[Frontiers in Bioscience, 4 d63-71. January 1, 1999]

DEVELOPING A SNAPSHOT OF THE ATP BINDING DOMAIN(S) OF AMINOGLYCOSIDE PHOSPHOTRANSFERASES

Michael H. Perlin[1], Scott A. Brown[1], and Jaydev N. Dholakia[2]

[1]Departments of Biology, and [2] Biochemistry, University of Louisville, Louisville, KY 40292 USA

TABLE OF CONTENTS

1. Abstract
2. Introduction- Resistance to aminoglycoside antibiotics
3. Aminoglycoside Phosphotransferase (APH) Enzymes
3.1. The best-characterized APHs
3.1.1. APH(3')-II
3.1.1.1. Site-directed mutagenesis
3.1.1.2. Photoaffinity labelling
3.1.2. APH(3')-III
3.1.2.1. Kinetic studies
3.1.2.2. Crystal structure
3.1.2.3. Protein kinase inhibitors
3.1.2.4. NMR spectroscopy
4. Perspectives
5. Acknowledgments
6. References

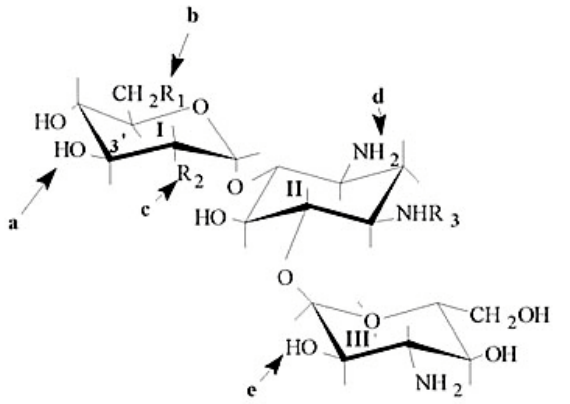
1. ABSTRACT

The aminoglycoside (AG) 3'-phosphotransferases [APH(3')s] are an important class of modifying enzymes which confer high-level resistance to those AGs actively modified by the enzymes. They catalyze the transfer of the terminal phosphate from ATP to the drug, thus preventing the AG's action at the 70S ribosome. These enzymes, which utilize ATP as a co-substrate, appear from amino acid alignments to be part of a much larger superfamily of kinases and ATP-binding proteins. Structure-function analyses have been initiated in our laboratory for APH(3')-II, whose gene was derived from transposon Tn5. Site-directed mutagenesis of the cloned APH(3')-II gene was used to genetically examine the residues in two highly-conserved motifs proposed to participate in ATP binding. Several of these residues, in fact, were shown to affect the enzyme's affinity for ATP. We have also initiated studies using photoaffinity labelling of APH(3')-II with the ATP analogs, 8-azido-ATP and 2-azido-ATP. We have shown that 8-N_3ATP and 2-N_3ATP can be substituted for ATP in the APH(3')-II catalyzed phosphorylation of kanamycin; such findings indicate that the interaction of these photoaffinity analogs of ATP with APH(3')-II is biologically relevant. One of the best-characterized of the APH(3') enzymes is APH(3')-IIIa, the first of the group whose structure has been analyzed by x-ray crystallography. Several studies have demonstrated that this enzyme functions by a Theorell-Chance mechanism. Moreover, the architecture of the enzyme, crystallized in the presence of ADP has revealed residues in the ATP-binding pocket which are likely to play important roles in catalysis. Once the results from biochemical analyses can be correlated with those from mutagenesis studies and x-ray crystallography, a clearer picture of the active site will be provided for an important class of AG-modifying enzymes and phosphotransferases. This picture will also allow a better understanding of these enzymes within the greater context of kinases and nucleotide-binding proteins.

2. INTRODUCTION- RESISTANCE TO AMINOGLYCOSIDE ANTIBIOTICS

The development of multiply-resistant strains of bacteria creates the potential for epidemics of infections which are incurable or which can only be treated by one or a few antibiotics. The aminoglycoside (AG) antibiotics are a class of drug used for the treatment of serious infections caused by gram-negative aerobic bacilli. Historically, they have been seen as a last reserve antibiotic, since infections which are refractive to treatment by other drugs have retained susceptibility to one or more of the AGs. However, the clinical effectiveness of these potent antibiotics has been seriously compromised by the emergence of strains which are resistant to their action. Three mechanisms of bacterial resistance to AGs have been observed (1). The first involves mutations which reduce generalized uptake of AGs into the bacterial cell. The second mechanism involves strains in which the ribosomal target of the AG has been mutationally altered. The mechanism which has the most clinical significance is the third mechanism, the presence of enzymes capable of modifying the antibiotic.

Resistance to antibiotics, while of obvious clinical concern, nevertheless poses interesting fundamental scientific questions for study and, moreover, provides



	R_1	R_2	R_3
Kanamycin A	NH_2	OH	H
Kanamycin B	NH_2	NH_2	H
Kanamycin C	OH	NH_2	H
Amikacin	NH_2	OH	-CO-CH(OH)CH_2NH_2

Figure 1. Structure of the kanamycins. Arrows indicate sites of O-phosphorylation (a), N-acetylation (b,c,d), and O-adenylylation (e).

important tools for molecular genetic and biochemical research. For example, antibiotic resistance genes, such as those conferring resistance to ampicillin, chloramphenicol, or kanamycin, are an invaluable source of dominant selectable markers for genetic engineering and cell transformation strategies. Resistance to aminoglycosides (AGs), e.g., kanamycin, is often conferred by genes encoding AG-modifying enzymes. These enzymes confer high-level resistance through the O-phosphorylation, O-adenylylation, or N-acetylation of specific sites on particular AGs, thereby rendering them inactive (1). In most cases, genes for these enzymes are plasmid-encoded (2) and in some cases, have been located on transposable elements (3, 4). Evidence has been found indicating that both intraspecies and interspecies transference of these genes has occurred (5, 6). Design of semi-synthetic AGs has sought to circumvent bacterial resistance due to modification. For example, the first semi-synthetic AG, amikacin (AK) was derived from kanamycin A (KM) by substitution of an hydroxyaminobutyric acid side-chain at the 1-amino position of the parent compound (1). This substitution was designed to interfere with modification by two enzymes (AAC(6'), a N-acetyltransferase, and APH(3')s, O-phosphoryltransferases) that confer resistance to KM. Nevertheless, bacterial resistance to these new derivatives also emerges, despite the fact that bacteria have not been exposed to the compounds before in nature. We have hypothesized (7) that existing AG-modifying enzymes serve as a reservoir for new AG resistance either via altered expression of an unchanged enzyme or by mutational alteration of the amino acid sequence of the enzyme. A careful analysis of the structure-function relationships for aminoglycoside modifying enzymes should help anticipate changes leading to new AG resistance; such insights may also provide the basis for synthesis of mechanistic inhibitors of these enzymes that could be used in conjunction with AGs for effective therapy.

Until the last few years, there has been a scarcity of structure-function information for any AG-modifying enzyme. The three-dimensional structure of ANT(4')-I (an O-adenylyltransferase) was described (8,9) and, most recently, the crystal structure was provided for one of the phosphotransferases (APH(3')-IIIa; 10). These studies have provided a much-needed resource for future work on these types of enzymes. In both cases, crystal structures have been used to elucidate the ATP-binding sites for the respective enzymes (9; 10). The AG phosphotransferases (APHs) share striking similarities both in selected regions of primary sequence and in structure to the larger superfamily of protein kinases and nucleotide-binding proteins. Given these similarities, this review will examine the available information on regions involved in nucleotide-binding for APH enzymes.

3. AMINOGLYCOSIDE PHOSPHOTRANSFERASE (APH) ENZYMES

There are over twenty distinct APHs (11) which have been reported from antibiotic-resistant bacteria. These enzyme families are identified by their respective sites for modification of the substrate AG molecules. Although APH(3"), APH(6) and APH(4) enzymes have been identified, by far the most common is a class of modifying enzymes which phosphorylates the 3'-hydroxyl on the amino-hexose I of deoxystreptamine AGs (figure 1) (1). This class is the most prevalent of the AG-modifying enzymes and, as such, should be a suitable model for studying evolution of such genes. Within this class of 3'-O-phosphotransferases there are 7 subclasses that can be distinguished from one another by their substrate specificity, although all the subclasses have in common the ability to modify kanamycin (11).

3.1. The best-characterized APHs

3.1.1. APH(3')-II

The AG 3'-phosphotransferase II [APH(3')-II; EC2.7.1.95] is one of the most well-known members of this group; its gene, *aphA-2* , encoded on Tn5, is among the most widely used selectable markers in the transformation of a range of organisms: e.g., mammals and plants, yeast, and *Dictyostelium discoideum*. We have carried out structure-function analysis for APH(3')-II and its gene (12-14). The DNA sequences for the genes of representatives for each subclass of 3'-AG phosphotransferase [APH(3')] are known (11, 15). Comparison of the deduced amino acid sequences revealed extensive homology in the C-terminal quarters of these enzymes. Additional experiments implicated the significance of the carboxy portion of the enzyme for catalysis; fusions of several amino acids to the N-terminus of Tn5-derived APH(3')-II were tolerated, whereas fusions at the carboxy terminus abolished enzyme function (3).

Serving as the focus for our work on APHs, APH(3')-II, was first purified to >95% homogeneity (7). The enzyme was estimated to have a molecular weight of

Figure 2. Alignment of APH(3') protein sequences. Motifs 1, 2, and 3 correspond to amino acid sequences highly-retained in the enzymes. Residues in APH(3')-IIa which have been modified by site-directed mutagenesis or which have been found in naturally-occurring mutants are in bold.

29.8 kD and it has a pH optimum of 7.0 - 7.5. Enzyme activity is inhibited by phosphate buffers and the enzyme's activity against AK is preferentially inhibited in Tris buffers (7). APH(3')-II was most stable if stored at pH 8.0. Siregar *et al.* (16) and Fuchs *et al.* (17) have since developed protocols which afford milligram quantities of homogeneous APH(3') enzymes. Our laboratory developed a more accurate assay for kinetic measurements with AG phosphotransferases. An assay which couples AG phosphorylation to NADH oxidation, using pyruvate kinase and lactate dehydrogenase was adapted for use in a spectrofluorometer as a decline in emission intensity (18). The assay is well-suited to kinetic measurements in the micromolar range. Using this assay, a number of kinetic parameters for the enzyme have been measured (16, 18) and it has been shown to modify neomycin (NM), kanamycin A (KM; K_m = 5.1 micromolar), amikacin (AK; K_m = 760 micromolar) and to have a K_m for ATP of 48 micromolar (12). We have previously found that although APH(3')-II modifies AK, the enzyme alone does not confer bacterial resistance to AK (7). This is primarily due to the poor catalytic efficiency (V_{max}/K_m) of the enzyme for this substrate (7).

Before X-ray crystallographic information about the three-dimensional structure of these enzymes, one could only make intelligent inferences about the functional domains of APH(3') enzymes through comparisons of their amino acid sequences (11, 19), analysis of naturally-occurring mutant enzymes (20, 21), characterization of mutant enzymes produced in the laboratory (12-14, 19), chemical treatment of purified enzyme (22), and inhibitor studies (23).

Our efforts at structure-function analysis for APH(3')-II have focused primarily on its ATP-binding domain(s) (13, 14). Comparison of the C-terminal portion of APH(3') enzymes with other nucleotide-binding enzymes have led to predictions that several highly-conserved regions (Motifs 1-3; 11, 15; See figure 2) might, by analogy, provide the functions of phosphate-binding and phosphoryl transfer. For example, of twelve residues conserved absolutely in Eukaryotic protein kinases (24, 25), ten are also retained in APH(3')-IIIa and seven are conserved throughout the entire APH family. The roles of highly-conserved residues in these regions were investigated by site-directed mutagenesis (13, 14), and their importance was examined in the affinity of APH(3')-II for ATP. We also began preliminary studies to chemically modify the ATP-binding domain with the photoaffinity analogs, 8-azido ATP and 2-azido ATP.

3.1.1.1. Site-directed Mutagenesis

We initially used residue-specific reagents to modify APH(3')-II. The results (22) implicated histidine in the binding of the AG substrate, tyrosine in binding ATP, and aspartic acid and/or glutamic acid residues also appeared to play roles. These results suggested candidates for us to change by site-directed mutagenesis. Residues were examined in the conserved "motifs 1 and 2" in the C-terminal quarter of APH(3') enzymes (13, 14). These residues were proposed to have similar functions to motifs found in protein kinases-i.e., direct involvement in either Mg^{+2}-ATP binding or catalysis as was suggested from affinity labelling and high resolution crystal structures of the enzymes (26, 27).

Other experiments suggested the importance of residues in the C-terminal portion of APH(3')-II that were conserved throughout the APH family. A deletion in the gene which eliminated the last 24 C-terminal amino acids produced a non-functional enzyme (3). Mutant enzymes were also isolated (20, 21) or produced (12-14, 19) so as to replace residues in this region. All strains bearing such mutant enzymes had reduced levels of resistance to KM. However, the effects of each mutation on enzyme activity were not actually determined. We examined several mutations in the evolutionarily-conserved region of APH(3')-II with regard to their effects on enzyme activity, predicted secondary structure, and conferred levels of resistance to AGs (13, 14). Dramatic changes in these characteristics were observed for a large number of the mutations. In particular, two invariant residues, Gly-210 and Arg-211, were predicted to be important due to their location: they reside in the motif of APH(3')-II which corresponds to the P loop or "glycine-rich flexible loop" (G..D.GR.R)(28). This motif is the one element found in all nucleotide binding regions; its apparent function is as a phosphate anchor (27, 28) The end of the loop is a basic amino acid. In the protein kinase family, the residue is most often a lysine, although in cAMP dependent protein kinase (29, 30) the corresponding amino acid is an arginine. In APH(3')-II the analogous glycine-rich region is also terminated by an arginine. Blazquez *et al.* (19) provided further evidence for the importance of this residue since replacement of Arg-211 with glycine greatly reduced conferred resistance to KM. Since the substituted residue (glycine) was uncharged, this may mean that the positively charged group at this end of the loop is critical for APH(3')-II function. In adenylate kinase, substitution of other amino acids for the Lys in the "glycine-loop" markedly reduced catalytic activity (30, 31). This Lys-21 was also suggested to interact directly with the alpha-phosphoryl group of bound ATP (28). The phenotypic consequences of

Table 1. Resistance profiles and enzyme activities for mutant APH(3')-IIs

Strains	MIC (micrograms/ml)									Rel. Enz. Activities
	KM	NM	AK	BT	RB	PM	GM A	TB	G418	
His-188 →Gln	16	16	8	16	16	8	>512	<4	8	0.028
Asp-190→Gly	<4	<4	<4	16	8	8	128	<4	4	0.080
Asp-208→Gly	<4	16	<4	<4	8	<4	128	<4	4	0.012
Asp-216→Gly	32	32	<4	16	8	<4	>512	<4	8	0.056
Asp-227→Gly	128	32	<4	8	16	16	>512	<4	8	0.640
Wildtype	>512	256	8	>500	>500	500	>512	<4	64	1.000
Gly-205→Glu	16	16	<4	16	--	32	--	--	16	0.110
Gly-210→Ala	16	32	<4	16	--	64	--	--	8	0.050
Arg-211→Pro	8	8	<4	8	--	64	--	--	16	0.025

KM, kanamycin; NM, neomycin; AK, amikacin; BT, butirosin; RB, ribostamycin; PM, paromycin; GM A, gentamicin A; TB, tobramycin; G418, geneticin

changing the corresponding amino acid (Arg-211) in APH(3')-II may imply a similar function for this cationic residue. Furthermore, the substitution of Ala for Gly-210 (another conserved member of the loop) greatly reduced both enzyme activity and conferred resistance.

The roles of a number of highly-conserved aspartic acid residues have been examined by mutational analysis. Three of those altered in our studies (Asp-190, Asp-208, and Asp-216) were conserved not only in all APHs, but also in quite a few additional phosphotransferases (25). Of these, Asp-190 and Asp-208, were proposed to bind the phosphate groups of ATP through Mg^{2+} and to participate in the activation of these phosphates (32). It was assumed from kinetic studies in the protein kinase family (33) that at least one of these residues was the phosphate acceptor. However, Asp-184 in cAMP-dependent adenylate kinase was shown to be located very close to the gamma-phosphate of the bound Mg^{2+}-ATP; thus, it was expected to participate in Mg^{2+} chelation from the complex. On the other hand, Ho *et al.* (34) showed that no phospho-enzyme intermediate occurs during catalysis and that, instead, catalysis occurs as a direct in-line transfer. Another conserved residue in this region was examined by Siregar *et al.* (16), who demonstrated that a Glu-182®Asp mutant (21) which conferred lower AG resistance *in vivo* also had an approximately 9-fold increase in the K_m for Mg^{2+}-ATP. And what of the mutations we have introduced into these invariant residues? Each yielded enzymes with greatly reduced enzymatic activity and each conferred severely reduced levels of resistance to various AGs (table 1). In fact, three of these mutant enzymes (Asp-190®Gly, Gly-205®Glu and Gly-210®Ala) had apparently increased K_ms for Mg^{2+}-ATP relative to the wildtype enzyme. In contrast, the change of a non-conserved residue in the same region (Asp-227) did not produce the severe effects of changing other conserved Asp residues. These results support a role for the conserved residues in ATP-binding.

Another residue which is retained in all APHs and phosphotransferases is His-188. It was suggested by Martin *et al.* (15) that this residue is the phosphate acceptor in a phospho-enzyme intermediate. Replacement of His-188 by Gln, in our experiments had a major impact on enzyme activity and bacterial resistance for major substrates and simultaneously increased the K_m for Mg^{2+}-ATP. This strongly suggests the importance of this residue in enzyme function, but does not necessarily support its role in phosphate binding.

To recap our findings so far with mutagenesis of APH(3')-II, we have found that several of the highly conserved residues, particularly those in the glycine-rich P loop, have importance in enzyme function. These may be involved in phosphate transfer or in ATP-binding. However, a detailed picture of the architecture of the ATP binding site requires the input of data from both affinity labelling and x-ray crystallography.

3.1.1.2. Photoaffinity Labelling

One of the approaches used to study the structure-function of an enzyme is to carry out the site-directed mutagenesis experiments as described above to change the amino acid residues conserved within the APH family and to study the resulting effect(s) on enzyme function. Another more direct and definitive approach is to biochemically identify the amino acid residues involved in the substrate binding and perform site-directed mutagenesis of these candidates to conclusively demonstrate their significance. Photoaffinity labelling has proven to be a successful method to determine nucleotide-binding domains of several proteins (35, 36). In order to be certain that the information obtained from these labelling experiments is reliable, several criteria must be met: most importantly, biological relevance. The successful substitution of 2-N_3ATP and 8-N_3ATP for ATP in the enzymatic phosphorylation of AGs by APH(3')-II would demonstrate that the photoaffinity analogs of ATP are interacting with the enzyme at the same biologically relevant site(s). Since the chemical species which cross-link these probes is generated from a relatively small azide group directly attached to the base of the nucleotide, this small addition is not expected to interfere with the interaction normally observed for true substrates in enzyme-catalyzed reactions. (35, 36). Upon exposure to UV light, the azido-analog is converted to the nitrene form, which is reactive and forms a covalent bond with any amino acid residue (i.e., it is chemically non-specific). The advantage of biological specificity and chemical non-

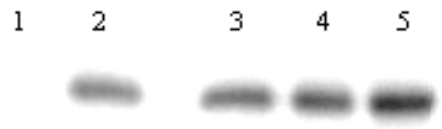


Figure 3. Phosphorimage of APH(3')-II after photolysis with 10 micromolar 8-N_3 gamma ^{32}P ATP. Lane 1, No UV treatment; lane 2, 1 min. UV exposure; lane 3, 2 min. UV exposure; lane 4, 5 min. UV exposure, and lane 5, 10 min UV exposure. Gel electrophoresis was performed on a 12% polyacrylamide gel.

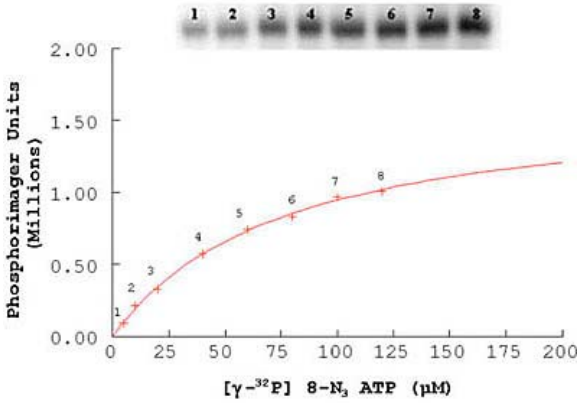


Figure 4. This figure shows the saturation binding curve for [gamma-^{32}P]-8-N_3ATP using a specific activity of 10,000 cpm/pmol. The concentrations are as follows: lane 1, 5 micromolar; lane 2, 10 micromolar; lane 3, 20 micromolar; lane 4, 40 micromolar, lane 5, 60 micromolar; lane 6, 80 micromolar; lane 7, 100 micromolar; and lane 8, 120micromolar. Counts were determined by the ImageQuant software for the phosphorimager and backgrounds were determined in each lane due to different amounts of [gamma^{32}P]-8-N_3ATP used in each sample.

specificity makes azido-nucleotides a potent tool for both identifying the nucleotide binding site(s) and investigating the amino acid sequence of the binding site peptides (35-37).

The photoaffinity reactions with APH(3')-II were carried out in 1.5 ml microcentrifuge tubes. The enzyme is usually purified and stored in buffers containing 2 mM dithiothreitol. However, dithiothreitol very rapidly reduces purine azides (37). Therefore, APH(3')-II preparations purified for photoaffinity labeling experiments were stored in buffers containing 1 mM beta-mercaptoethanol, instead of dithiothreitol, and the enzyme solutions were diluted at least 10-fold in photoaffinity reactions. A typical reaction mixture contained in 20 microliters: 30 mM PIPES, pH 7.0, 10 mM Mg-acetate, 25 mM KCl, 1.5 micrograms APH(3')-II, 10 micromolar [gamma-^{32}P]2-N_3ATP (5,000 cpm/pmol) and other nucleotides to test the specificity of the reaction. After 5 min incubation at 0° C, the tubes were irradiated at 0° C with a UV lamp at a wavelength of 254 nm (150 microwatts/cm^2) at a distance of 1 cm from the samples for different time intervals.

Preliminary results showed the optimum time of photolysis (UV exposure) was 2 min. There was no significant difference between 2 and 5 min UV exposure times, however, longer exposure to photolysis of 10 min showed an increase in the crosslinking, presumably due to non-specific binding (See figure 3) (counts were determined using Molecular Dynamics ImageQuant). Since the nitrene form of azido probes, generated upon UV exposure, is of very short half-life and the photolysis reaction is usually completed within 2 min of UV exposure, 2 min was chosen as the photolysis incubation time for additional experiments. The results in figure 3 also indicate that the binding of the photolabel is completely dependent on UV light. Inclusion of unlabeled ATP, but not GTP or CTP, in the reaction mixture, competed for photolabelling by 2-N_3ATP and 8-N_3ATP, demonstrating the chemical specificity of the UV crosslinking.

Photolabelling with either 2-N_3ATP and 8-N_3ATP was saturable, as shown in figure 4 and figure 5, again suggesting that the crosslinking of the azido analogs was specific. The apparent K_D values estimated from the saturation kinetics (35 micromolar for 2-N_3ATP and 69 micromolar for 8-N_3ATP) were similar to the reported K_m of ATP (48 micromolar) for APH(3')-II-catalyzed phosphorylation of kanamycin (7, 18).

The results in figure 6 show the utilization of [gamma-^{32}P]ATP analogs for the phosphorylation of KM by APH(3')-II. Each reaction was carried out in a reaction mixture of 20 microliters containing: 100 mM PIPES, pH 7.0, 7.5 mM Mg-acetate, 1.5 micrograms APH(3')-II, 0.2 mM KM, and a designated amount of ATP or ATP analog (100 cpm/pmol) as indicated. After incubation at 25° C for 5 min, a 10 microliters aliquot was spotted onto phosphocellulose filter paper that was washed with 0.85% phosphoric acid followed by dH_2O. The phosphorylation of KM was determined by estimating the radioactivity retained on the filter. These results of the photoaffinity labelling experiments demonstrate that the crosslinking of either 2- or 8-N_3ATP is both specific and biologically relevant and provides evidence that further experiments using these analogs should lead to the successful elucidation of the residues in the ATP binding domain of APH(3')-II.

Site-directed mutagenesis affords an important follow-up for photoaffinity labelling studies. Photoaffinity labelling of APH(3')-II may identify residues in the region of the active site but not in it. Further use of site-directed mutagenesis will allow us to ask questions concerning the role of specific individual amino acids. Both approaches are necessary together to identify the true active site, since mutagenesis of a particular amino acid could change APH(3')-II activity without the residue itself being in the active site, i.e., by changing the tertiary structure of APH(3')-II.

3.1.2. APH(3')-III

APH(3')-IIIa, carried by opportunistic enterococci and staphylococci, is perhaps the best studied of the AG-modifying enzymes. Elegant studies from the

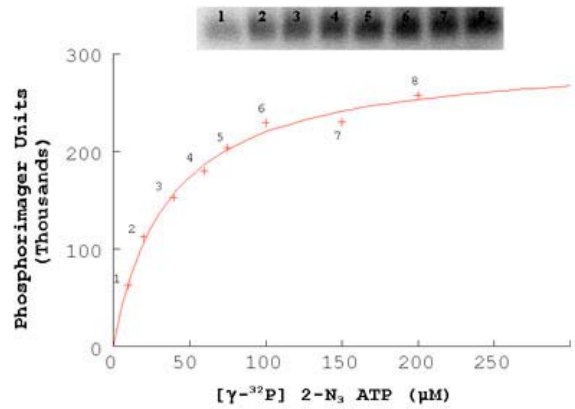


Figure 5. This figure shows the saturation binding curve for [gamma-^{32}P]-2-N_3ATP using a specific activity of 10,000 cpm/pmol. The concentrations are as follows: lane 1, 10 micromolar; lane 2, 20 micromolar; lane 3, 40 micromolar; lane 4, 60 micromolar, lane 5, 75 micromolar; lane 6, 100 micromolar; lane 7, 150 micromolar; and lane 8, 200micromolar. Counts were determined by the ImageQuant software for the phosphorimager and backgrounds were determined in each lane due to different amounts of [gamma-^{32}P]-2-N_3ATP used in each sample.

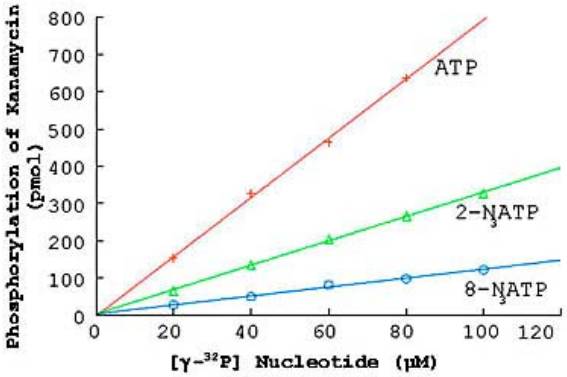


Figure 6. Biological relevance of azido analogs. APH(3')-II activity was estimated by quantitating the phosphorylation of KM as described previously (7) with either ATP, 2-N_3ATP or 8-N_3ATP as indicated.

labs of G. McKay and G. Wright have provided the bulk of information regarding this enzyme's kinetic properties, enzymatic mechanism, and three-dimensional structure (10, 38-42). The 264 amino acid enzyme exists as a monomer or as a covalent dimer through linked disulfide bridges between Cys-19 and Cys-156 in a head-to-tail arrangement. Although there is currently no evidence for such dimer formation in the other APH(3')s described, and while none share Cys residues in exactly the same locations at those involved in disulfide formation in APH(3')-III, several contain Cys residues nearby that could participate in such interactions (APH(3')-II: Cys31, Cys 131 (uniformly conserved); APH(3')-I: Cys10, Cys137, Cys139 (uniformly conserved); APH(3')-VII: Cys25, Cys32, Cys119 (uniformly conserved), Cys 165). Apparently, although the active sites of the respective molecules in the APH(3')-IIIa dimer face each other, they act independently and there is neither cooperation nor antagonism in the dimer state (10). Siregar *et al.* (16) examined the roles of the 5 Cys residues in APH(3')-II by S-cyanylation of >93% of the free Cys thiols. Under saturating conditions there was approximately 50% loss of activity comparing the unmodified to the cyanylated enzyme. Thus, the Cys residues apparently did not play a critical role in APH(3')-II phosphotransferase activity.

3.1.2.1. Kinetic studies

The kinetic mechanism of APH(3')-IIIa function was examined (McKay and Wright 1995, 1996). Positional isotope-exchange results were consistent with a direct phosphoryl transfer to the AG substrate and not with a phosphoenzyme intermediate. This was an important finding, since it had been proposed (15) that the invariant His-188 could serve as the acceptor for the phosphate**.** This residue is part of Motif I, which contains the conserved HGDXXXN that is similar to a domain found in Eukaryotic protein kinases. Further studies (using product and analog inhibitors, as well as alternative substrates) demonstrated that the reaction catalyzed by APH(3')-IIIa occurs by sequential binding first, of ATP, next of the AG and its subsequent phosphorylation, then release of the modified AG, and dissociation of ADP, which is the rate-limiting step. Moreover, the enzymatic rates in the presence of various viscous agents showed that ATP binding and release of ADP were controlled by diffusion and that the rate-limiting step for the reaction of APH(3')-IIIa was exclusively the release of ADP. Further, since the enzyme exhibited small solvent and thio effects, this demonstrated that nucleophilic attack on the ATP and deprotonation of the hydroxyl were not the major factors controlling the overall steady-state reaction rate. Together, the results of these experiments support the Theorell-Chance enzymatic mechanism. In such a mechanism, the k_{cat} reflects only the first-order rate-dissociation of ATP from the Enzyme-ADP complex.

3.1.2.2. Crystal structure

The first (and, to date, only) three-dimensional structure for an APH(3') enzyme was provided recently by Hon *et al.* (10). The APH(3')-IIIa crystallized in the presence of ADP was shown to exist as two lobes: an N-terminal lobe, consisting of 94 amino acids and equivalent to a similar lobe found in Eukaryotic Ser/Thr protein kinases; and a C-terminal lobe of 157 residues. A deep cleft was found to hold these two lobes together. The cleft is composed of twelve amino acids which form a short beta-strand and alpha-helix and are apparently the ATP (or at least, ADP) binding site (10) Comparison of this proposed structure with that for Eukaryotic protein kinases showed that a loop found in such enzymes between beta- strands 1 and 2 of the N-terminal lobe was absent in APH(3')-IIIa at that location. Hon *et al.* (10) suggest that a one-residue insertion causes a different conformation there for APH(3')-IIIa relative to that for Eukaryotic protein kinases. However, the motif normally found in this lobe, a "glycine-rich loop", G-X-G-X-X-G, as mentioned above, does appear in Motif 2 of APH(3')-IIIa and the rest of the APH(3') enzymes. In fact, Asp-208, one of the residues in

this motif whose role was examined by site-directed mutagenesis of APH(3')-IIa (see above), was seen to form interactions with both ADP and two Mg^{2+} ions in APH(3')-IIIa (10).

The crystal structure of APH(3')-IIIa confirmed several predictions made from other studies. Site-directed mutagenesis of APH(3')-IIa (13) showed that where Asp-190 was replaced with Gly the respective enzyme had reduced affinity for Mg^{2+}-ATP. The replacement of Asp-190 with Ala in APH(3')-IIIa caused a 650-fold decrease in k_{cat} for the mutant enzyme (10). Further evidence for the importance of Asp-190 (or at least the domain in which it resides) comes from analysis of the bifunctional acetyltransferase-phosphotransferase AAC(6')-APH(2''). Although the overall primary amino acid similarity of this enzyme compared to the other APHs is low, the C-terminal portion containing the APH(2'') function does share similarities with the other APHs. In particular, it contains the sequence H^{372}NDFSCN, similar to the H^{188}GDLGDSN, which contains Asp-190. This residue corresponds to Asp-166 in c-AMP dependent adenylate kinase, a residue which serves as the catalytic residue for deprotonation of the AG hydroxyl to allow efficient attack on ATP. The crystal structure of APH(3')-IIIa shows this amino acid to be positioned for interaction with the incoming hydroxyl and thus, as expected from mutagenesis studies, its role is critical for catalysis: either by providing the proper orientation for the hydroxyl or by increasing the electrophilicity of the gamma-phosphate of ATP through direct or indirect hydrogen bonding (10).

Based on the results of inactivation of APH(3')-IIIa by an ATP analog, McKay *et al.* (23) predicted that both Lys-33 and Lys-44 would reside in the ATP-binding region. However, site-directed mutagenesis of Lys-33 did not support its importance in ATP binding. This is not so surprising, considering that this residue is only found in APH(3')-IIIa and APH(3')-VIIa. On the other hand, Lys-44 is found in all APH(3')s; substitution of Ala for this residue in APH(3')-IIIa affected the steady-state kinetics for the enzyme, but only the K_m for ATP was significantly increased. The crystal structure for the enzyme shows that although Lys-33 is in the predicted ATP-binding pocket and is above the adenoside, it is located too far away for direct interactions with the nucleotide. In contrast, Lys-44 is found to be in beta-strand 3, above the bound ADP, with the expectation of direct interaction with the alpha and beta (and, by inference, gamma) phosphates.

3.1.2.3. Protein Kinase Inhibitors

Since APHs and Eukaryotic protein kinases share many structural similarities, Daigle et al. (43) examined the effects of several inhibitors of the latter group on the activity of two APH enzymes, APH(3')-IIIa and the bifunctional AAC(6')-APH(2). The isoquinolinesulfonamide group of inhibitors were found to be especially effective as inhibitors of the APH enzymes. They displayed competitive inhibition, with the best compounds yielding inhibition constants in the <100 micromolar range with respect to ATP; some of the compounds (e.g., N-(2-aminoethyl)-5-isoquinolinesulfonamide) were also non-competitive inhibitors of KM. Although the effects of these inhibitors were only observed in *in vitro* enzymatic studies (i.e., they had no effect on reducing resistance of bacterial strains in grown in culture), the authors suggested that the results supported the efficacy of designing and employing similar inhibitors as part of a strategy to combat resistant strains of bacteria.

3.1.2.4. NMR Spectroscopy

Transferred nuclear Overhauser effect spectroscopy (an NMR method) has recently been used to detect intra- and inter-ring nuclear Overhauser effect measurements for two AG substrates in their respective ternary complexes with ATP and APH(3')-IIIa (44). These studies suggest a better picture of the electrostatic and three-dimensional interactions of AG substrates bound to an APH enzyme and ATP, and, along with the previous studies and crystal structure, provide the basis for the design of mechanistic inhibitors of such enzymes.

4. PERSPECTIVES

APH(3')s are an important class of AG-modifying enzymes which share many features with both protein kinases and nucleotide-binding proteins. A variety of methodologies have been employed to study the structure-function relationships for these proteins, although three-dimensional structure data has only quite recently been available for one of the APH(3')s. Independently, these methods have provided insights into selected aspects of the structure and function for enzymes in the APH(3') family. Together, they allow correlation of inferences made from each method alone. In collaboration with Clyde Smith of Massey University, we are trying to determine the crystal structure of APH(3')-IIa, as well as those of the mutants we have produced with altered K_ms for ATP. Recently we have made a change in APH(3')-IIa of Lys-50®Arg of APH(3¢)-IIa that resulted in decreased MICs for several aminoglycoside antibiotics. The characterization of this mutant for its affinity for ATP and in photoaffinity labelling experiments as described in this article should provide the functional significance of this lysine and its relationship with other protein kinases. As more details of such structure-function relationships become available, we will better understand how the APHs fit into the overall paradigm of kinases and nucleotide-binding proteins. More importantly, these studies and others will help us to appreciate in their own right, an important class of enzymes which will continue to participate in the evolution of bacterial resistance to AG antibiotics.

5. ACKNOWLEDGMENTS

The authors would like to express their gratitude to Boyd Haley and Ashok Chavan for their technical expertise and advice for photoaffinity labeling of APH(3')-II. This work was supported, in part, by an IRIG-URG grant to M.H.P. from the Office of the Vice President for Research at the University of Louisville.

6. REFERENCES

1. Foster, T. J.: Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiological Reviews* 47, 361-409 (1983)
2. Cox, D. A., K. Richardson, and B. C. Ross: The Aminoglycoside In: Topics in Antibiotic Chemistry Eds: Sommers P.G., Horwood E., Sussex, England, V.I.:2-90 (1977)

3. Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller: Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene* 19,327-336 (1982)

4. Oka, A., H. Sugisaki, and M. Takanami: Nucleotide sequence of the kanamycin resistance transposon Tn903. *J. Mol. Biol.* 147,217-226 (1981)

5. Trieu-Cuot, P. and P. Courvalin: Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3'5'-aminoglycoside phosphotransferase type III. *Gene* 23,331-341 (1983)

6. Trieu-Cuot, P., G. Gerband, T. Lambert, and P. Courvalin: *In vivo* transfer of genetic information between gram-positive and gram-negative bacteria. *EMBO J.* 4,3583-3587 (1985)

7. Perlin, M. H. and S. A. Lerner: High-level resistance to amikacin in *Escherichia coli* due to phosphorylation and reduced uptake of the drug. *Antimicrob. Ag. Chemother* 29,216-224 (1986)

8. Sakon, J., H.H. Liao, A.M. Kanikula, M.M. Benning, I. Rayment, and H.M. Holden: Molecular structure of kanamycin nucleotidyltransferase determined to 3.0 D resolution. *Biochemistry* 32,11977-11984 (1993)

9. Pedersen, L.C., M.M. Benning and H.M. Holden: Structural investigation of the antibiotic and ATP-binding sites in kanamycin nucleotidyltransferase. *Biochemistry* 34,13305-13311 (1995)

10. Hon, W.C., G.A. McKay, P.R. Thompson, R.M. Sweet, D.S.C. Yang, G.D. Wright, and A.M. Berghuis: Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. *Cell* 89,887-895 (1997)

11. Shaw, K.J., P.N. Rather, R.S. Hare and G.H. Miller: Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* 57,138-163 (1993)

12. Kocabiyik, S. and M. H. Perlin: Altered substrate specificity by substitutions at Tyr218 in bacterial aminoglycoside 3'-phosphotransferase II [APH(3')-II]. *FEMS Microbiol. Lett.* 93,199-202 (1992)

13. Kocabiyik, S. and M. H. Perlin: Site-specific mutations of conserved c-terminal residues in aminoglycoside 3'-phosphotransferase II: phenotypic and structural analysis of mutant enzymes. *Biochem. Biophys. Res. Commun*. 3,925-931 (1992)

14. Kocabiyik, S. and M. H. Perlin: Amino acid substitutions within the analogous nucleotide binding loop (P-loop) of aminoglycoside 3'-phosphotransferase II: functional and structural implications of the mutations. *Int. J. Biochem.* 26,61-66 (1993)

15. Martin, P., E. Jullien, and P. Courvalin: Nucleotide sequence of *Acinetobacter baumannii aphA-6* gene: evolutionary and functional implications of sequence homologies with nucleotide-binding proteins, kinases and other aminoglycoside-modifying enzymes. *Mol. Microbiol.* 2,615-625 (1988)

16. Siregar, J.J., S.A. Lerner and S. Mobashery: Purification and characterization of aminoglycoside 3'-phosphotransferase type IIa and kinetic comparison with a new mutant enzyme. *Antimicrob. Ag. Chemother.* 38,641-647 (1994)

17. Fuchs, R.L., R.A. Heeren, M.E. Gustafson, G.J. Rogan, D. E. Bartnicki, R. M. Leimgruber, R.F. Finn, A. Hershman, and S. A. Berberich: Purification and characterization of microbially expressed neomycin phosphotransferase II (NPTII) protein and its equivalence to the plant expressed protein. *Biotechnology* 11,1537-1547 (1993)

18. Perlin, M. H., S. McCarty, and J. P. Greer: Coupled spectrofluorometric assay for aminoglycoside phosphotransferases. *Anal. Biochem*. 171,145-149 (1988)

19. Blazquez, J., J. Davies, and F. Moreno: Mutations in the *aphA-2* gene of transposon Tn5 mapping within the regions highly conserved in aminoglycoside phosphotransferases strongly reduce aminoglycoside resistance. *Mol. Microbiol*. 5,1511-1518 (1991)

20. Lee, K.Y., J. D. Hopkins, and M. Syvanen: Evolved neomycin phosphotransferase from an isolate of *Klebsiella pneumoniae*. *Mol. Microbiol*. 5,2039-2046 (1991)

21. Yenofsky, R. L., M. Fine, and J. W. Pellow: A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selective pressure. *Proc. Nat. Acad. Sci. USA* 87,3435-3439 (1990)

22. Perlin, M. H: Analyzing the molecular components of aminoglycoside resistance. *SAAS Bull. Biochem. and Biotech.* (1989)

23. McKay, G.A., R.A. Robinson, W.S. Lane and G.D. Wright: Active-site labelling of an aminoglycoside antibiotic phosphotransferase (APH(3')- IIIa). *Biochemistry* 33,14115-14120 (1994)

24. Hanks, S.K. and T. Hunter: Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic)

domain structure and classification. *FASEB J.* 9,576-596 (1995)

25. Hanks, S.K., A.M. Quinn and T. Hunter: The protein kinase family conserved features and deduced phylogeny of the catalytic domains. *Science* 241, 42-52 (1995)

26. Bramson, H. N., E. T. Kaiser, and A. S. Mildvan: *CRC Crit. Rev. Biochem*. 15,93 (1984)

27. Knighton, D. R., J. Zheng, L. F. Ten Eyck, V. A. Ashford, N. H. Xuong, S. S. Taylor, and J. M. Sowadski: Crystal structure of the catalytic subunit of cyclic AMP-dependent protein kinase. *Science* 253,407-413 (1991)

28. Fry, D. C., S. A. Kuby, and A. S. Mildvan: ATP-binding site of adenylate kinase, mechanistic implications of it s homology with ras-encoded p21, F1-ATPase, and other nucleotide-binding proteins. *Proc. Nat. Acad. Sci. USA* 83,907-911 (1986)

29. Saraste, M., P. R. Sibbald, and A. Wittinghofer: The P-loop of Protein Kinases. *TIBS* 15,430-434 (1990)

30. Reinstein, J., I. Schlichting, and A. Wittinghofer: Structurally and catalytically important residues in the phosphate binding loop of adenylate kinase of E*scherichia coli*. *Biochem* 29,7451-7459 (1990)

31. Sigal, I. S., T. B. Gibbs, J. S. D'Alonzo, G. L. Temeles, B. S. Wolanski, S. H. Socler and E. M. Solnick: Identification of effector residues and a neutralizing epitope of Ha-ras-encoded p21. *Proc. Nat. Acad. Sci. USA* 83,952-956 (1986)

32. Brenner, S: Phosphotransferase sequence homology. *Nature* 329,21 (1987)

33. Yoon, M. Y. and P. F. Cook: Chemical mechanism of the cyclic 3'5' AMP-dependent protein kinase from pH studies. *Biochem*. 26,4118-4122 (1987)

34. Ho, M.F., H. N. Bramson, D. E., Hansen, J. R. Knowles, and E. T. Kaiser: Stereochemical course of the phospho group transfer catalyzed by cAMP-dependent protein kinase. *J. Am. Chem. Soc.* 110,2680-2686 (1988)

35. Haley, B.E.: Nucleotide photoaffinity labeling of protein kinase subunits. *Methods Enzymol.* 200, 477-487 (1991)

36. Dholakia J.N., Francis, B.R., Haley, B.E. and Wahba, A.J.: Photoaffinity labeling of the rabbit reticulocyte guanine nucleotide exchange factor and eukaryotic initiation factor 2 with 8-azidopurine nucleotides. *J. Biol. Chem.* 264, 20638-20642 (1989)

37. Haley, B.E.: Development and utilization of 8-azidopurine nucleotide photoaffinity probes. *Fed. Proc.* 42, 2831-2836 (1983)

38. McKay, G.A., and G.D. Wright: Kinetic mechanism of aminoglycoside phosphotransferase type IIIa: evidence for a Theorell-Chance mechanism. *J. Biol. Chem.* 270,24686-24692 (1995)

39. McKay, G.A., and G.D. Wright: Catalytic mechanism of enterococcal kanamycin kinase (APH(3')- IIIa): viscosity, thio, and solvent isotope effects support a Theorell-Chance mechanism. *Biochemistry* 35,8680-8685 (1996)

40. McKay, G.A., P.R. Thompson and G.D. Wright: Broad spectrum aminoglycoside phosphotransferase type III from Enterococcus: overexpression, purification, and substrate specificity. *Biochemistry* 33,6936-6944 (1994)

41. Thompson, P.R., D.W. Hughes, and G.D. Wright: Mechanism of aminoglycoside 3'-phosphotransferase type IIIa: His188 is not a phosphate-accepting residue. *Chem. Biol*. 3,747-755 (1996)

42. Thompson, P.R., D.W. Hughes, and G.D. Wright: Regiospecificity of aminoglycoside phosphotransferase from Enterococci and Staphylococci (APH(3')-IIIa). *Biochemistry* 35,8686-8695 (1996)

43. Daigle, D.M., G.A. McKay, and G.D. Wright: Inhibition of aminoglycoside antibiotic resistance enzymes by protein kinase inhibitors. *J. Biol. Chem*. 272,24755-24758 (1997)

44. Cox, J.R. and E.H. Serpersu: Biologically important conformations of aminoglycoside antibiotics bound to an aminoglycoside 3'-phosphotransferase as determined by transferred nuclear Overhauser effect spectroscopy. *Biochemistry* 36,2353-2359 (1997)

Keywords: Drug Resistance; Gram-Negative Aerobic Bacilli; Aminoglycoside Phosphotransferases; Site-Directed Mutagenesis; Photoaffinity Labelling

Send correspondence to: Dr Michael H. Perlin, Department of Biology, University of Louisville, Louisville, KY 40292 USA, Tel: 502-852-5939, Fax:502-852-0725, E-mail: mhperl01@athena.louisville.edu

Received 3/3/98 Accepted 7/14/98