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Photoaffinity labeling of Aminoglycoside Phosphotransferase 3'-II with azido ATP confirms the involvement of conserved lysine in ATP binding

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Abstract

Of the mechanisms bacteria use to evade the effects of aminoglycoside (AG) antibiotics, enzymatic modification has the most clinical relevance due to the promiscuous nature of the DNA encoding the genes for these enzymes. One such enzyme, aminoglycoside 3'-phosphotransferase IIa (APH(3')-IIa), is used as a model for understanding this modification at a molecular level along with anticipating the evolution of AG resistance. To study the structure-function relationships of this enzyme, we previously determined the crystal structure of APH(3')-IIa and modeled its ATP binding site. We identified a lysine that appeared to be involved in the binding of ATP and generated a conserved lysine to arginine mutant to better assess this residue's functional contributions. We determined the binding kinetics for ATP to this mutant and employed active-site labeling using an azido ATP analog to confirm the importance of this conserved lysine. Competition experiments with tryptophan during active-site labeling revealed that a conserved tryptophan residue in the amino-terminus of the enzyme may be involved directly or by association with ATP binding. Together, these data give us a clearer picture of how ATP associates with APH(3')-II and could aid in the development of small chemical compounds which specifically inhibit its activity.

Keywords: aminoglycoside phosphotransferase; azido-ATP analogues; photoaffinity labeling; kanamycin; ATP binding; structure-function analyses.

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Since the discovery of the first aminoglycoside (AG) antibiotic over 50 years ago, AGs have played an important role in the clinical treatment of serious infections caused by aerobic gram-negative bacilli. The clinical effectiveness of these potent antibiotics, however, has been seriously compromised by the emergence of strains that are resistant to their action. Of the mechanisms bacteria use to evade the effects of aminoglycoside (AG) antibiotics, enzymatic modification has the most clinical relevance due to the promiscuous nature of the DNA encoding the genes for these 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 (Beck et al. 1982). In most cases, genes for these enzymes are plasmid-encoded but they have also been located on transposable elements (Beck et al. 1982; Cox et al. 2000; Oka et al. 1981). In either case, it is not difficult to transfer this genetic material from one bacterium to another. Evidence has been found indicating that both intraspecies and interspecies transference of these genes has occurred (Salvucci et al. 1992; Salvucci et al. 1994; Trieu-Cuot and Courvalin 1983; Trieu-Cuot et al. 1985).

APH(3′)-IIa is a member of a class of enzymes which phosphorylates the 3'-hydroxyl on the amino-hexose I of deoxystreptamine AGs (Foster 1983). This enzyme class is the most prevalent of the AG-modifying enzymes and, as such, should be a suitable model for studying evolution of genes responsible for conferring resistance to AGs. Within this class of 3′-AG phosphotransferases (APH(3')s) there are 8 subclasses that can be distinguished from one another by their substrate specificity, although all the subclasses have in common the ability to modify kanamycin (KM) (Martin et al. 1988). The DNA sequences for the genes of representatives for each subclass of APH(3') are known (Shaw et al. 1993). Comparison of the deduced amino acid sequences has revealed extensive homology in the C-terminal quarters of these enzymes which is responsible for catalysis. To aid in the understanding of the enzymatic activity, previous studies altered APH(3')-IIa by fusing several amino acids to the N-terminus or the C-terminus. Fusions at the N-terminus were tolerated, but C-terminus fusions abolished enzyme function (Beck et al. 1982).

Our earlier work with APH(3')-IIa used sequence homology and site-directed mutagenesis in conserved domains to discern

residues involved in the ATP-binding site (Kocaciyik and Perlin 1992; Kocabiyik and Perlin 1994; Perlin et al.1999). Following this work, we were then able to generate a crystal for APH(3′)-IIa in the presence of Kanamycin (KM), but not ATP (Nurizzo et al. 2003). Since the crystal lacked ATP, we compared it with a close family member, APH(3')-IIIa, as well as other kinases, to help identify any residues that we had not focused on in previous studies that may be important in ATP binding or phosphate transfer (Hon et al. 1997). The comparison of the amino acid sequences of APH(3')-IIa and APH(3')-IIIa, displays a 31.5 % identity and a 43.5% similarity. Based on the crystal comparisons, we hypothesized the involvement of $lysine_{50}$ of APH(3')-IIa for ATP utilization and will be the focus of this paper.

Photochemical labeling with the azido nucleotide analogs has been successfully used to characterize the nucleotide-binding domain(s) of several proteins (Salvucci et al. 1992; Salvucci et al. 1994; Dholakia et al. 1989; Mann et al.1999; Olcott et al. 1994; Pfister et al. 1984). Since the chemical species that cross-link these probes is generated from a relatively small azide group directly attached to the base of the nucleotide, there is less of a chance to interfere with the interaction normally observed for true substrates in enzyme-catalyzed reactions (Haley 1991). Upon exposure to UV light, the azido-analog is converted to a nitrene form, which is reactive and forms, with any nearby amino acid residue, a covalent linkage (i.e., it is chemically non-specific). The advantage of biological specificity and chemical non-specificity makes azido-nucleotides a potent tool for identifying and characterizing the nucleotide binding domain(s).

We report that the $2N_3ATP$ analog binds to APH(3')-IIa with similar efficiency as ATP and can be substituted for ATP to phosphorylate AG antibiotics, indicating that the interaction of 2-azido ATP is catalytically relevant. We also use this analog to confirm the vital role of $lysine_{50}$ in the binding of ATP for the phosphorylation of aminoglycoside antibiotics.

Materials and Methods

sAPH(3')-IIa^{wt} and sAPH(3')-IIa^{K50R}

sAPH(3')-IIa^{wt} and sAPH(3')-IIa^{K50R} were engineered to have the StrepTag-II peptide on the N-terminus of each protein and were purified as previously described by a one-step purification method using Strep-Tactin® Sepharose® column (Sigma-Genosys, The Woodlands, TX) (Nurizzo et al. 2003). Briefly, both wild type and mutant (K50→R) APH(3′)-IIa genes were cloned into the pASK-IBA7 vector for expression and purification in *E. coli* strain BL21(DE)pLysS. Purified untagged APH(3')-IIa was a kind gift from R.L Fuchs (Monsanto, St. Louis, MO).

Phosphocellulose Paper Binding (PPB) Assay

The transfer of [γ-^{32}P] of ATP to the aminoglycoside KM-A (Sigma Chemical Co.) was quantitated as previously described (Martin et al. 1988; Nurizzo et al. 2003). A typical reaction mixture contained in 45 µl, 30 mM PIPES (pH 7.0), 10 mM magnesium acetate, 25 mM potassium acetate, 2.5 mM dithiothreitol, 40 nM APH(3')-IIa and [γ-^{32}P]ATP (2000 cpm/pmol) as indicated. Reactions were incubated at 25º C for 30 seconds to 2 minutes. Under these conditions, the reaction was linear for at least the first 2 minutes, and less than 5% of the ATP or ATP analog was used in each time point. A 10 µl aliquot was removed at different time intervals as indicated and spotted on phosphocellulose paper. The paper squares were then twice washed with 0.85% phosphoric acid for 10 min followed by one wash in distilled water for 10 min. The samples were dried and the radioactivity measured in a Packard Scintillation Counter.

Photoaffinity labeling with 2-azido ATP analog

Photoaffinity labeling was performed on ice in a 0.7 ml microcentrifuge tube in a 20 µl reaction mixture containing 30 mM PIPES (pH 7.0), 10 mM magnesium acetate, 25 mM potassium acetate, [γ-^{32}P]$2N_3ATP$, and 1 µg of sAPH(3')-II^{wt} or sAPH(3')-II^{K50R} as indicated. The reaction mixture was incubated at 25º C for 1 min and then transferred to ice for the crosslinking using a 254-nm hand-held ultraviolet lamp (150 µW/cm^2) held 1 cm above the sample for 1 min unless stipulated otherwise. The reactions were terminated by the addition of SDS (0.1%) and ditheothreitol (2.5 mM), boiled for 1 min and electrophoresed in a 12.5% SDS-polyacrylamide gel, followed by phosphorimaging (Storm Phosphorimager, Molecular Dynamics) to determine the relative ^{32}P incorporation.

Enzyme digestion

To identify the ATP-binding site, peptide mapping of photoaffinity labeled APH(3')-IIa was performed employing endoproteinase Lys-C (Roche Lab). Proteolysis was carried out for 24 hr at 37º C with 1:1 molar ratio of endoproteinase Lys-C and APH(3')-IIa in reactions containing 1 M urea (pH 8.5). The reactions were terminated by adding an equal volume of SDS-gel buffer and the peptides were fractionated by tris-tricine polyacrylamide gel electrophoresis as previously described (Schagger et al. 1987).

We used 16.5% total (T) acrylamide and 6% crosslinker (C) in a 13 cm separating gel, a 2.5 cm "spacer gel" with 10% T and 3% C and a 1 cm stacking gel of 4% T and 3% C. The molecular weight standards (2.5 kDa to 16.9 kDa) used for this gel were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ).

Results

Kinetic Data

To achieve rapid and efficient recovery of photoaffinity labeled APH(3')-IIa, we used an engineered protein that contains the Strep-Tag® II affinity tag on the N-terminus [sAPH(3')-II]. The addition of this 15 amino acid tag does not affect the function of the enzyme (Nurizzo et al. 2003).

The K_m values for ATP have been previously reported and included in this report for comparison (Nurizzo et al. 2003). K_ms for ATP and $2\text{-}N_3ATP$ were calculated using excess KM-A (100 µM) as the AG substrate. highlights the difference between the mutant and the wild type enzymes in their affinity for ATP, with an eight fold increase in Km for the sAPH(3')-II^{K50R} mutant. There was very little difference, however, in the turnover rate (k_{cat}) between the two enzymes: k_{cat} for wild type APH(3')-IIa being 35.31±0.79 min^{-1} and that for the mutant K50R being 42.15±1.56 min^{-1}.

$2\text{-}N_3ATP$ was also used in kinetic analysis with the two enzymes with slightly different results. To conserve a limited supply of the $2\text{-}N_3ATP$, only three concentrations were used, but each point was determined in duplicate. Therefore, only apparent K_ms were determined for $2\text{-}N_3ATP$. The graphical representation of these data is shown in Figure 1. Wild type APH(3')-II showed an increase in the apparent K_m value as compared to ATP, however, there was almost a twofold decrease in apparent K_ms for K50R

Table 1: Kinetic constants for sAPH(3')-II^{wt} and sAPH(3')-II^{K50R}

	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}min^{-1}$)
sAPH(3')-II^{wt}				
	ATP*	23.29 ± 2.97	35.31 ± 0.79	1.52 X 10^6
	2-N_3ATP	157.2 ± 18.31	20.03 ± 1.21	1.28 X 10^5
sAPH(3')-II^{K50R}				
	ATP*	195.4 ± 21.49	42.15 ± 1.56	2.16 X 10^5
	2-N_3ATP	118.3 ± 21.61	3.87 ± 0.44	3.27 X 10^4

k_{cat} is a measure of the turnover rate of the enzyme, and k_{cat}/K_m is a measure of the catalytic efficiency. * - These data were previously reported (Nurizzo, et al. 2003).

mutant. This slight increase in affinity for the ATP analog in the mutant enzyme did not translate into a higher k_{cat}. The turnover capacity, shown in Table 1 and graphically in Figure 1B, for sAPH(3')-II^{K50R} enzyme using 2-N_3ATP and KM-A as substrates was only 3.87 ± 1.44 min^{-1}. This represents an approximately eleven fold reduction in the catalytic activity and suggests that the combination of the azido group in the ATP analog combined with the substitution of arginine at position 50 in APH(3')-IIa almost completely destroys the function of the enzyme towards this substrate.

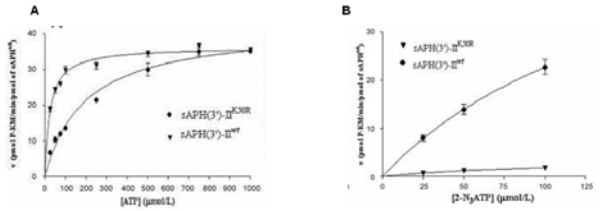

Figure 1. Representative Michaelis-Menten plots for sAPH(3')-II^{wt} and sAPH(3')-II^{K50R}. Reaction conditions were as described in the text with an excess of KM-A (100 μM) and either ATP (A) or 2-N_3ATP (B) as substrates. The lines drawn in all graphs were generated from fitting the data to the Michaelis-Menten equation.

Azido Labeling

The above kinetic experiments show that 2-N_3ATP can be substituted for ATP in the enzymatic reaction catalyzed by the wild type APH(3')-IIa. Preliminary experiments with APH(3')-IIa demonstrated that [γ-^{32}P]2-N_3ATP photoinserts in a UV dependent and saturating fashion and this crosslinking decreases in the presence of increasing concentrations of ATP. Figure 2 shows that the maximal photolabeling of APH(3')-IIa with [γ-^{32}P]2-N_3ATP is achieved in one minute UV exposure.

It is important to know if irradiating the probe under our experimental conditions can produce any long-lived chemically reactive intermediates that could lead to non-specific labeling of APH(3')-IIa (Salvucci et al. 1994; Perlin et al. 1999;Nurizzo et al. 2003). In order to check this, a reaction was set up as described above, but without the addition of the enzyme. The reaction mixture was exposed to UV and then the enzyme was added. As seen in lane 3 in Figure 3 no reactive chemical intermediates were produced that would react with the enzyme non-specifically in the second stage when reactions were pre-irradiated for 1 min. Moreover, when such reactions were re-irradiated (Figure 3, lane 4), only a small amount of probe crosslinked to the enzyme after the second exposure, suggesting that the majority of the azido groups were converted to a short-lived nitrene during the first exposure to UV. In an attempt to increase the photolabeling efficiency (which was about 10%), we included the second substrate, KM-A, and kept the reaction on ice for one minute. However, as seen in Figure 3, lane 5, the addition of KM-A did not increase the photolabeling of APH(3')-IIa but the enzyme phosphorylated KM-A. Thus, even on ice the 2-N_3ATP analog could be used as a substrate by APH(3')-IIa, confirming its binding in the active site of the enzyme.

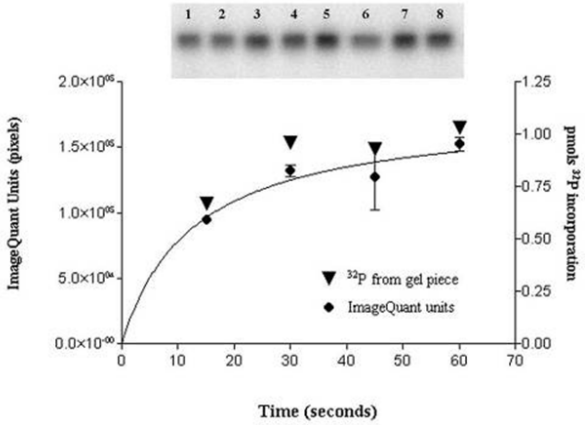

Figure 2. Time-dependent photoaffinity labeling of APH(3')-IIa with 2-N_3ATP. The insert is a phosphorimage of 1 μg (32.5 pmols) of sAPH(3')-II^{wt} after photolysis with 25 μM [γ-^{32}P]2-N_3ATP (775 CPMs/pmol) for 15 sec (lanes 1 and 2), 30 sec (lanes 3 and 4), 45 sec (lanes 5 and 6), and 1 min (lanes 7 and 8)UV exposure. Saturation kinetics were determined from the ImageQuant units using the Michaelis-Menten equation.

As we previously observed, the crosslinking of 2-N_3ATP on APH(3')-IIa exhibits saturation at increasing analog concentrations (Perlin et al. 1999). This further suggests that photolabeling is occurring at a specific site rather than randomly labeling the enzyme. A dose dependent photoaffinity labeling of sAPH(3')-II^{wt} and sAPH(3')-II^{K50R} were carried out using 2-N_3ATP (Figure 4). Non-linear least squares analysis was used to estimate V_{max} and apparent K_D values. The apparent K_D values were 77.4 μM and 105.8 μM for sAPH(3')-II^{wt} and sAPH(3')-II^{K50R}, respectively. The V_{max} values, however, differed by 3.7 fold with values of 3.87 pmols/min for sAPH(3')-II^{wt} versus 1.06 pmols/min for competitor; lane 3, 250 μM tryptophan; lane 4, 500 μM tryptophan; lane 5, 1 mM ATP; lane 6, 2 mM ATP; lane 7, 1 mM ADP; and lane 8, 2 mM ADP.

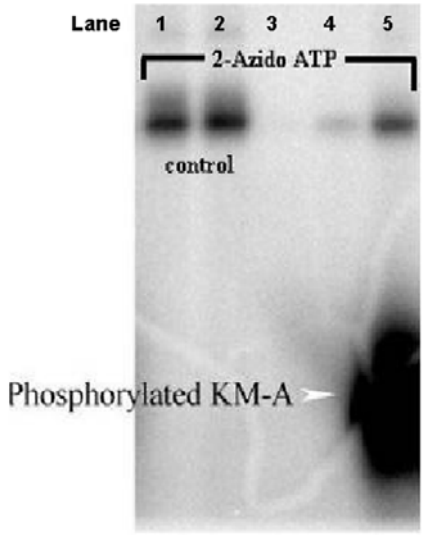

Figure 3. Specificity of photolabeling of APH(3')-IIa. 1 μg (32.5 pmols) of sAPH(3')-IIa^{wt} was incubated in each reaction for 1 minUV exposure. Lanes 1 and 2 were the control reactions containing APH(3')-IIa and 25 μM [γ-^{32}P]2-N_3ATP. In lane 3, the reaction mixture was exposed to UV light prior to the addition of the sAPH(3')-II^{wt}. Lane 4 contains the UV-exposed reaction mixture as in lane 3 followed by the addition of sAPH(3')-IIa^{wt} and another 1 min UV exposure in second stage. Lane 5 contained the same reaction mixture as in lane 1 with the addition of 10 μM KM-A.

sAPH(3')-II^{K50R}. This can also be seen in Figure 5 in a side by side comparison of a photolabeling of the sAPH(3')-II^{wt}, sAPH(3')-II^{K50R} and a third mutant, sAPH(3')-II^{E111I}, which was

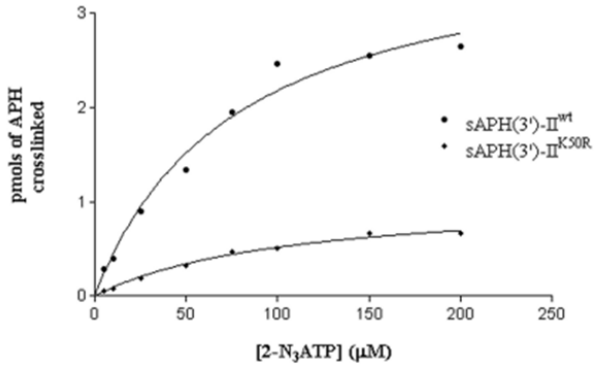

Figure 4: Saturation analyses using 2-N_3ATP with sAPH(3')-IIa^{wt} and sAPH(3')-IIa^{K50R}. The enzymes were incubated with increasing concentrations of 2-N_3ATP (5 μM, 10 μM, 25 μM, 50 μM, 75 μM, 100 μM, 150 μM and 200 μM), exposed to UV light for 1 min and analyzed by SDS-polyacrylamide gel electrophoresis. The amount of radioactivity was determined by liquid scintillation counting of the gel pieces containing APH(3')-II^{a}. Non-linear least squares analysis was used to determine the predicted lines.

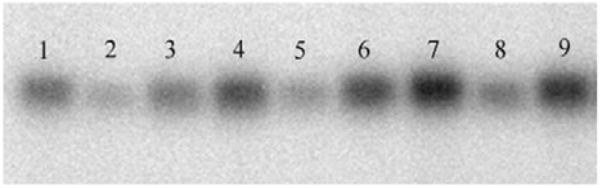

Figure 5. Photoaffinity labeling of 2-N_3ATP to crosslink sAPH(3')-IIa^{wt}, sAPH(3')-IIa^{K50R} and mutant sAPH(3')-II^{E111I}, whose mutation was not in the proposed ATP binding domain. Lanes 1-3 contained 25 μM; Lanes 4-6 contained 50 μM; and Lanes 7-9 contained 100 μM 2-N_3ATP. The enzyme in each reaction were 1 μg sAPH(3')-IIa^{wt} (Lanes 1, 4, and 7); or sAPH(3')-II^{K50R} (Lanes 2, 5, 8); or sAPH(3')-II^{E111I} (Lanes 3, 6 and 9).

used as a control. Based on the crystal structure, the E111I substitution is not located near the proposed ATP binding domain, and it appears that it binds 2-N_3ATP with similar affinity as observed for sAPH(3')-II^{wt}.

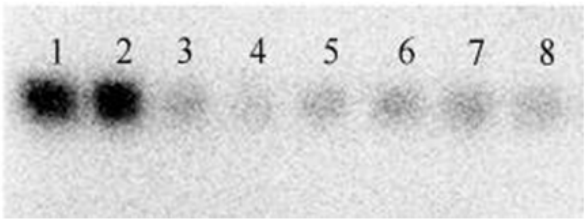

Figure 6. Effect of ATP, ADP and Trp on photoaffinity labeling of APH(3')-IIa. Each reaction contained 25 μM 2-N_3ATP. The crosslinking was performed at 25º C with 1 min UV exposure. Lanes 1 and 2, no competitor; lane 3, 250 μM tryptophan; lane 4, 500 μM tryptophan; lane 5, 1 mM ATP; lane 6, 2 mM ATP; lane 7, 1 mM ADP; and lane 8, 2 mM ADP.

The specificity of photoinsertion of 2-N_3ATP was further characterized by studying the effects of unlabeled ATP or ADP in the photolabeling reaction. If the analog is binding to the biological active site, addition of ATP or ADP will compete for the binding and decrease the photoaffinity labeling. As seen in figure 6, both ATP and ADP significantly reduced crosslinking of 2-N_3ATP. Based on a previous observation by van de Loo and Salvucci (1996) where inclusion of tryptophan in the reaction abolished crosslinking by an azido ATP analog and our identification of a conserved tryptophan in the crystal structure of APH(3')-IIa near the proposed ATP binding domain, we also decided to examine competition by tryptophan for photoinsertion of 2-N_3ATP (Perlin et al. 1999; van de Loo et al. 1996). As seen in Figure 6, the inclusion of tryptophan in the reaction also abolished crosslinking of 2-N_3ATP to APH(3')-IIa, and at concentrations less than that of ATP or ADP.

Endoproteinase Lys-C digestion

In order to determine the residue(s) that has been modified by the [γ-^{32}P]2-N_3ATP, proteolytic digestion was used to isolate the radiolabeled peptide of the enzyme. For complete protein digestion, it was necessary to use a 1:1 molar ratio of Lys-C protease to APH(3')-IIa and its variants. Only then was a single labeled peptide observed (Figure 7). It is clear that the crosslinking of APH(3')-IIa was specific for one labeled peptide of the predicted size that contains Lys_{50} as determined by proteotic map of APH(3')-IIa with Lys-C protease. It was not

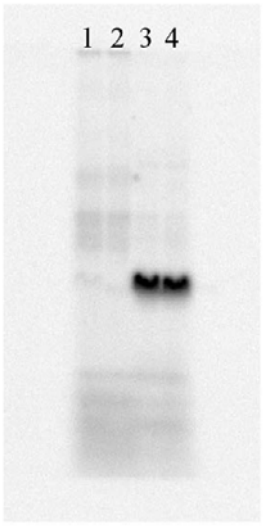

Figure 7. Proteolytic analysis of photolabeled APH(3')-IIa and sAPH(3')-IIa^{K50R}. Photoaffinity labeling was performed as described in the text, using 50 μM 2-N_3ATP. After photolabeling, the reactions were adjusted to 1 M urea and pH 8.5. 1.0 μg of endoproteinase Lys-C was added and the mixture incubated at 37° C for 24 hours. The lanes above contain duplicate reactions: 1 and 2, 1 μg sAPH(3')-II^{K50R}; and 3 and 4, 1 μg APH(3')-IIa.

surprising that a similar peptide was not observed for sAPH(3')-II^{K50R} since the ability of the mutant to utilize 2-N_3ATP in the enzymatic reaction was extremely low.

Discussion

The APH family of enzymes uses ATP to phosphorylate and inactivate aminoglycoside antibiotics such as neomycin and kanamycin. These enzymes are thus responsible for bacterial antibiotic resistance. A detailed understanding of both ATP and antibiotic binding to these enzymes is essential to design more effective enzyme inhibitors or broad-spectrum antibiotics. As part of a program to define the molecular basis for aminoglycoside recognition and inactivation by such enzymes, we have previously determined the crystal structure of APH(3')-IIa in complex with kanamycin (Nurizzo et al. 2003). We also modeled an ATP binding site of APH(3')-IIa based on the comparison of its crystal structure with those of ATP bound APH(3')-III and cAMP-dependent protein kinase, that proposed a critical role of a conserved Lys_{50} in the ATP binding to APH(3')-IIa. In this report, we directly examined this hypothesis and futher characterized the ATP binding domain of APH(3')-IIa. A conservative $K_{50}R$ mutation, within the ATP binding site of APH(3')-IIa proposed to assist in alignment of the phosphates, produced a greater than 8-fold increase in the K_m of ATP, but this did not change the k_{cat}, or the turnover rate. This mutation was modeled after a similar change made by Hon et al. (1997) in the conserved Lys at position 44 in APH(3')-IIIa. While the Lys44 mutant showed approximately a three times greater effect on the K_m for ATP, the change introduced was to a non-conservative Ala. The mutation introduced in the current study was more conservative making it less likely that the results seen may be due to improper folding of the enzyme. Hon et al. also did not see a difference in k_{cat}, a result that was similar to our own (Hon et al. 1997).

Since the azido analog used for affinity labeling can also be used as substrate, steady-state kinetics were carried out with it as well. From the results, it is clear that 2-N_3ATP which specifically photoinserts in the ATP binding domain is also a substrate for sAPH(3')-II^{wt}. The sharp decrease in enzymatic activity of sAPH(3')-II^{K50R} suggests that the problem seen with its utilization of ATP may be compounded by the addition of the azido group on the purine ring. In addition to its poor utilization of 2-N_3ATP as a substrate in the enzymatic reaction, the mutant APH could not be labeled as efficiently as wildtype. Initial experiments suggested only a 3- to 4-fold difference in crosslinking efficiencies when comparing the mutant to wild type enzymes. However, upon protease digestion of the mutant, there were no specific bands modified, suggesting that the photoaffinity labeling of the mutant protein observed was due to non-specific interactions. This also supports a change in the binding properties of nucleotides with sAPH(3')-II^{K50R} as seen with the increase in K_m for ATP.

Competition experiments suggested that conserved tryptophan residue(s) may also influence the binding of 2-N_3ATP, and ATP to sAPH(3')-II^{wt}. Van de Loo and Salvucci also observed such a competition by tryptophan in their azido labeling experiments of tobacco Rubisco activase (van de Loo et al. 1996). They attributed the decrease in crosslinking to a Trp residue on the protein interacting with free Trp in solution through a base stacking phenomenon (Nurizzo et al. 2003; van de Loo et al. 1996). This implicated an invariant tryptophan present in the family of activases that was directly involved in the activation of the enzyme. There is also an invariant tryptophan found in the N-terminus of the APH(3') family of enzymes, Trp 69, and additionally a relatively conserved Trp at position 91 (numbering according to APH(3')-IIa). These two putative sites may be further explored through site-directed mutagenesis to generate second site suppressors specific for this group of antibiotic modifying enzymes.

Conclusions

With the data compiled regarding the specific crosslinking of 2-N_3ATP to sAPH(3')-II^{wt}, it appears that lysine at position 50 plays a critical role in binding of ATP. This residue does not appear to affect the ability of the enzyme to catalyze the reaction, however, because when ATP concentration is in excess, the K50R enzyme's catalytic turnover is the same as wildtype. In the absence of a crystal structure which includes ATP in the active site of APH(3')-IIa, our result strongly establishes the role for K_{50} in the binding of ATP.

The protein biochemistry of the aminoglycoside 3'-phosphotransferases continues to advance. As the exact mechanism for AG modification becomes unraveled, so too can the development of powerful inhibitors which, when used in conjunction with these drugs, allow for the continued use of these life saving compounds. In addition to inhibitors, synthetic AGs could also be designed from molecular modeling that would optimize their bactericidal effects while minimizing their ability to be modified enzymatically. Regardless of the method, circumventing the resistance to these drugs continues to be the ultimate goal of scientists and the medical profession. By characterizing the structure-function relationships of this very important class of enzymes, we can lend insight into the evolution of resistance to AG antibiotics and secure their effective use for decades to come.

Acknowledgements

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Contributions

Dr. S. Brown was responsible for conducting experiments, analyzing data, and writing the manuscript.

Dr. J. Dholakia provided insights into the photoaffinity labeling experiments and participated in the preparation of the manuscript.

Dr. M. Perlin provided commentary and analysis of the molecular aspects of the data, including StrepTagII purification of the proteins and significance of the findings with regards to aminoglycoside susceptibility. He also participated in the preparation of the manuscript and had the overall editing responsibilities.

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