Structure and Function of APH(4)-Ia, a Hygromycin B Resistance Enzyme*§•

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The aminoglycoside phosphotransferase (APH) APH(4)-Ia is one of two enzymes responsible for bacterial resistance to the atypical aminoglycoside antibiotic hygromycin B (hygB). The crystal structure of APH(4)-Ia enzyme was solved in complex with hygB at 1.95 Å resolution. The APH(4)-Ia structure adapts a general two-lobe architecture shared by other APH enzymes and eukaryotic kinases, with the active site located at the interdomain cavity. The enzyme forms an extended hydrogen bond network with hygB primarily through polar and acidic side chain groups. Individual alanine substitutions of seven residues involved in hygB binding did not have significant effect on APH(4)-Ia enzymatic activity, indicating that the binding affinity is spread across a distributed network. hygB appeared as the only substrate recognized by APH(4)-Ia among the panel of 14 aminoglycoside compounds. Analysis of the active site architecture and the interaction with the hygB molecule demonstrated several unique features supporting such restricted substrate specificity. Primarily the APH(4)-Ia substrate-binding site contains a cluster of hydrophobic residues that provides a complementary surface to the twisted structure of the substrate. Similar to APH(2) enzymes, the APH(4)-Ia is able to utilize either ATP or GTP for phosphoryl transfer. The defined structural features of APH(4)-Ia interactions with hygB and the promiscuity in regard to ATP or GTP binding could be exploited for the design of novel aminoglycoside antibiotics or inhibitors of this enzyme.

With rising incidences of multi-drug resistant bacteria and a decline in the discovery of new antibiotics, attention is returning to “old” antibiotics that in the past were not selected for clinical development by the pharmaceutical industry. These compounds represent chemical matter that may provide essential scaffolds that could be altered with modern medicinal chemical approaches or may be suitable to be reintroduced into the clinic in this current era of pressing need. For example, the lipopeptide antibiotic daptomycin (Cubicin®) was discovered in the 1980s by Eli Lilly but was not brought to the clinic until 2003 because of the efforts by Cubist Pharmaceuticals, which resulted in Food and Drug Administration approval of this older drug (1).

Currently anologs of the known aminoglycoside antibiotics such as gentamicin and kanamycin are not seen as favorable drug candidates by the pharmaceutical industry. Discovered in the 1950s, these compounds were acclaimed for their broad spectrum and potent bactericidal activity. However, they lost favor as front line drugs as a result of toxicity and poor oral availability and as newer agents with improved pharmacology entered the market. Furthermore, the emergence of resistance provided an additional challenge to the clinical utility of these drugs. Resistance to these antibiotics is most frequently conferred by aminoglycoside-modifying enzymes encoded by transferable gene cassettes. Nevertheless, in this era of acute antibiotic need, the aminoglycoside compounds, particularly the ones that were clinically “underused” in the past, are re-emerging as promising drug leads. This in turn necessitates the detailed analysis of mechanisms of resistance to aminoglycosides as an essential part of developing antimicrobial therapies involving this class of antibiotics.

Aminoglycoside-modifying enzymes act through three major chemical mechanisms: acetyltransfer, nucleotidyltransfer, and phosphoryltransfer. Each of these classes of enzymes is highly diverse, with numerous representatives featuring different substrate profiles and regiochemical selectivities resulting in a plethora of enzymes with varied and distinct features. The ∼30 enzymes within the aminoglycoside phosphotransferase class of enzymes typically show less than 35% pairwise identities (2).

Hygromycin B (hygB),4 isolated from Streptomyces hygroscopicus (3) represents a promising underused aminoglycoside antibiotic. It has atypical chemical features in comparison with more common representatives of this antibiotic class. A key structural feature of these common aminoglycosides is the 2-deoxystreptamine core, which is substituted by two

4 The abbreviations used are: APH, aminoglycoside phosphotransferase; hygB, hygromycin B; AMPPPN, adenosine 5’-(β,γ-imino)triphosphate; AMPPCP, adenosine 5’-(β,γ-methylene)triphosphate; NPL, nucleotide positioning loop.

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amino sugar rings at the 4 and 6 positions (as in gentamicin or tobramycin), or the 4 and 5 positions (as in neomycin or butirosin). hygB is unique in that it is comprised of an N-methyl 2-deoxystreptamine ring (hyosamine), linked through the C5-OH to a talose sugar, which in turn forms an orthoester with the unusual amino acid destomatic acid (Fig. 1), resulting in a distinctive fused ring structure. This unique structure translates into a novel mode of action. Where antibiotics like gentamicin bind to the 30 S ribosomal subunit, resulting in mistranslation of mRNA, hygB binding to this subunit primarily blocks mRNA and tRNA translocation (4, 5). Furthermore, hygB has been shown to also impact translocation in eukaryotic ribosomes (6), increasing the spectrum of this antibiotic to eukaryotic pathogens and resulting in its application in agricultural settings as an anthelmintic agent in poultry and swine.

Like all antibiotics, resistance to hygB has emerged. Plasmid-encoded hygB resistance in Escherichia coli was reported some 30 years following its introduction (7, 8). This resistance was linked to the aminoglycoside phosphotransferase-(4)-Ia (APH(4)-la) enzyme, which catalyzes the ATP-dependent phosphorylation of the hydroxyl moiety on C4 of the hyosamine ring (Ring I) of hygB (Fig. 1) (8). This mechanism differs from the self-protection one described in the hygB-producing Streptomyces hygroscopicus, which involves phosphorylation at the 7&superscript;′-OH of the desatic acid ring (Ring IV) (9, 10). The enzyme responsible for this activity, APH(7&superscript;′)-la, shares only 37% amino acid identity with APH(4)-la. This implies that APH(4)-la may have evolved distinctly from self-resistance in the producing organism.

The three-dimensional structures of a number of APHs have been reported (11–15). These structures have demonstrated that despite their sequence diversity, APH enzymes share a common fold that is similar to that of eukaryotic protein kinases. This common fold features a bilobed structure comprised of a primarily β-sheet-containing N-terminal domain and a mixed α+β C-terminal domain, with the two domains connected by a linker region. Relative to eukaryotic protein kinases, APH enzymes contain an insertion in the C-terminal domain, which plays an important role in aminoglycoside binding (12). Despite structural information about a number of APH enzymes and advances in understanding the mechanism of phosphorylation reaction (16), the basis of different substrate specificity toward diverse aminoglycosides between the representatives of APH enzymes remains poorly understood. Low sequence similarity between APH enzymes precludes effective modeling of uncharacterized APH family members and necessitates further structural studies of diverse members of this family.

Given the importance of hygB as a potential scaffold for new drug discovery, we embarked on structural characterization of the APH(4)-Ia-hygB binary complex and investigated the enzymatic properties of this enzyme. The three-dimensional structure shows that although the APH(4)-Ia enzyme adopts the general features of other APH enzymes, such as the overall eukaryotic protein kinase-like fold and the location of the hygB ligand-binding site, it reveals unique structural features that explain its selectivity for this atypical aminoglycoside.

**EXPERIMENTAL PROCEDURES**

*Expression and Purification of APH(4)-Ia—The aph(4)-Ia gene (NCBI accession number V01499) from the retroviral protein expression vector pQCCXI (Clontech) was used as the template for preparation of an overexpressing construct. An internal NdeI restriction endonuclease site was removed by creating a silent mutation at the site using the Stratagene QuikChange strategy (oligonucleotide primers are shown in supplemental Table S1). The gene was then amplified with oligonucleotide primers that introduced flanking 5’ NdeI and 3’ HindIII sites that were used to clone the gene into vector pET28a to create the expression plasmid pET-APH(4)-Ia.*

APH(4)-Ia was expressed in E. coli BL21(DE3) (Novagen) and grown overnight in the presence of 50 μg/ml kanamycin A in Luria Bertani (LB) broth. A 10-ml overnight culture was used to inoculate 1 liter of fresh LB broth with antibiotic selection and was grown to OD<sub>600</sub> ~0.6 at 37 °C. It was then induced for 16 h at 16 °C with 1 μM isopropyl-β-thiogalactoside. The cells were harvested by centrifugation at 6000 × g for 10 min. The cell pellet was then resuspended in 15 ml of 50 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pancreatic bovine DNase. The resuspended pellet was passed through a T-S series cell disrupter (Constant Systems Inc.) twice at 35,000 p.s.i. to create a lysate. The insoluble and soluble fractions were separated by centrifugation at 48,000 × g for 30 min. The supernatant was then applied to a 1-ml nickel-nitrilotriacetic acid column (Qiagen) equilibrated in 10 column volumes of 50 mM HEPES, pH 7.5, 300 mM NaCl, 10 mM imidazole (Buffer A). A stepwise gradient over five column volumes of each 10, 25, 50, and 100% Buffer B (50 mM HEPES, pH 7.5, 300 mM NaCl, 250 mM imidazole) was used to elute the enzyme. The APH(4)-Ia-containing fractions were identified by SDS-polyacrylamide gel electrophoresis and were subsequently pooled and dialyzed against 50 mM HEPES, pH 7.5, overnight at 4 °C with stirring. The protein was then quantified using the Bradford protein assay and was either concentrated or diluted to 0.5 mg/ml and stored in 15% glycerol at −20 °C.

For crystallographic purposes, the aph(4)-Ia gene was subcloned from pET-APH(4)-Ia into the vector pI5TV-LIC, which codes for a N-terminal His<sub>6</sub> tag, a TEV protease cleavage site, and an ampicillin resistance gene and allows ligase-independent cloning. The expression vector was utilized for expression of selenomethionine-substituted protein using standard M9 high yield growth procedure according to the manufacturer’s instructions (Shanghai Medicilon; catalog number MD045004-50), with E. coli BL21(DE3) codon plus cells.

The cells were lysed by sonication in 0.3 M NaCl, 50 mM HEPES, pH 7.5, 5 mM imidazole, 5% glycerol, 0.5 mM tris(2-carboxyethyl)phosphine, and 0.25 mM phenylmethanesulfonyl fluoride. Protein was bound to nickel-nitrilotriacetic acid resin at 4 °C for 2 h, washed with lysis buffer with 30 mM imidazole, and then eluted with lysis buffer with 250 mM imida-
ole. Eluted protein was supplemented with 1 mM EDTA. The His$_6$ tag was removed by cleavage with TEV protease overnight at 4 °C in dialysis with the buffer 0.3 M NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, and 0.5 mM tris[2-carboxyethyl]phosphine, followed by binding to nickel-nitrilotriacetic acid resin and capture of flow-through. Protein was concentrated to at least 10 mg/ml. Protein was flash frozen in liquid nitrogen and stored at −70 °C.

Crystallography, Data Collection, and Structure Determination—APH(4)-Ia crystals were grown at 22 °C using the hanging drop method, by mixing 1 μl of protein at 6.5 mg/ml with 1 μl of reservoir solution containing 0.2 M diammonium hydrogen citrate, 16% polyethylene glycol 3350, and 2 mM hygB. The crystals were cryo-protected with reservoir solution supplemented with 20% paratone prior to flash freezing. Diffraction data at 100 K at the wavelength corresponding to the anomalous scattering peak wavelength of selenomethionine (0.97915 Å) were collected at Beamline 19-BM at the Structural Biology Center, Advanced Photon Source, Argonne National Laboratory. Diffraction data were reduced with HKL2000 (17).

The structure was solved by single-wavelength anomalous dispersion using the AutoSol module of Phenix (18). Seven of the eight selenomethionine sites in the asymmetric unit were found as judged by occupancy values greater than 0.50. An initial model of the protein was built using Phenix AutoBuild, followed by rounds of manual model building and refinement with Coot (19) and Phenix.refine. Final rounds of refinement were completed with BUSTER (20) with TLS parameterization (TLS groups were residues 8–99 and 100–301). The final atomic model includes residues 8–301 of APH(4)-Ia, all 25 atoms of hygB, 27 ammonium ions, one chloride ion, and seven polyethylene glycol molecules (with varying numbers of atoms per polyethylene glycol molecule visible in the electron density). Procheck (21) was utilized for the Ramachandran analysis. An iterative composite omit map for verification of position of hygB was generated with Phenix.autobuild with default parameters.

Structure similarity searches of the Protein Data Bank were performed using the Dali server (22). Structure superpositions were performed with the SSM algorithm in Coot or also using the LIGPLOT program (23).

Electrostatic potential surfaces were calculated using the APBS PyMOL plugin (24). Red is negative, white is neutral, blue is positively charged, and surfaces were contoured between −20 and +20 $k_B T/e$, where $k_B$ is the Boltzmann constant, $T$ is temperature, and $e$ is the electronic charge. Protein structure images were produced with PyMOL (Delano Scientific).

Protein Data Bank Coordinates—The structure of APH(4)-Ia has been submitted to the Protein Data Bank with the accession number 3OVC.

Site-directed Mutagenesis of APH(4)-Ia—Site-directed mutagenesis was carried out using the Stratagene QuickChange strategy in the pET-APH(4)-Ia plasmid. The sequences of the oligonucleotide primers can be found in supplemental Table S1. DNA sequencing using primers for the T7 promoter and terminator were used to verify the success of the mutants. All of the mutants were purified as described previously above.

Steady State Kinetic Analysis of APH(4)-Ia—The phosphorylation of antibiotics was monitored by coupling the release of ADP or GDP following antibiotic modification with pyruvate kinase/lactate dehydrogenase (25). The oxidation of NADH (ε = 6220 M$^{-1}$ cm$^{-1}$) was monitored at 340 nm using a SpectraMax plate reader in a 96-well format. A typical reaction contained 195 μl of reaction buffer (50 mM HEPES, pH 7.5, 40 mM KCl, 10 mM MgCl$_2$, 0.3 mM NADH, 3.5 mM phosphoenolpyruvate, 0.00125 units pyruvate kinase/lactate dehydrogenase, and −0.1 μM APH(4)-Ia). A 5-μl solution of the appropriate antibiotic was added to the reaction and allowed to incubate for 5 min at 25 °C. The reaction was initiated with a 50-μl solution of nucleotide and monitored for 5 min at 340 nm. The initial rates ($v$) were determined by utilizing the linear portion of the progress curve and analyzed by nonlinear least squares fitting to Equation 1.

$$v = \frac{V_{\text{max}} S}{(K_m + S)} \quad (\text{Eq. 1})$$

Structure Determination of Phosphohygromycin B—Large-scale preparation of phosphohygromycin B was carried out enzymatically using a 50-ml reaction comprised of 50 mM HEPES, pH 7.5, 40 mM KCl, 10 mM MgCl$_2$, 3 mM hygB, 5.4 mM ATP, and 2.1 μM APH(4)-Ia. The reaction was monitored using thin layer chromatography with a mobile phase comprised of 5:2 MeOH:NH$_4$OH and visualized with ninhydrin. The reaction mixture was passed through a 0.2-μm Millipore filter and a 10-kDa Millipore centrifugal filter to remove precipitated and soluble enzyme, respectively. The filtrate was then lyophilized overnight and resuspended in 4 ml of H$_2$O and applied to a 48-ml AG50W × 8 200–400 mesh column (78 × 28 mm) that had been charged with 1% NH$_4$OH. The product was recovered with a wash of 0.1% NH$_4$OH and lyophilized.

RESULTS AND DISCUSSION

Substrate and Regiospecificities of APH(4)-Ia—Previous enzyme-based assays indicated that APH(4)-Ia is highly specific for hygromycin B and close analogs (8). Using purified recombinant APH(4)-Ia, we screened a diverse panel of aminoglycoside antibiotics as potential substrates including the 4,6-disubstituted 2-deoxystreptamine-based aminoglycosides (amikacin, kanamycin A, gentamicin C complex, and tobramycin), the 4,5-disubstituted 2-deoxystreptamine-based aminoglycosides (butirosin, neomycin B, paromomycin, ribostamycin, and lividomycin) and the atypical aminoglycosides (neamine, apramycin, streptomycin, spectinomycin, and hygromycin B). Only hygB was susceptible to phosphorylation by APH(4)-Ia (see Table 2). Such narrow substrate specificity is unusual for APHs, which often have very broad aminoglycoside substrate profiles (26–30). In contrast, APH(4)-Ia demonstrated the ability to utilize both ATP and GTP as a phosphate donor with ~5-fold preference toward ATP based upon the $k_{cat}/K_m$ values of 5.42 × 10$^5$ and 1.04 × 10$^5$, respectively. Similar nucleotide promiscuity has been previously
observed for the APH(2') class of enzymes (31, 32). In eukaryotic protein kinases, the capacity to utilize GTP is rare, and this difference could be exploited in the development of specific inhibitors.

High resolution mass spectrometry of phosphorylated hygB gave a mass of 607.1990 consistent with monophosphorylation of hygB. Regiospecificity of phosphoryl transfer was confirmed to be at position 4 by multidimensional NMR as predicted (Fig. 1) (8).

Overall Structure of APH(4)-Ia—APH(4)-Ia shares relatively low sequence similarity with previously characterized APH enzymes (maximum of 16% identity across the full-length enzymes) (11–15). To gain further understanding of the unique substrate selectivity of this enzyme, the structure of APH(4)-Ia was solved by the single-wavelength anomalous dispersion method using selenomethionine-derivatized APH(4)-Ia protein co-crystallized with hygB. The final model contained one polypeptide chain in the asymmetric unit, with residues 8–301 resolved in the electron density, along with all atoms of the antibiotic (structure statistics are summarized in Table 1).

The analysis of the APH(4)-Ia structure demonstrated that like previously characterized APH enzymes, APH(4)-Ia adopts a general eukaryotic protein kinase fold. Thus to simplify comparative structural analysis, we labeled the secondary structure elements of the APH(4)-Ia structure according to those in the original APH structure, APH(3')-IIIa (12). The N-terminal domain (residues 7–92) and the C-terminal domain (residues 99 and 301) are connected by a short loop (residues 93–98) also known as a hinge (Fig. 2A). The C-terminal domain can be further divided into two subdomains, each comprised of two noncontiguous sequences. The core subdomain spans residues 99–143 and 190–258. The second subdomain is exclusively α-helical and is made up of residues 144–189 and 259–301.

Pairwise superposition of APH(4)-Ia with the full-length structures of APH(2')-IIa (15), APH(2')-IVa (14), APH(3')-IIa (13), APH(3')-IIIa (12, 33), and APH(9)-Ia (11) shows root mean square deviation values between 2.6 and 3.3 Å (over 182–229 matching Ca atoms), reflecting the conservation of the general APH fold in the APH(4)-Ia structure. However, the C-terminal core subdomain of APH(4)-Ia shows particularly high fold conservation and superimposes with the equivalent domain of other APH enzymes with a lower range of root mean square deviation values (between 2.0 and 2.6 Å, over 89–95 matching Ca atoms). This similarity is reflected in the strict spatial conservation of common catalytic residues, many of which are localized to the C-terminal core subdomain. The N-terminal domain of APH(4)-Ia is also more highly conserved in structure than the full enzyme structure overall and superimposes with the equivalent domain of other APH enzymes with root mean square deviation values between 2.1 and 2.6 Å (over 67–75 matching Ca atoms). This is reflected in the high conservation of the ATP-binding site, which is largely formed by this subdomain. Taken together, these observations suggest that the structural variation in APH enzymes can be attributed to the C-terminal helical subdomain. This domain is involved in substrate recognition, thus leading to the analysis of the topology and conformation of the APH(4)-Ia C-terminal helical subdomain.

Role of the C-terminal Helical Subdomain in the Narrow Substrate Specificity of APH(4)-Ia—Primary sequence alignment suggested that residues 156–171 of APH(4)-Ia should correspond to the aminoglycoside-binding loop of APH(3')-IIIa, but according to tertiary structure analysis, the region corresponding to these residues is largely displaced away from the antibiotic-binding site. Instead, the space corresponding to the aminoglycoside-binding loop in APH(3')-IIIa structure is occupied by α9 and α10 helices in the APH(4)-Ia (Fig. 3). A similar feature had been shown for the APH(2')-IIa structure (15); our superposition verified that the C-terminal helical subdomain of APH(2')-IIa is a better match to this subdomain of APH(4)-Ia (Fig. 3).

Although substitution of the aminoglycoside-binding loop by helices α9 and α10 in APH(4)-Ia positions amino

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**TABLE 1**

| X-ray diffraction data collection and structure refinement statistics |
|---------------------------------------------------------------|
| **Data collection**                                            |
| Wavelength (Å)                                               | 0.9798 (peak) |
| Resolution range (Å)                                        | 50.0–1.95 |
| Space group                                                 | P3_2 |
| a = b (Å)                                                   | 70.64 |
| c (Å)                                                       | 125.88 |
| R_{free} (Å)                                                 | 0.068 (0.697)* |
| Completeness (%)                                             | 52.2 (3.46) |
| Redundancy                                                   | 99.9 (100) |
| Number of non-hydrogen atoms                                 | 10.3 (7.6) |
| **Refinement**                                               |
| Resolution range (Å)                                        | 23.54–1.95 |
| Number of reflections                                       | 23.54–1.95 |
| Working set                                                  | 25,785 |
| Test set                                                     | 1360 |
| R_{free} (Å)                                                 | 0.176/0.218 |
| Number of reflections                                       | 0.176/0.218 |
| Protein                                                      | 36.8 |
| Substrate                                                   | 29.6 |
| Ion/solvent                                                  | 52.8 |
| Water                                                        | 48.2 |
| Root mean square deviation                                   |
| Bond length (Å)                                              | 0.010 |
| Bond angle (°)                                               | 0.98 |
| Average thermal factor (Å^2)                                 | 3.86 |
| Ramachandran analysis (%)                                    |
| Most favored regions                                         | 92.1 |
| Additionally allowed regions                                 | 7.5 |
| Generously allowed regions                                   | 0.4 |
| Disallowed regions                                           | 0 |

*The values in parentheses refer to highest resolution shell of 1.98–1.95 Å.

* R_{free} was calculated by randomly omitting 5% of the observed reflections from the refinement.

* According to the Ramachandran plot in Procheck (21).
acids largely in the same vicinity in the aminoglycoside-binding site, this replacement introduces numerous differences. The helical periodicity and the specific residue content of α9 and α10 result in different residues accessible for interaction with the substrate. These structural features may contribute to the narrow substrate specificity of APH(4)-Ia. Notably, APH(3′)-Ia Asp-159, which interacts with the C ring of kanamycin, corresponds to Trp-288 in the APH(4)-Ia structure. The presence of this bulky side chain would preclude binding of 4,6-disubstituted aminoglycosides to APH(4)-Ia, a notion consistent with the substrate specificity studies. Furthermore, modeling of aminoglycosides with additional chemical groups at the position 1 of the 2-deoxystreptamine ring, such as the 4-amino-2-hydroxy-butyryl group of butirosin, into the APH(4)-Ia structure. The presence of this bulky side chain would preclude binding of 4,6-disubstituted aminoglycosides. These structural observations are consistent with the steady state kinetic studies presented here.

APH(4)-Ia Contains a Unique Cluster of Hydrophobic Residues That Complements the Twisted Shape of hygB—Having established that the active site of APH(4)-Ia is not comprised of an aminoglycoside-binding loop structure, we analyzed the specific interactions between the enzyme and hygB. As with other APH enzymes (11–15, 33), the substrate-binding site in the APH(4)-Ia structure is found in a negatively charged pocket between the C-terminal core and helical subdomains (Fig. 2B). In the structure, the hygB adopts a conformation such that rings II, III, and IV are not co-planar, in contrast to the equivalent rings of 4,6-disubstituted ligands when bound to APH(2′) or APH(3′) enzymes. The plane defined by ring I of hygB is nearly perpendicular to a plane through rings II and III. Ring I is also nearly perpendicular in the other axis to the plane of ring IV, because of the twist introduced by the orthoester between rings III and IV. Interestingly, rings I and IV of hygB superimpose well with rings A and C of the spectinomycin bound to APH(9)-Ia (11). This is due to the fact that ring IV of hygB is twisted relative to rings II and III, in the same way as the twist of the spectinomycin ring C relative to rings A and B.

Ring I of hygB is prominently separated from the other rings with the C4-OH (O11) phosphorylation site positioned at a distance of 2.2 Å from catalytic Asp-198 (Fig. 4). When other APH-aminoglycoside complex structures are superimposed onto the APH(4)-Ia/hygB structure, ring I of hygB occupies the position corresponding to the phosphorylated rings of spectinomycin bound to APH(9)-Ia, and ring C or ring A of 4,6-disubstituted aminoglycoside ligands bound to APH(2′) enzymes and APH(3′) enzymes, respectively. On the enzyme side, this feature is reflected in
FIGURE 3. Domain architecture of APH enzymes and secondary structure topology of the C-terminal helical subdomain. A, domain architecture of APH enzymes and a representative eukaryotic protein kinase (ePK) enzyme cAMP-dependent protein kinase (CAPK) (39). The space occupied by the aminoglycoside-binding loop from APH(3′)- enzymes (shaded red) is replaced by the C-terminal region shaded pink in the APH(4)-Ia, APH(2″)-Ia, and APH(9)-Ia enzymes, as indicated by dashed lines connecting these regions. B, zoom-in view of the C-terminal helical subdomain showing that the aminoglycoside-binding loop of APH(3′)-Ila (residues 150–165) is colored red, and the region of APH(4)-Ia (residues 278–300) that fills the equivalent volume is colored dark blue. Right panel, superposition of the structures of APH(3′)-Ila (residues 125–182; pink) and APH(4)-Ia (residues 125–189 and 278–300; blue). Aminoglycoside-binding loop of APH(3′)-Ila (residues 150–165) is colored red, and the region of APH(4)-Ia (residues 278–300) that fills the equivalent volume is colored dark blue. Right panel, superposition of the structures of APH(3′)-Ila (residues 122–184 and 275–291; gray) and APH(4)-Ia (residues 125–189 and 278–300; blue).
the strict conservation of the C-terminal core subdomain conformation and that of the catalytic residues across all APH enzymes.

The APH(4)-Ia enzyme forms a pocket that is complementary to the twisted shape of hygB (Fig. 4). There are 13 direct hydrogen bonds between hygB and eight residues of this APH enzyme (Fig. 4C). These interactions are nearly equally distributed between the two “faces” of the substrate: an “A face” that interacts with six residues from the catalytic and NTP-binding sites and a “B face” that interacts with five residues from the C-terminal helical subdomain.

The interaction between the enzyme and hygB is bridged by a water molecule and three ammonium ions. The water molecule interacts with the phosphorylation site O11 and forms six interactions with the A face of the ligand. The ammonium ions interact with Gln-273 and atoms in rings I and IV of hygB.

Specific interactions between hygB and APH(4)-Ia Ser-201 appear to be responsible for straining of the backbone $\phi/\psi$. 

The phosphorylation site on hygB is labeled with a black star. Carbon, nitrogen, and oxygen atoms and water and ammonium molecules are colored black, blue, red, gray, and blue, respectively. The interactions from Asp-126 and Ser-128, residues from a symmetry-related chain in the crystal, are indicated by apostrophes.
angles of this residue (Table 1; Ser-201 is the only residue in the “generously allowed” region of the Ramachandran plot). This strained nature of Ser-201 does not have an analog in previously characterized APH substrate complex structures; thus this residue plays a unique role in this APH enzyme.

In addition to an extensive network of electrostatic/h-bonding interactions, the APH(4)-Ia-binding pocket also supplies a significant hydrophobic surface to complement the structure of the substrate (Fig. 4B). Residues Trp-235, Trp-238, Leu-239, and Met-242 provide hydrophobic and stacking interactions with rings II, III, and IV of hygB. Trp-235 is positioned directly under and stacks with ring II of hygB. The edge of Trp-238 defined by atoms NE1 and CD1, along with Leu-239 form a surface accommodating ring IV of hygB. The CE atom of Met-242 interacts with an edge of ring II. Trp-235 and Trp-238 in turn pack against Trp-288 from α9 of the enzyme. The hydrophobic patch formed by these three tryptophan residues represents a distinctive feature of the APH(4)-Ia structure when compared with other structures of APH enzymes. The three tryptophan residues likely contribute to the conformation of the other residues making up this hydrophobic patch, therefore determining selectivity for the atypical chemical architecture of hygB. Interestingly, hygB occupies only a portion of the large cleft between the APH(4)-Ia N- and C-terminal domains. This cleft contains residues Pro-135, Tyr-144, Phe-150, Ala-197, Met-221, Tyr-227, Asn-231, Arg-268, and Ile-269, belonging to both C-terminal subdomains.

Nucleotide-binding Site of APH(4)-Ia Resembles That of APH(2')-IIa—At least seven residues involved in nucleotide binding in APH(2')-IIa (15), APH(3')-IIIa (12, 33), and APH(9)-Ia (11) are also conserved in the APH(4)-Ia. These include the Brenner motif, a sequence that is distinctive of enzymes that catalyze phosphoryl transfer (34), and the Asp-Phe-Gly (DFG) sequence, which is conserved in eukaryotic protein kinases. APH(4)-Ia Glu-288 corresponds to a highly conserved Asp/Glu residue in α7 that forms interactions with the backbone amides of the His-Xaa sequence of the Brenner motif. APH(4)-Ia contains a conserved glycine (Gly-31) in the β2-β3 loop corresponding to the nucleotide positioning loop (NPL)/Gly-rich loop. The enzyme contains the conserved Leu-48–Arg-49 sequence in β3, shown to be involved in interactions with bound nucleotides and forming a salt bridge with a highly conserved acidic residue in α2. The enzyme also contains the highly conserved IDWS sequence in the β7–β8 loop; side chains of the ID pair form interactions with bound nucleotides.

APH(4)-Ia did not crystallize in complex with ATP or its nonhydrolyzable analogs such as AMPPNP or AMPPCP. To gain some insight about APH(4)-Ia interactions with this substrate, the ATP molecule was manually docked in the nucleotide-binding site of APH(4)-Ia using a superposition with the structure of APH(2')-IIa in complex with ATP and streptomycin (15). This superposition was accomplished by aligning Ca atoms in the region between β6 and β9, comprising the Brenner and DFG motifs (root mean square deviation = 0.85 Å). This superposition showed that the APH(4)-Ia and APH(2')-IIIa N-terminal domains differ by a rotation of ~10° about the hinge region, and we therefore adjusted the ATP position to compensate for this variation.

Based on this positioning of the nucleotide, most of the conserved nucleotide-binding elements in the APH(4)-Ia structure are properly positioned for interactions with ATP except for the side chains of Arg-49 and Asp-216. In ATP-bound forms of APH enzymes, the positively charged group of the conserved Lys/Arg residue is positioned to interact with nonbridging oxygen atoms of the α- and β-phosphates of ATP (11, 15, 33, 35). APH(4)-Ia Asp-216 corresponds to the residue that would interact with Mg$^{2+}$ and a nonbridging oxygen of the α-phosphate of ATP in an APH(4)-Ia-Mg-ATP complex. In the APH(4)-Ia ATP binding model, changes in the side chain rotamers of Arg-49 and Asp-216 would be needed to initiate such interactions.

The model also indicated that the NPL of APH(4)-Ia would form interactions with the nucleotides that resemble those seen between the NPL of APH(2')-Ia and ATP (15). The NPL sequence is conserved between APH(4)-Ia and APH(2')-IIa and includes two serines involved in interactions with phosphates of ATP. Therefore, the NPL of APH(4)-Ia likely does not undergo significant conformational changes and would not clamp over the nucleotide as seen in the nucleotide-bound APH(3') and APH(9) complexes (11, 33, 36).

This similarity between the nucleotide-binding pocket of APH(4)-Ia and APH(2')-IIa could also explain why APH(4)-Ia shows promiscuity toward the nucleotide substrates ATP and GTP. APH(2')-IIa was recently shown to utilize both nucleotides in its reaction (32). It has been suggested that a larger nucleotide-binding pocket in the APH(2') enzymes could offer an alternative binding mode for GTP as compared with ATP (14). APH(3')-IIIa on the other hand shows exclusivity for ATP (31), which could be due to a tighter binding pocket, and a large conformational change of the NPL upon nucleotide binding (33).

Site-directed Mutagenesis of APH(4)-Ia—To confirm the results of the structural analysis, several APH(4)-Ia active site residues were probed by site-directed mutagenesis. Eleven active site residues: Gln-101, Asp-126, Ser-201, Asn-231, Thr-238, Gln-273, and Asp-285 (all involved in hygB binding); Arg-49, Asn-203, and Asp-216 (participating in nucleotide binding); and Asp-198 (involved in γ-phosphate transfer from ATP to hygB) were selected. Each of these residues was individually mutated to an alanine, with the exception of Trp-238, which was converted to leucine to retain similar hydrophobic and volume properties. Of the 11 mutations studied, the four expected to participate in ATP binding and catalysis (R49A, D216A, N203A, and D198A) showed the greatest level of impairment in hygB modifying activity as compared with the wild-type enzyme (Table 2). Among these, the D216A, N203A, and D198A variants showed complete loss of activity. The R49A variant showed no change in its ability to recognize hygB but was severely impaired in ATP binding with a 30-fold increase in its $K_m$ as compared with the wild type. The residues involved in nucleotide binding are well conserved across all of the APH enzymes with the exception of the substitution of the α-β phosphate coordinating lysine with an arginine (Arg-49). This substitution is also seen in APH(2')-IIIa and...
the macrolide phosphotransferases MPHα and MPHβ. Arg-49 shows the same level of indispensability as compared with its lysine counterparts in other APH enzymes (12, 37), where its removal leads to severe impairment in ATP utilization.

The seven APH(4)-Ia variants with alanine substitutions of residues involved in hygB binding showed nominal changes (no greater than a 2-fold increase) in $K_m$ and $k_{cat}/K_m$ as compared with the wild-type enzyme for hygB utilization. This reflects the extensive and distributed hydrogen-bonding network used to bind hygB. In such a network, removal of one or even two hydrogen bonds in the network of 13 total bonds is not predicted to have a significant effect.

Conclusions—This study presents a structural and functional analysis of APH(4)-Ia interactions with its substrate, the atypical aminoglycoside antibiotic hygromycin B. APH(4)-Ia adopts the common eukaryotic protein kinase-like fold seen in all APH enzyme structures. However, the APH(4)-Ia structure also demonstrates unique features that provide molecular insight into the high substrate specificity of this enzyme. Primarily, the APH(4)-Ia substrate-binding site is lined by hydrophobic residues, including a cluster of Trp residues, absent in previously characterized APH enzymes. These residues provide a structural and chemical complement to the structure of hygB and also restrict binding of 4,6- or 4,5-disubstituted 2-deoxystreptamine-based aminoglycosides.

Through comparative structural analysis, it was shown that the aminoglycoside-binding site of APH(4)-Ia is made up of residues found toward the C-terminal portion of the C-terminal helical subdomain, and not the region corresponding to the “aminoglycoside-binding loop,” found in the N-terminal portion of the C-terminal helical subdomain in otherwise homologous APH(3′) enzymes. This topology is shared with APH(2′) and APH(9) groups of enzymes. APH(4)-Ia can also utilize GTP in its phosphorylation reaction, which is a property previously observed only for APH(2′) enzymes (32).

HygB has broad antimicrobial activity and is only targeted by two known modifying enzymes, APH(7′)-Ia and APH(4)-Ia, making it a good scaffold for novel antibiotic development in comparison with other aminoglycoside antibiotics such as kanamycin A, which is targeted by over 18 different modifying enzymes. The presented structural and mutational analysis opens the possibility for the design of hygB derivatives that would be less prone to modification by APH(4)-Ia, further improving the prospects for generation of novel phosphorylation-proof aminoglycoside antibiotics.

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