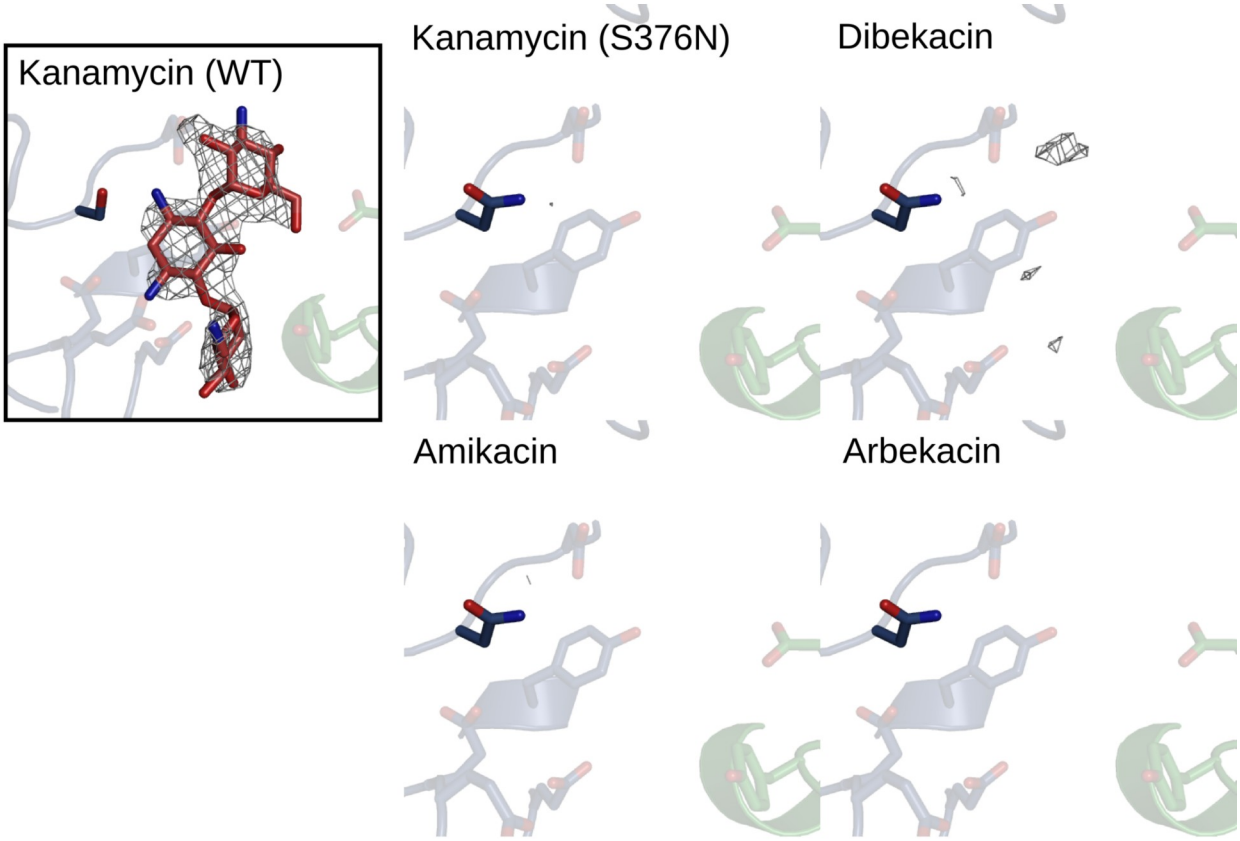

***Figure 5.8*: Difference density in the active site of the S376N mutant enzyme soaked with aminoglycoside antibiotics**

F_o-F_o difference electron density for crystals of S376N mutant APH(2")-Ia soaked with N1-unsubstituted (kanamycin, dibekacin) and N1-substituted (amikacin, arbekacin) aminoglycoside antibiotics. Difference maps are calculated as the difference of the aminoglycoside-soaked and S376N APH-GMPPNP data sets, using the phases from the unsoaked structure. All images represent chain D of the respective structure. Maps are carved at 2 Å around the position of the kanamycin in the kanamycin-bound wildtype structure (inset for comparison), displayed at σ = 3.0.

5.3.5 S376N is compatible with and alternate binding mode of amikacin, may support increased binding and catalysis of the compounds.

Mutation of S376 does not induce structural changes that allow accommodation of the N1-AHB group of compounds through the typical binding mode that other aminoglycosides use. In fact, the larger asparagine side-chain provides an additional obstruction to binding, making this interaction even less favourable. This is borne out in soaking experiments where this mutation

appears to completely block binding of N1-substituted and -unsubstituted compounds to the aminoglycoside-binding site, at least in crystals.

The S376N mutation also doesn't introduce any chemical changes that would improve activity toward N1-substituted compounds, either. While the N376 mutation introduces a new amino acid that contacts the enzyme's catalytic D374 base, pKa calculations indicate that this contact decreases the pKa of this residue slightly (6.2 in the wildtype enzyme, 5.6 in the S376N mutant as calculated by PropKa), which would actually make the residue slightly less reactive. So, this interaction is unlikely to introduce a greater rate of catalysis in the S376N mutant.

Modelling of the S376N mutation in the co-crystal structure of amikacin bound to APH(2")-Ia indicates that the binding mode observed in chain A of the APH-GMPPNP-Amikacin co-crystal structure can tolerate changes to the S376 residue, with the larger asparagine residue easily accommodated (Figure 5.9). Despite a lack of effective soaking into crystals of S376N APH(2")-Ia, it is possible that this mutation supports the atypical binding mode of amikacin, through water-coordinated interactions. Such an interaction could facilitate the binding and subsequent modification of N1-substituted compounds, by contributing interactions that help stabilize this alternate means of aminoglycoside binding in the enzyme.

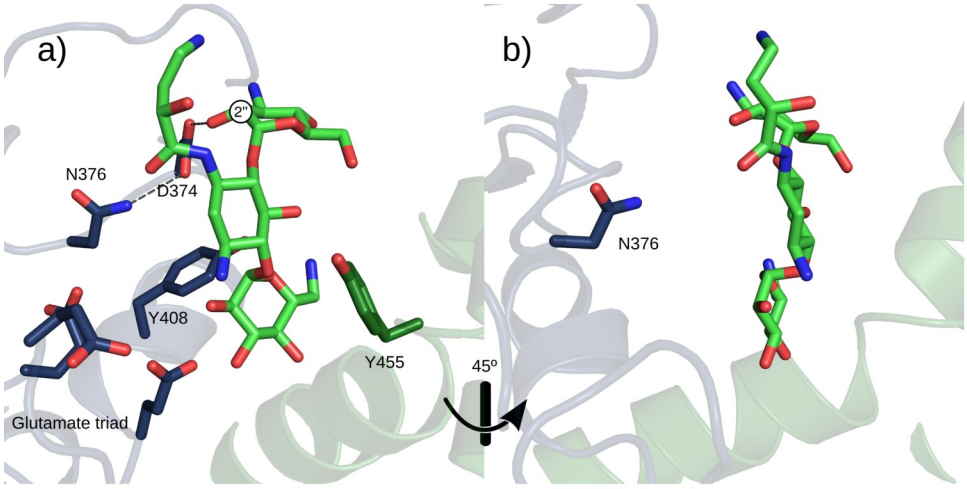

Figure 5.9: **Superimposition of amikacin bound to wildtype APH(2'')-Ia in the aminoglycoside-binding site of the S376N mutant**

a) The weak alternate binding mode of amikacin observed in chain A of the co-crystal structure easily tolerates substitution of S376 to N. b) The introduction of an asparagine at this site may help facilitate amikacin binding, although the binding interactions are too far to be direct, possibly facilitated by active site water molecules.

5.4 Discussion

5.4.1 N1-modification blocks neamine-based binding interactions of aminoglycosides, although alternate binding modes appear possible

Relative to the N1-unsubstituted aminoglycosides kanamycin and dibekacin, the N1-substituted aminoglycosides amikacin and arbekacin do not bind in the crystallized APH(2")-Ia enzyme. This does not preclude binding to these compounds at all, but in the crystals where easy binding to unsubstituted compounds takes place, the AHB group added to N1 of amikacin and arbekacin completely blocks this interaction.

Reports have described the regioselective 2"-phosphorylation of arbekacin by both the wildtype (Kondo et al., 1993) and S376N mutant (Ishino et al., 2004) enzymes. This occurs despite high K_M values (Daigle et al., 1999a), which indicate weak binding of the substrate in a catalytically-competent conformation.

In contrast with earlier observations that unsubstituted aminoglycosides conserve binding interactions with APH(2")-Ia though the neamine-like core elements, amikacin indicates a weak alternate means by which it may bind the enzyme. This alternative means of binding may be responsible for the low rates of 2"-phosphorylation observed with amikacin and arbekacin. Even if the enzyme confers feeble rates of phosphorylation toward N1-substituted aminoglycosides, there may be instances where it is advantageous. This phosphorylation would not happen at high rates, but could confer resistance in cases where expression of the enzyme is greatly up-regulated, as in one clinical arbekacin resistant strain (Matsuo et al., 2003).

5.4.2 S376N mutation blocks prototypical aminoglycoside binding but may improve alternate amikacin binding mode

The S376N mutation also doesn't introduce any chemical changes that could easily affect interactions with N1-substituted aminoglycosides. While the N376 mutation introduces a new amino acid that contacts the enzyme's catalytic D374 base, p*Ka* calculations indicate that this contact decreases the pKa of this residue, which would make the residue slightly less reactive. This interaction is unlikely to introduce a greater rate of catalysis in the S376N mutant.

All four compounds soaked into crystals of APH(2")-Ia S376N showed no visible binding in the Fo-Fo difference map (Figure 5.7). This indicates that just as the N1 group blocks binding to the wildtype enzyme, the S376N mutation blocks binding of all aminoglycosides to the enzyme in the crystalline form.

While this mutation blocks compounds binding to the typical site, it may help accommodate the alternate mode of binding in chain A of the APH(2")-Ia-GMPPNP-Amikacin co-crystal structure. The fact that this interaction only occurs in co-crystals may reflect the necessity for this compound to bind to the free enzyme before forming crystals, or alternatively that slightly higher concentrations of amikacin in the crystallization trays compared to soaking of the compound (3 mM versus ~1 mM) drives more binding to the enzyme. In either case, the binding is still weak and not particularly well resolved in comparison to the N1-unsubstituted aminoglycosides introduced into crystals by soaking like dibekacin (Section 5.3.1) and other neamine-based aminoglycosides (Section 3.3.3).

5.4.3 Promiscuity and specificity in aminoglycoside binding to resistance enzymes

It seems likely that the modification of amikacin and arbekacin by APH(2")-Ia is a result of a weak, secondary binding mode which is not easily studied by standard enzyme methodologies. Despite our findings presented in Chapter 3 that unsubstituted aminoglycosides use a conserved platform that binds the neamine rings of these compounds, there is evidence of an alternate means by which the N1-substituted amikacin and arbekacin might bind the enzyme and still be

modified. This is not an uncommon occurrence, as multiple enzymes, including APH(2")-Ia, have been shown to have some promiscuity in their aminoglycoside binding mechanisms (Section 1.4.6).

Our findings of lividomycin binding to the enzyme showed that this compound exhibited multiple binding modes in the aminoglycoside binding site (Section 3.3.6), while evidence for modification of neomycin on an alternate site (Daigle et al., 1999a) necessitates some alternative means of placing the 5" hydroxyl group in contact with the enzyme's catalytic centre. In AAC(1) and the Eis acetyltransferase modification of compounds can occur at multiple sites (Chen et al., 2011; Sunada et al., 1999). APH(3') phosphorylates at the 5" position of some compounds (Thompson et al., 1996), while ANT(4')-Ia also phosphorylates some compounds at the 4" position (Gerratana et al., 2001). These nonspecific interactions typically confer weak resistance when compared to the highly evolved, dedicated means of resistance that enzymes are honed toward over time.

However, these alternate functions can become native functions with mutation and selection. Mutations that stabilize the interactions and promote this activity, even with marginal rates, can provide an advantage when selective pressures are sufficient to tolerate the fitness costs of the adaptation.

5.4.4 Mutation to S376N reflects the messy emergence of a new function in a resistance factor

Modification of N1-substituted aminoglycosides might be considered a “moonlighting” function (Moore, 2004) of the APH(2")-Ia kinase enzyme. Such a function, in this case a similar catalytic reaction accomplished through a secondary means of binding the substrate, can confer new functions to a protein, and can provide selective advantages if it occurs in the appropriate context.

The weak catalytic activity toward N1-substituted aminoglycosides in APH(2")-Ia and promotion of this activity by the S376N mutation may reflect the first step in converting a moonlighting function of the enzyme to the optimized native activity. This new function indicates that while a resistance factor can be optimized, it is still not static and subject to change.

Increased activity of APH(2")-Ia toward amikacin is consistent with what we might expect for the emergence of a *de novo* feature in an antibiotic resistance enzyme. Without time to develop additional compensatory mutations, any selective benefit provided by a new mutation will be offset by selective losses, in this case large losses in the lack of activity toward gentamicin and other aminoglycosides now precluded from binding. Additional epistatic changes are probably necessary for the enzyme to improve its selectivity and specificity for a new substrate (Schenk et al., 2013). These changes can bring about catalytic optimization of the enzyme.

This echoes that of AAC(6')-Ib-cr, a mutant of AAC(6')-Ib that gained two point mutations that added ciprofloxacin resistance to the enzyme, a completely different class of antibiotic (Robicsek et al., 2006). In this case, the enzyme bound its new substrates 100 fold less strongly than its native substrates and destroyed the enzyme's activity toward neomycin, but the new resistance form was still strong enough for it to be advantageous to the bacterium (Vetting et al., 2008).

Changes to APH(2")-Ia also echo the mutagenesis studies of APH(2")-IIa that found no single mutation provided increased resistance to amikacin, but two double-mutants were sufficient to generate resistance, although the mutants generated did not create obvious new contacts for the N1-substituted compound (Toth et al., 2010). It is possible that in this case the mutations also helped the enzyme stabilize an already weak binding interaction.

5.5 Conclusions

APH(2")-Ia shows weak to no binding to N1-substituted aminoglycosides when compared with their unsubstituted counterparts. This confirms that the addition of an AHB group to the compounds blocks them from binding to the enzyme by the same means as unsubstituted neamine-based compounds. The semisynthetic aminoglycoside dibekacin, which does not possess an N1-substitution, binds in a manner fully consistent with neamine-based binding described in Chapter 3.

The N1-substituted aminoglycoside amikacin exhibits two weak secondary binding modes consistent with 2"-*O*-phosphorylation. Different conformations in different protein chains reflect importance of the protein conformation in determining how the the antibiotic interacts with the binding site. The first of these binding conformations is similar to that of kanamycin bound to the enzyme, where the N1 group is accommodated by adopting an unfavourable *cis*-amide conformation. The second of these modes is primarily facilitated by the 6'-linked ring and N1-AHB group. Poor definition for the other rings indicate that most of the binding interactions are driven by the non-neamine-based parts of the molecule.

The S376N mutant of APH(2")-Ia blocks binding of aminoglycosides containing the neamine rings of the antibiotics. This arbekacin-resistant mutation does not provide structural changes that lead to alteration of the aminoglycoside binding site, or through facilitation of a well-defined alternate binding mode. Soaking experiments support that this mutation does not stabilize binding to N1-substituted compounds or N1-unsubstituted compounds.

Of the two conformations of amikacin modelled in the co-crystal structure determined from this compound, one is compatible with the S376N mutation. The catalytic benefit of the N376 residue could be in stabilizing this alternative conformation of the aminoglycoside. If true, this indicates that the APH(2")-Ia enzyme exhibits potential for the emergence of new antimicrobial resistance activities, even in this catalytically optimized and established antibiotic resistance factor.

6 Summary and outlook

These studies collectively illustrate aspects of antibiotic resistance caused by a widespread resistance factor. The structural characteristics of this protein are consistent with a fine-tuned machine that confers resistance to a broad swath of compounds, and does so with a minimized impact upon its host cell. At the same time, this resistance factor is still subject to change and can adapt to new challenges as they are presented.

AAC(6')-Ie/APH(2'')-Ia illustrates that antibiotic resistance is an ancient process with versatility and dynamism. However, even finely-tuned resistance factors can still be opportunistic and change in response to the innovations we use to fight them.

6.1 AAC(6')-Ie/APH(2'')-Ia as an exemplar of antibiotic resistance

Study of any resistance factor teaches us about the nature of resistance as a whole. While a common understanding of antibiotic resistance involves *de novo* mutations that emerge and are selected in response to antibiotic use, dedicated resistance factors, pre-existing in environmental samples, present finely-tuned and optimized antibiotic resistance. These ancient resistance elements will continue to emerge in clinical populations despite our best concerted efforts to stop them. Improving our understanding of the mechanisms of resistance and their common features found within unrelated resistance factors allow us to be better prepared for the inevitable emergence of new and previously unseen forms of resistance.

AAC(6')-Ie/APH(2'')-Ia is one of these resistance factors, both refined to high activity, but also with a breadth of range that makes it broadly active toward many compounds. This protein is an excellent microcosm of the forces that shape antibiotic resistance, and the innovations and paradoxes within it. The structural experiments of AAC(6')-Ie/APH(2'')-Ia described in this thesis illustrate the influence of competing forces upon the evolution of AAC(6')-Ie/APH(2'')-Ia.

Resistance in AAC(6')-Ie/APH(2'')-Ia is dedicated. In Chapter 2, it was found that the bifunctional enzyme shows a rigid global architecture and the two enzymatic domains are fixed relative to each other. Furthermore, binding of ligands did not appreciably change this arrangement. This structural arrangement between domains implies the enzyme has a long

evolutionary history as a bifunctional particle, required to allow the adaptive change necessary to make a rigid association between the domains. This also requires a function that is selected for in arranging these domains together, which could be through small-scale structural rearrangements that allow communication between domains, or through interactions such as steering or channelling of substrates that increase the efficiency of one or both enzymatic domains.

Resistance in AAC(6')-Ie/APH(2'')-Ia is versatile. In Chapter 3, it was found that the APH(2")-Ia domain of the enzyme binds both 4,5-disubstituted and 4,6-disubstituted aminoglycosides using the same conserved scaffold. The binding of both 4,5-disubstituted and 4,6-disubstituted aminoglycosides to the enzyme illustrates a conserved platform for antibiotic binding that allows these compounds to interact with the enzyme using their conserved rings. This combination of specificity to the conserved nucleus of the antibiotic compounds while toleration of differences continues a common theme in dedicated antibiotic resistance enzymes. Antibiotic resistance is specific but accommodates variability.

Resistance in AAC(6')-Ie/APH(2'')-Ia is thrifty. In Chapter 4, a mechanism of energy conservation in this enzyme was identified. APH(2")-Ia also shows adaptation and optimization in its catalytic mechanism. The enzyme contains a catalytic switch between two conformations of the active site triphosphate substrate. This antibiotic resistance enzyme has developed ways of reducing its impact on the host organism. In observing this conformational switch it is possible to track multiple structural steps that occur in the enzyme to transition from unbound and open to closed, activated, and catalytically competent.

Resistance in AAC(6')-Ie/APH(2'')-Ia is malleable. In Chapter 5, a potential weak alternative binding mode of N1-substituted aminoglycosides in the active site of APH(2")-Ia was identified. This binding mode is compatible with a clinically observed mutant, S376N, which improves resistance to N1-substituted compounds, and so may be promoted by this mutation. This mutant enzyme is an example of the development of a new function in a resistance enzyme, but has not had evolutionary time to adapt and optimize the enzyme, leading to low activity and the loss of native activity toward 4,6-disubstituted aminoglycosides. These observations illustrate that APH(2")-Ia remains capable of adaptation toward new substrates, although this comes at a loss of efficiency and native activity.

AAC(6')-Ie/APH(2'')-Ia illustrates that antibiotic resistance is finely-tuned, regulated, and judicious, but also opportunistic with the potential to change in response to the innovations we use to counteract it.

6.2 Future study of the AAC(6')-Ie/APH(2'')-Ia protein

These studies on a widespread clinical antibiotic resistance factor illustrate important features of a unique protein, but also leave many new areas of inquiry in their wake.

While the structure of the full-length enzyme can be modelled against SAXS data, and this was independently validated by another group (Smith et al., 2014) a high-resolution structure of the intact two-domain particle has not yet been determined. This determination that the enzyme exhibits a rigid bi-domain structure where the domains pack against each other indicates that a complete structural model should still be possible through the structural study of the full-length protein. While I was not successful in determining conditions for X-ray diffraction quality crystals of the full-length enzyme, I remain optimistic that crystal leads I identified will one day yield quality crystals, and the full-length AAC(6')-Ie/APH(2'')-Ia structure will be determined by X-ray crystallography. In addition, other techniques such as electron microscopy, nuclear magnetic resonance spectroscopy (especially utilizing residual dipolar couplings) (Fischer et al., 1999), spin-labelled electron paramagnetic resonance spectroscopy, site-directed mutagenesis interface mapping, mass spectrometry footprinting could also be applied to the study of domain interactions in this bifunctional enzyme to probe interactions between domains outside of a crystal lattice.

The 4,5-disubstituted aminoglycoside-binding behaviour of the enzyme indicates how these compounds bind the APH(2'')-Ia domain, but leave the question of why. If the function of this binding activity is to sequester the antibiotic from the ribosome, then a catalytically inert enzyme should confer resistance to the antibiotics, which can be tested. If the aminoglycoside binding to the enzyme is strictly an accident of evolution, then the binding of these compounds might be expected to exhibit a fitness cost to the microbe, which is not easily tested in the context of the full-length enzyme which also modifies these compounds at the AAC(6')-Ie domain, but could be evaluated in the APH(2'')-Ia in isolation. While some studies have proposed that other APH(2'') enzymes can not bind to 4,5-disubstituted aminoglycosides due to steric factors (Shi et al., 2011),

the amount of flexibility observed in APH(2")-Ia accommodating aminoglycoside binding would indicate that this could also be possible in other APH(2") enzymes.

The mode of antibiotic binding observed indicates a possible inhibitor development strategy, as neamine rings and even larger compounds are still bound competitively by the enzyme and they would displace the modifiable substrates like gentamicin and tobramycin from the active site. Use of an aminoglycoside-based APH(2")-Ia inhibitor could be possible, or simply a cocktail of 4,5-disubstituted and 4,6-disubstituted aminoglycosides. Any strategy of this sort must also contend with the AAC(6')-Ie domain of the enzyme, so compounds with no 6' amino group are probably a wise starting point.

The catalytic switch between stabilized and activated triphosphate conformations indicates an adaptive behaviour of the enzyme, which could be involved in mitigating the enzyme's fitness cost. This process can be further probed through study of the active-site mutants that influence the triphosphate, although this is complicated by the fact that S214 and Y237 also appear to be involved in a separate process: stabilizing the Gly-loop for catalysis. The induction of a higher rate of catalysis upon aminoglycoside binding explains some puzzling previous findings, but also raises the question of what role an increased hydrolysis rate in 4,5-disubstituted aminoglycoside bound APH(2")-Ia could play in biology. This remains an open question, intrinsically linked to the study of enzymatic fitness in AAC(6')-Ie/APH(2")-Ia.

If there is indeed an adaptive mechanism in APH(2")-Ia to reduce off-target hydrolysis, this mechanism could be exploited in two ways in antibiotic adjuvant design. Compounds that trigger inappropriate activation and hydrolysis in the enzyme (as 4,5-disubstituted compounds appear to do) can act as anti-resistance agents, by increasing the fitness cost toward the enzyme. Alternatively, the inactive stabilized triphosphate is not reactive, and so any compounds that trap the stabilized state of the enzyme-triphosphate complex will become effective allosteric inhibitors for the enzyme. Screening for this activity is easily accomplished by tracking reduction in the rate of background hydrolysis in the wildtype enzyme.

The S376N mutant enzyme provides a sobering note, as it illustrates that even a historically optimized enzyme is still subject to evolutionary change and the emergence of new resistance properties. The good news from this is that this new form of resistance does not come fully-formed. It does not bind any aminoglycosides with high affinity, and its effectiveness toward its native substrates is eliminated. While this enzyme gains activity toward N1-substituted aminoglycosides, its activity toward others is lost, so alternation of antibiotics could help prevent this resistance mutation from gaining hold. While *de novo* forms of resistance in AAC(6')-Ie/APH(2")-Ia are possible, they are also easily countered, at least at present.

6.3 The ongoing race against antimicrobial resistance

Antibiotic resistance remains a pressing public health crisis with dire predictions for the future unless dramatic change occurs (Wellcome Trust and UK Department of Health, 2016). Countering the spread of resistance requires concerted action on many fronts (World Health Organization, 2015). To find new and better solutions to this problem, contributions are needed in many areas including public health, sanitation, development of new antimicrobials, antibiotic adjuvants, and novel therapeutic modalities. Key to many of these strategies is a more thorough understanding of the mechanisms of resistance, and the evolutionary change that occurs to generate resistant bacterial isolates.

Strategies to fight the onward march of antibiotic resistance require knowledge of mechanisms of resistance (Section 1.2.6). An understanding of resistance factors is necessary to develop antibiotics that evade resistance, inhibitors of resistance factors, and to develop novel modalities that might be deployed alongside antibiotics. The AAC(6')-Ie/APH(2")-Ia enzyme is an excellent example of an antibiotic resistance enzyme and insights we learn through the study of this factor extend other antibiotic resistance factors as well.

AAC(6')-Ie/APH(2")-Ia is the first and best studied bifunctional antibiotic resistance factor. The principles that apply for AAC(6')-Ie/APH(2")-Ia may also be extended toward other bifunctional aminoglycoside-modifying enzymes (Zhang et al., 2009). Chapter 2 of this thesis found that the domains have a rigid association which implies an adaptation to a functional bi-domain particle. If this finding holds for other multifunctional or multidomain antibiotic resistance enzymes, we can expect that they also gain function through multimerization, which

can lead to more complex resistance factors that act more efficiently and are more difficult to inhibit.

Binding of aminoglycosides to APH(2")-Ia conserves the shared neamine elements of the aminoglycosides, which continues the pattern of antibiotic resistance enzymes that bind substrates in a way that mimics their natural site of action. Antibiotic resistance by target mimicry allows the resistance factor to converge upon the same elements that are essential to the antibiotic action (Fong and Berghuis, 2002), which drastically reduces our ability to find compounds that bind the resistance enzyme but not the antibiotic target site selectively (Bassenden et al., 2016). Indication that APH(2")-Ia may bind non-substrate compounds also provides an example where resistance by enzymatic action and resistance by simple antibiotic sequestration overlap. While inefficient, resistance by sequestration could be more widespread means of resistance than we recognize in cases such as this one.

A structural switch between activated and inactive forms of the enzyme co-substrate, which is influenced by antibiotic binding, implies that APH(2")-Ia has developed an novel means of regulation from other known aminoglycoside kinases or more broadly eukaryotic protein kinase-like enzymes. This mechanism suggests a means by which the enzyme could reduce its fitness cost, a challenge for all antibiotic resistance factors. As the fitness cost of many antibiotic resistance factors has proven to be less strong than initially expected, closer scrutiny of the structural and biochemical features of these enzymes, transporters, and other proteins may reveal that they may have also developed means of mitigating the fitness burden they impose upon their host.

The emergence of a mutation in AAC(6')-Ie/APH(2")-Ia that confers increased resistance toward semisynthetic compounds indicates that even a well-established factor that acts on environmental antibiotics can adapt to man-made compounds. It appears that this resistance is still inefficient, as it does not tightly bind its new substrates, but nevertheless still escapes the action of antibiotics in cells that carry the resistance factor. Like studies on other factors of antibiotic resistance, it seems that multiple changes are necessary for an enzyme to develop an efficient new function (Toprak et al., 2011), but it remains possible, even in clinical settings, for a resistance factor to make a jump to a new activity. This must give us pause. The selective

environment we impose on a resistance factor can promote the emergence of new resistance variants, even if these variants remain inefficient.

AAC(6')-Ie/APH(2'')-Ia teaches us lessons about antibiotic resistance in an established resistance factor. The competing forces of effective antibiotic detoxification and activity toward a broad and diverse group of compounds shape this resistance factor into a complicated machine that is fine-tuned to its targets.

AAC(6')-Ie/APH(2'')-Ia is one resistance factor among many, and even within this one protein, many evolutionary innovations that combine to make it a formidable resistance machine. There are many other potentially problematic antibiotic resistance factors extant in the antibiotic resistome (Wright, 2007). They can transfer to human pathogens and lead to resistance. In order to anticipate and plan for the emergence of these resistance factors, we must better understand the forces at play, and the innovations they can develop. We must construct the environment in a way that minimizes the selection and spread of new resistance functions. There is no silver bullet for antibiotic resistance. Antibiotic resistance has always existed and will always exist. Mechanisms of resistance are dynamic and sophisticated and simple attempts to select against them are unlikely to work. Resistance innovates in response to our actions. The race against resistance continues.

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