Aminoglycoside 2’’-Phosphotransferase IIla (APH(2’’)-IIla) Prefers GTP over ATP

STRUCTURAL TEMPLATES FOR NUCLEOTIDE RECOGNITION IN THE BACTERIAL AMINOGLYCOSIDE-2’’ KINASES

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Clyde A. Smith,1 Marta Toth,2 Hilary Frase3, Laura J. Byrnes1,2, and Sergei B. Vakulenko5

From the 1Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Contrary to the accepted dogma that ATP is the canonical phosphate donor in aminoglycoside kinases and protein kinases, it was recently demonstrated that all members of the bacterial aminoglycoside 2’’-phosphotransferase IIla (APH(2’’)) aminoglycoside kinase family are unique in their ability to utilize GTP as a cofactor for antibiotic modification. Here we describe the structural determinants for GTP recognition in these enzymes. The crystal structure of the GTP-dependent APH(2’’)-IIla shows that although this enzyme has templates for both ATP and GTP binding superimposed on a single nucleotide specificity motif, access to the ATP-binding template is blocked by a bulky tyrosine residue. Substitution of this tyrosine by a smaller amino acid opens access to the ATP template. Similar GTP binding templates are conserved in other bacterial aminoglycoside kinases, whereas in the structurally related eukaryotic protein kinases this template is less conserved. The aminoglycoside kinases are important antibiotic resistance enzymes in bacteria, whose wide dissemination severely limits available therapeutic options, and the GTP binding templates could be exploited as new, previously unexplored targets for inhibitors of these clinically important enzymes.

Bacteria use kinase-like enzymes, known as aminoglycoside kinases or aminoglycoside phosphotransferases (APHs),3 for self-protection against clinically important aminoglycoside antibiotics. The aminoglycoside antibiotics, produced by soil microorganisms as part of an arsenal in the ongoing battle waged between soil bacteria, include the clinically relevant drugs gentamicin and kanamycin, (supplemental Fig. S1). The aminoglycosides are a family of broad-spectrum antibiotics targeting the bacterial ribosome and disrupting protein synthesis (1–3) and are used for the treatment of infections caused by both Gram-positive and Gram-negative bacteria (4). They all have a similar structure, consisting of a central aminocyclitol ring (the B ring) with aminoglycan groups (the A and C rings) attached to the B ring (supplemental Fig. S1). Several subfamilies of APH enzymes are known, named according to the position on the aminoglycoside at which they act, and their substrate/cofactor specificity (5–9), with the APH(3’’) and APH(2’’’) families being the most prevalent. Enzymes in these two families have the capability of conferring resistance to a wide range of aminoglycosides, yet no two enzymes have quite the same substrate specificity profile, raising important questions with regard to what structural features are important for this selectivity. The APH enzymes phosphorylate free hydroxyl groups on the antibiotics, which interferes with the subsequent binding of these molecules to their ribosomal target. The phosphate group is typically derived from ATP and the phosphoryl transfer mechanism involves a conserved aspartate residue that initially orients the substrate hydroxyl group (10) and subsequently acts as a proton acceptor once cleavage of the phosphorus-oxygen bond has begun (11). The triphosphate moiety of the nucleotide is stabilized by the presence of two magnesium ions that bridge the nucleotide and the enzyme and neutralize some of the negative charge on the triphosphate.

Structurally, the APHs are similar to the eukaryotic protein kinase (ePK) catalytic domain, and although there is limited overall sequence identity, the APHs contain a number of
kinase-specific sequence motifs related to nucleotide binding and phosphoryl transfer. It is generally accepted that the highly regulated ePKs evolved from a simpler non-regulated ancestral kinase that may have resembled the APHs (12). Bacterial and archaeal genomes also contain a wide range of protein kinases (bacterial protein kinases (bPKs) and archaeal protein kinases (aPKs)) that share sequence and structural similarities with the ePKs (13–18), and phosphorylation by protein kinases is now seen as a universal process in all kingdoms of life (19).

The protein kinases (PKs; collectively the ePKs, bPKs, and aPKs) almost always use ATP as a cofactor for phosphoryl transfer (20) with few exceptions, notably casein kinase II (CK2) (21), calcium/calmodulin-dependent protein kinase II (22), mst-3 kinase (23), and protein kinase Cδ, which shows an increase in autophosphorylation with GTP (24). Our recent work on the APHs from the aminoglycoside-2′′-phosphotransferase [APH(2′′)] subfamily (25) have shown that not only are these enzymes capable of using GTP as the phosphate source but that two members of this family (APH(2′′)-Ia and APH(2′′)-IIa) utilize GTP exclusively in vivo. The ability of the APH(2′′) enzymes to use GTP as a cofactor is unexpected, as APHs were also considered to be exclusively ATP-dependent (20, 26). This raises a number of questions related to nucleotide binding and catalysis in the aminoglycoside kinases and in the protein kinases in general. What are the structural determinants of nucleotide selectivity in these enzymes, and why is it that the eukaryotic kinases have evolved to be almost exclusively ATP-dependent, whereas some bacterial aminoglycoside kinases can be exclusively GTP-dependent, exclusively ATP-dependent, or possess the ability to utilize both of these substrates? In an attempt to answer some of these questions we have solved the crystal structure of APH(2′′)-IIa, an aminoglycoside kinase that uses only GTP as the phosphate source. We provide evidence for the existence of a structural template for GTP recognition in all bacterial aminoglycoside kinases from the APH(2′′) subfamily, a template that is also conserved in other APH enzymes yet is poorly conserved in the majority of bacterial Ser/Thr kinases and eukaryotic protein kinases.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Kinetic Analysis**—The wild-type aph(2′′)-IIa gene and the mutant F108L were cloned and expressed, and the resultant proteins were purified as previously described (27). The Y92A mutant APH(2′′)-IIa was cloned into the NdeI and HindIII sites of the pET22b expression vector, the recombinant plasmid was transformed to *Escherichia coli* BL21(DE3), and the transformants were selected on LB agar supplemented with 100 μg/ml ampicillin. For protein purification the bacterial culture was grown until it reached an optical density of ∼0.7 (A_{600} ∼ 0.7). Protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and cells were grown at 10 °C for 2 days. Cells were disrupted by sonicnation, centrifuged (20,000 × g/30 min), and the Y92A mutant enzyme was purified from the supernatant by Gentamicin-Affi-Gel 15 (Bio-Rad) affinity chromatography. The enzyme was eluted with a NaCl gradient in 25 mM Hepes, pH 7.5, and fractions were analyzed by enzyme activity and subsequently by SDS-PAGE. Protein concentration was measured with the BCA kit (Pierce). Phosphorylation of kanamycin by the Y92A mutant of APH(2′′)-IIa was monitored with a continuous spectrophotometric assay in the presence of various concentrations of ATP and GTP. Reactions were initiated by enzyme addition. The K_{m} values for ATP and GTP substrates were determined by fitting the kinetic data to the Michaelis-Menten equation using the program Prism 5 (GraphPad Software, Inc.), v = V_{max}[S]/K_{m} + [S], where v is the initial velocity, and S and K_{m} are the substrate concentration and the Michaelis constant of the variable substrate.

**Protein Crystallography**—Two forms of the APH(2′′)-IIa enzyme were crystallized as the Mg_{5}GDP complex, the wild-type form and a point mutant F108L. Preliminary analyses and characteristics of the F108L crystals have been described (27). The Mg_{5}GDP complex of wild-type APH(2′′)-IIa was prepared by preincubation of 10 mM GTP and 20 mM MgCl_{2} with the enzyme at ~1 mg/ml in 20 mM HEPES, pH 7.5, and then concentrating the resultant complex to ~8 mg/ml. Crystals were grown by sitting drop vapor diffusion from 0.2 mM lithium nitrate and 20% PEG 3350. The wild-type GDP-APH(2′′)-IIa data were collected on beam line BL9-2 at the Stanford Synchrotron Radiation Lightsource from a single crystal flash-cooled in a cryoprotectant composed of crystallization buffer and 20% glycerol. The crystal belonged to space group P1 with two molecules in the asymmetric unit. Data were processed with XDS/XSCALE (28). A native F108L GDP-APH(2′′)-IIa data set was collected at Stanford Synchrotron Radiation Lightsource beamline BL9-2 to a nominal resolution of 1.70 Å. The data were processed with XDS/XSCALE. Data collection statistics are given in Table 1.

The F108L crystals were used for heavy metal soaking experiments. They were transferred to crystallization buffer (0.25 mM MgCl_{2}, 0.1 M Tris-HCl, pH 8.5, and 20% (w/v) PEG 4000) containing varying concentrations (20-75 mM) of the gadolinium complex Gd-HPDO3A (29) and for varying time intervals (1-24 h). The data from a Gd-HPDO3A-soaked F108L APH(2′′)-IIa crystal were collected at Stanford Synchrotron Radiation Lightsource beamline BL9-1. A total of 120 images were collected at the peak and the inflection point of the gadolinium III absorption edge and at a high energy remote wavelength. The three data sets were processed with XDS/XSCALE.

| TABLE 1 | |
|---|---|---|---|
| Peak | Inflection | Remote |
| **Wavelength (Å)** | 1.7117 | 1.7197 | 1.20369 |
| **Resolution limit (Å)** | 2.5 | 2.5 | 2.5 |
| **Unique reflections** | 21,385 | 21,399 | 21,612 |
| **R_{merge} (%)** | 6.5 (23.5)* | 9.8 (57.8) | 5.7 (23.7) |
| **I/σ(I)** | 10.0 (3.7) | 12.6 (3.7) | 7.7 (1.6) |
| **Average redundancy** | 3.5 (3.2) | 3.4 (2.8) | 3.7 (3.7) |
| **Completeness (%)** | 96.6 (96.6) | 96.0 (97.6) | 96.9 (98.5) |
| **MAD resolution limit (Å)** | 3.0 | 3.0 |
| **FOM from SOLVE** | 0.70 |
| **FOM from SOLVE** | 0.70 |
| **Residues autobuilt** | 0.81 |
| **R_{merge}/R_{free}** | 205 (55) |
| **R_{merge}/R_{free}** | 0.437/0.477 |

* Numbers in parentheses relate to the highest resolution shells, 2.6-2.5 Å.
* After density modification.
* The number in parentheses is the residues placed according to the sequence.
TABLE 2

APH(2’’)-IIIa native data collection and refinement statistics

|                      | F108L Mg,GDP complex | Wild-type Mg,GDP complex |
|----------------------|----------------------|--------------------------|
| **Data collection**  |                      |                          |
| Space group          | C2                   | P1                       |
| Cell dimensions, (Å³) | 82.48, 54.33, 77.56, β = 107.9 | 41.78, 54.74, 77.30, 89.9, 103.8, 106.9 |
| Molecules in the AU  | 1                    | 2                        |
| Resolution (Å)       | 46.0-1.7 (1.75-1.7)   | 80.6-2.2 (2.25-2.2)       |
| Reflections, observed/unique | 135,226/36,432 | 60,213/30,488 |
| Rmerge (%)           | 6.6 (59.2)           | 7.4 (33.5)               |
| Completeness (%)     | 12.8 (2.3)           | 9.6 (2.51)               |
| Redundancy           | 99.6 (99.8)          | 95.1 (94.3)              |
|                      | 3.7 (3.7)            | 2.0 (1.9)                |
| **Refinement**       |                      |                          |
| R_factor/R_factor (%)| 17.4/22.0            | 20.4/29.1                |
| Protein atoms/solvent atoms | 2478/403        | 4833/291                |
| Average B, protein (Å²) | 23.8             | 31.4/28.8               |
| Average B, solvent (Å²) | 34.7             | 28.8                    |
| r.m.s.d. bonds (Å)   | 0.007                | 0.009                   |
| r.m.s.d. angles (%)   | 1.14                 | 1.18                    |
| Ramachandran statistics |                  |                          |
| Favored region       | 94.0%                | 91.7                    |
| Number disallowed     | 0                    | 0                       |

*Numbers in parentheses relate to the highest resolution shells.

Data collection statistics and some structure solution statistics are given in Table 2.

The F108L Mg,GDP-APH(2’’)-IIIa structure was solved by multiwavelength anomalous diffraction methods with data to 2.5 Å resolution using the PHENIX suite of programs (30). The density-modified electron density maps from PHENIX were of excellent quality, and the presence of the nucleotide molecule was clearly evident. Interactive model building with COOT (31) was used to add the majority of the residues, but no nucleotide, magnesium ions, or water molecules were added at this stage. Refinement was transferred to the 1.7 Å resolution native F108L data, and Mg,GDP was modeled into the active site. The final model contained 290 residues (5–302), 403 water molecules, a GDP molecule, and four magnesium ions, with a crystallographic R-factor of 0.174 and an R_free of 0.220. The structure of the wild-type GDP complex was solved by molecular replacement with the program MolRep from the CCP4 suite (32) using the F108L Mg,GDP-APH(2’’)-IIIa structure as a starting model. The structure was refined with REFMAC and PHENIX to a final R-factor and R_free of 0.204 and 0.29, respectively, at 2.2 Å resolution (see Table 1).

RESULTS

Crystal Structure of APH(2’’)-IIIa—Two crystal forms of APH(2’’)-IIIa were obtained from initial crystallization trials, those of a wild-type Mg,GDP complex diffracting to ~2.2 Å resolution in space group P1 and a binary Mg,GDP complex of a F108L mutant identified as a “second round” mutation in a study of the effects of random mutagenesis on the aph(2’’)-IIIa gene with respect to increased levels of aminoglycoside resistance (33). The structure was solved by multiwavelength anomalous diffraction methods at 2.5 Å resolution (Table 1). The APH(2’’)-IIIa structure consists of two domains (Fig. 1), with the N-terminal domain (residues 1–92) comprising a five-stranded β-sheet flanked by two α-helices. A six-residue “hinge” peptide joins this domain to the C-terminal domain, the latter further subdivided into two structural subdomains; the core sub-domain (residues 98–142, 189–252) composed of 2 roughly parallel α-helices flanked by 2 shorter α-helices and capped by 4 β-strands formed into a β-finger motif and the helical subdomain (residues 143–188, 253–303) comprising four α-helices. During structure refinement, residual F_o – F_c electron density in the cleft between the N-terminal domain and the core subdomain was modeled as GDP with two magnesium ions coordinating to the α- and β-phosphate groups (supplemental Fig. S2).

The overall fold of APH(2’’)-IIIa is similar to that of the other APH enzymes whose structures are known including APH(2’’)-Ia (34), APH(2’’)-Va (35), APH(3’’)-Ia (36), APH(3’’)-IIIa (37), APH(4)-Ia (38), APH(9)-Ia (39), and Rv3168 from Mycobacterium tuberculosis (40) along with three other unpublished structures from structural genomics initiatives, APH(3’’)-Ia (PDB code 2R78) and two putative APHs (PDB codes 3CSV and 3DXP) (supplemental Fig. S3). Superposition of APH(2’’)-IIIa onto the other known APH structures (supplemental Table S1) indicates that the topology of the APH(2’’) enzymes along with APH(4)-Ia, APH(9)-Ia, Rv3168, and the two putative APHs are essentially identical. However, the APH(3’’) enzymes show significant differences in their helical subdomains in that they have a long loop joining the first two helices of this sub-domain (helices a5 and a6 in APH(2’’)-IIIa numbering; see Fig. 1), which has been implicated in aminoglycoside binding (36, 37), whereas in the APH(2’’) enzymes this loop is very short (2 residues). The APH(3’’) enzymes are also shorter than the other APHs by ~30–60 residues, primarily due to the presence of only 3 helices in the helical subdomain, whereas the other APHs have 4 and sometimes 5 helices. In this regard the APH(3’’) enzymes could perhaps be considered as archetypal or minimal aminoglycoside phosphotransferase, being significantly shorter than the other APHs yet still having full activity and a broad substrate selectivity. The presence of additional α-helices in the other APHs could serve to fine-tune their substrate profiles making them more selective; for example, APH(9)-Ia is the largest of the APHs at 339 residues with 5 helices in the helical subdomains, yet it has a very restricted substrate preference, phosphorylating only spectinomycin (39).
The APH(2′′)-IIIa enzyme also shows considerable structural similarity to the catalytic domains of the PKs (Fig. 2), indicative of their common ancestry. Superposition of APH(2′′)-IIIa onto cyclic-AMP-dependent kinase (CAMPK) (41), CK2 (21), and the bacterial Ser/Thr kinase PknB from *M. tuberculosis* (15), gives r.m.s.d. of 3.4 Å (for 172 matching Ca positions), 3.3 Å (for 176 Ca), and 3.3 Å (for 169 Ca), respectively, with sequence identities of between 15 and 17%.

**Nucleotide Specificity Motif of APHs**—The close structural similarity between APH(2′′)-IIIa, APH(2′′)-IIa, and APH(2′′)-IVa coupled with the observed differences in nucleotide utilization raises the inevitable question, Why is APH(2′′)-IIIa unable to effectively use ATP? The Mg2GDP complex of APH(2′′)-IIIa represents the first structure of an aminoglycoside kinase with a guanine nucleotide bound, and the structure provides us with a unique insight into how GTP is accommodated by this enzyme and pinpoints the structural features that make this enzyme highly GTP-selective. The GDP cofactor is bound between the two structural domains of the enzyme, close to the site used by other APH enzymes to bind ATP (34, 42). The GTP pocket is composed of two distinct binding sites, one for the triphosphate and another for the nucleotide base. A loop, structurally homologous with a glycine-rich G-loop (consensus sequence GXGXXG) seen in the PKs (43), forms hydrogen-bonding interactions with the diphosphate of the GDP, whereby the diphosphate adopts a similar configuration and occupies the same position as the phosphate groups of ATP in other APH enzymes and in the PKs. Two magnesium ions coordinate with the diphosphate and bind to residues conserved in both the APHs and PKs (Fig. 3A). The ribose moiety makes no direct hydrogen bonding contacts with the enzyme. The binding site for the guanine ring is delineated by three β-strands (β1, β3, and β5), the interdomain hinge peptide, and loops on the core subdomain. The guanine ring is sandwiched between a number of hydrophobic side chains including Leu-27 and Val-43 from the N-terminal domain and Ile-99, Leu-203, and Leu-217 from the C-terminal domain. The base is anchored to the hinge by hydrogen bonds with the amide nitrogen and the carbonyl oxygen atoms of Val-95 and the side chain of Tyr-92 (Fig. 3, A and B). There is an additional hydrogen bond to a well-ordered water molecule immobilized by the carbonyl oxygen atoms of Val-95 and Gly-97. The hydrogen-bonding pattern involving Val-95 and this immobilized water molecule is highly reminiscent of the canonical Watson-Crick hydrogen bonding pattern observed between guanine and cytosine in DNA and RNA (Fig. 3B).

Examination of the nucleotide binding region of APH(2′′)-IIIa along with APH(2′′)-IIa and APH(2′′)-IVa demonstrates that the hinge region of these enzymes provides two overlapping binding templates, one for guanine and another for adenine. The Mg2AMPPCP-streptomycin ternary complex of APH(2′′)-IIa represents the only currently known structure of an APH(2′′) enzyme complexed with an ATP analog (34), so this structure has been used in a direct comparison with APH(2′′)-IIIa to highlight the differences leading to nucleotide selectivity in this family of enzymes. Our analysis shows that the general scaffold for purine nucleotide selectivity (the nucleotide selectivity motif (NSM)) (Fig. 4A) has an adenine-specific template comprising the carbonyl oxygen of the residue at the N-terminal end of the hinge region (designated residue L1; Arg-93 in APH(2′′)-IIIa; Fig. 3B) and the amide nitrogen of the residue two positions farther along (residue L3; Val-95). The guanine-specific template is formed by the amide nitrogen and carbonyl oxygen of residue L3 and the water molecule anchored by the carbonyl oxygen atoms of residues L3 and L5 (Gly-97). Thus the guanine- and adenine-specific templates overlap such
that residue L3 participates in the binding of both nucleotides. The L3-L5 water molecule observed in APH(2′′)-IIIa is also present in the ternary AMPPCP-streptomycin-APH(2′′)-IIa complex, two of the three apo forms of APH(2′′)-IVa (35) and in the tobramycin-APH(2′′)-IVa complex (44). The need for overlapping specificity templates for the two purine bases is necessitated by the different arrangement of the hydrogen bond donor and acceptor atoms on adenine and guanine. ATP has an amine donor at position 6 of the adenine ring and a ketimine acceptor at position 1 (Fig. 4B), and these can in turn donate a hydrogen bond to the L1 carbonyl oxygen and accept a hydrogen bond from the L3 amide nitrogen, respectively (Fig. 4A). Conversely GTP has a keto acceptor at position 6 and amino donors at positions 1 and 2 (Fig. 4C), which means the guanine cannot bind in the same location as adenine, requiring a shift of the base across the binding pocket in a direction roughly parallel to the hinge peptide to place the hydrogen bond donor and acceptor atoms back in register (Fig. 4A).

Detailed analysis of the NSMs of APH(2′′)-IIIa, APH(2′′)-IIa, and APH(2′′)-IVa also provide evidence for why the two latter enzymes have the ability to use both ATP and GTP as phosphate donors, whereas ATP is a very poor substrate for APH(2′′)-IIIa. Although both adenine and guanine binding templates are conserved in all three aminoglycoside kinases, access to the adenine-specific template in APH(2′′)-IIIa is blocked by the side chain of Tyr-92 (Figs. 3B and 4A). In the ePKs, the residue at the same position as Tyr-92 is known as the “gatekeeper” (supplemental Fig. S4) as it regulates the availability of the active site for a range of inhibitors (45, 46). In APH(2′′)-IIa it appears that Tyr-92 also plays a “gatekeeper” role by preventing productive binding of ATP to the hinge, entirely consistent with the enzyme strong preference for GTP over ATP. The Tyr-92 side chain is flanked on one side by the main chain of Arg-93 and on the other by the side chain of Arg-45, such that the phenolate ring has very limited degrees of freedom (Fig. 3B). The side chain of Arg-45 is further restricted in its movement by electrostatic interactions with a conserved glutamate (Glu-59) from the α2 helix, which runs along the back of the nucleotide binding site and is equivalent to the C helix in the kinases that plays a key role in enzyme activation.

**FIGURE 2. Molecular surface representations of APH(2′′)-IIIa (A), cyclic-AMP dependent protein kinase, CAMPK (B), casein kinase 2, CK2 (C), and the bacterial protein kinase PknB from *M. tuberculosis* (D).** The color scheme for these molecules is the same used in Fig. 1.
Thus, the gatekeeper residue Tyr-92 is effectively trapped by Arg-45 in the conformation it occupies in APH(2\')-IIIa. The gatekeeper residues in APH(2\')-IIa and APH(2\')-IVa are Met and Phe, respectively, and in both structures the adenine-specific template is fully accessible. Moreover, the gatekeeper residue in APH(2\')-Ia (whose structure has yet to be determined), which utilizes exclusively GTP as a cofactor (25), is also a tyrosine (supplemental Fig. S4), and it is likely that a similar mechanism may be involved in its nucleotide selectivity whereby the tyrosine gatekeeper prevents access to the adenine-specific template.

To test our hypothesis that the bulky side chain of Tyr-92 is blocking access to the adenine-specific template in APH(2\')-IIIa, we mutated this residue to alanine. Cells producing the Y92A APH(2\')-IIIa mutant protein retain their resistance to kanamycin, and kinetic analysis of the purified Y92A mutant enzyme shows an 8-fold decrease in the $K_m$ for ATP (from 1.6 to 0.2 mM). This makes the Y92A mutant APH(2\')-IIIa enzyme similar to the naturally occurring APH(2\')-IVa enzyme, which is capable of using both ATP and GTP as a phosphate source with almost equal preference (25) (Table 3). Although APH(2\')-IVA also has a bulky gatekeeper residue (phenylalanine), in the two independently determined structures of this enzyme (35, 44), the phenylalanine side chain (5°) toward Arg-45 would result in a steric clash (at a van der Waals contact distance of 3.2 Å, as indicated). In the other direction, the C$_\beta$ atom of the Tyr-92 ring is about 3.4 Å from the carbonyl oxygen of Arg-93, and a similar rotation (6°) of Tyr-92 in this direction brings the phenolate ring into van der Waals contact (3.2 Å, as indicated) with the Arg-93 carbonyl oxygen. The location of the hydrophobic pocket above the Tyr-92 side chain is indicated by the blue phenylalanine side chain as observed in the two independent structures of APH(2\')-IVA (35, 44).

Comparison of APH(2\')-IIIa with the known structures of APHs from other families shows that the conformation of the NSMs in these enzymes is essentially identical with respect to the main chain torsion angles (supplemental Table S2), their adenine-specificity templates being almost superimposable (supplemental Fig. S5). Moreover, the guanine specificity template also seems to be intact in the other APHs, including the water binding site formed by the carbonyl oxygen atoms of L3.

FIGURE 3. A, shown is a stereoview of the nucleotide binding site in the GDP-APH(2\')-IIIa complex showing the N-terminal domain (pink, upper) and the core subdomain (green, lower). The interdomain hinge peptide (cyan) is shown on the left, and the location of the L3 residue (Val-95) is indicated. The G-loop is on the right and is colored gray. The GDP molecule is in yellow, the final $F_o - F_c$ electron density (contoured at 1$\sigma$) is shown in blue for the nucleotide, the two Mg$^{2+}$ ions are shown as gold spheres, and the five coordinated water molecules are shown as red spheres. Hydrogen bonding and electrostatic interactions are shown as dashed lines. B, a stereoview of the NSM in APH(2\')-IIIa shows the bound GDP molecule as yellow sticks. The location of an ATP analog is also indicated (thin gray sticks), as observed in the ternary AMPCPP-streptomycin-APH(2\')-IIIa structure (34) (superimposed on APH(2\')-IIIa based upon residues in the G-loop), showing the very close approach it would make to the side chain of Tyr-92. The restricted movement of tyrosine is shown as transparent gray representations of the phenolate ring. The O$_\beta$ oxygen atom of the Tyr-92 is 3.8 Å from the C$_\beta$ atom of the Arg-45 side chain, and a small rotation of the tyrosine side chain (5°) toward Arg-45 would result in a steric clash (at a van der Waals contact distance of 3.2 Å, as indicated). In the other direction, the C$_\beta$ atom of the Tyr-92 ring is about 3.4 Å from the carbonyl oxygen of Arg-93, and a similar rotation (6°) of Tyr-92 in this direction brings the phenolate ring into van der Waals contact (3.2 Å, as indicated) with the Arg-93 carbonyl oxygen. The location of the hydrophobic pocket above the Tyr-92 side chain is indicated by the blue phenylalanine side chain as observed in the two independent structures of APH(2\')-IVA (35, 44).
A GTP Recognition Template in Kinase Enzymes

and L5, with the exception of the putative APH from Silicibacter sp. TM1040 (PDB code 3CSV) that has the L3 carbonyl oxygen pointing away from the guanine binding pocket and the L4 and L5 residues turning back in toward the pocket. Apart from the members of the APH(2’)-Ia family, there is only one other APH enzyme that has been shown to unequivocally use GTP. APH(4)-Ia is able to use both GTP and ATP in the phosphorylation of hygromycin, albeit with a 5-fold preference for the latter (38). Although the structure of APH(4)-Ia has no nucleotide bound, it does have a water molecule bound in the L3/L5 pocket, and modeling of a GTP into the binding site (data not shown) suggests that a guanine nucleotide could readily be bound in a productive manner. Although the accepted dogma is that the other APH enzymes are ATP-dependent, their GTP binding ability has never been tested. Structural comparisons with the APH(2’)-Ia enzymes suggests it is plausible that they could bind and utilize GTP (supplemental Fig. S5), and additional biochemical and structural studies on members of other APH families will be necessary to test this possibility.

Guanine Specificity Template in the PKs—We have further extended our analysis to the known structures in the wider protein kinase superfamily. CK2 is the only PK enzyme that is capable of using both ATP and GTP with roughly equal propensity and whose structure is available (21). The conformation of the NSM from L1 to L3 is identical in CK2 and APH(2’)-Ila (supplemental Table S2), and in CK2 the guanine makes two hydrogen bonds to the main chain nitrogen and carbonyl oxygen atoms of L3 in a similar fashion as in APH(2’)-Ila. However, the motif deviates markedly at the L4 and L5 residues (Fig. 5A) such that the L5 carbonyl oxygen is unavailable to form the water binding site. In CK2 the lack of a hydrogen bond between guanine and a water molecule does not appear to be critical as CK2 binds GTP tightly and productively (the $K_m$ and $k_{cat}/K_m$ values are 22 $\mu$M and $4 \times 10^4$ M$^{-1}$s$^{-1}$, respectively). When CK2 and APH(2’)-Ila are superimposed based upon the GDP molecules, their G-loops overlap almost perfectly, with the hinge regions in the two structures deviating in such a way that the L1-L3 segments of the motif point in different directions (Fig. 5A). However, quite remarkably, these segments intersect at residue L3 such that the two protein atoms that are involved in hydrogen bonding to the guanine are in the same location and orientation in both enzymes.

We also examined the x-ray crystal structures of a set of 79 PKs (75 ePKs, 2 bPKs, and 2 aPKs) from the PDB that contain bound nucleotides (ATP, ADP, AMPPNP, AMPPCP or ATP$\gamma$S) (supplemental Table S3). CAMPK, the most studied of all kinases (47), was taken as the canonical reference molecule. All other kinases and APH(2’)-Ila were superimposed onto CAMPK based upon strands $\beta$1 and $\beta$2 and the G-loop (with an overall average r.m.s.d. of 0.8 Å for 15 matching Cα atoms). Examination of the bound nucleotide showed that in the majority of these kinases (>72%) the overall conformation of the NSM is similar to that seen in CAMPK, and in almost 60% of the cases the triphosphate moiety (or the diphosphate in the case of ADP) adopted a similar configuration, with the $\alpha$- and $\beta$-phosphates sitting against the G-loop and the $\gamma$-phosphate invariably projecting out into the active site (Fig. 5B). All PKs studied retain the adenine-specific template on the hinge region, with the same main chain conformation as in APH(2’)-Ila. In the majority of these enzymes the NSM also has a similar conformation at residue L3; however, differences at the L4 and L5 residues alter the structure of the putative guanine-specific template compared with APH(2’)-Ila.

TABLE 3

| Enzyme            | $K_m$ for ATP ($\mu$M) | $K_m$ for GTP ($\mu$M) | NTP dependence |
|-------------------|------------------------|------------------------|----------------|
| APH(2’)-Ila       | 870                    | 3.5                    | GTP-specific   |
| APH(2’)-Ila       | 16                     | 70                     | ATP and GTP   |
| APH(2’)-Ila       | 1600                   | 4                      | GTP-specific   |
| APH(2’)-Ila       | 101                    | 137                    | ATP and GTP   |
| APH(2’)-Ila Y92A  | 216                    | 94                     | ATP and GTP   |

FIGURE 4. A, the APH(2’)-Ila NSM is on the left (residues L1-L5), with the residue specific for adenine recognition (L1) highlighted in pink, and the residue contributing to both adenine and guanine recognition (L3) in blue. The GTP molecule is represented by thick blue lines and ATP by thinner red lines. The specific hydrogen bonding interactions of the atoms on the NSM with the guanine and adenine are indicated. The gatekeeper residue (Tyr-92 in APH(2’)-Ila) is shown as dashed lines. Steric interactions are indicated by thick dashed lines. B, an ATP molecule shows the location of the hydrogen bond donor and acceptor atoms on the adenine ring. C, a GTP molecule shows the different dispensation of the donor and acceptor atoms on the guanine ring.
DISCUSSION

The bacterial aminoglycoside kinases (APHs) include several families of clinically important enzymes that produce resistance to aminoglycoside antibiotics by the transfer of phosphate groups to free hydroxyl groups on the drugs. Structurally, the APHs are similar to the ePKs, enzymes that catalyze protein phosphorylation leading to the regulation of multiple cellular processes. Until recently, it was widely accepted that ATP is the source of phosphate for these enzymes in vivo, and structural elements involved in ATP binding were extensively studied in ePKs and to a lesser degree in bacterial APHs. For APH(3')-IIIa it was established that in conjunction with hydrogen bonding to the hinge peptide and \pi-\pi stacking interactions with aromatic residues, a methionine residue in the gatekeeper position played a key role in adenine recognition in this enzyme. Significantly more information is available on the structural determinants of ATP binding in the ePKs. Comparative analysis of the ePKs has demonstrated that in addition to the adenine recognition site on the hinge peptide, which has long been used as a template for the development of kinase inhibitors (45, 46, 48, 49), these enzymes contain a number of very highly conserved sequence motifs related to the binding and orientation of the triphosphate of ATP and to phosphoryl transfer (50). These motifs include the glycine-rich G-loop, the catalytic loop, and the activation segment. The G-loop is critical for activity and correctly positions the $\gamma$-phosphate of ATP for attack on the hydroxyl acceptor (43, 51) by firmly anchoring the \alpha- and \beta-phosphates. The catalytic loop (HRDXXXN) contains the essential aspartate residue (the underline in the consensus sequence), which acts as the catalytic base and ultimately accepts the proton from the hydroxyl substrate. The activation segment (XDFGXXAPE) is a loop of variable length and sequence, where the DFG and APE motifs represent anchor points at each end of the flexible loop. This loop undergoes a large scale conformational change generally in response to phosphorylation (12, 52, 53).

These three sequence motifs are also conserved in the APHs (supplemental Fig. S4). First, all of the aminoglycoside kinases have a G-loop equivalent to the PK G-loop. There is some variation in the G-loop sequence between the APHs and the PKs, but structurally the loop plays the same role, folding over the triphosphate, positioning the $\gamma$-phosphate correctly, and pro-
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motivating phosphoryl transfer (54). The catalytic aspartate residue in the DXXXN motif is also spatially conserved (Asp-196 in APH(2′″)-IIIa) and is essential for activity in the APH enzymes (37, 55). Once again the sequences show some variation particularly at the C-terminal end where some of the APHs (including APH(2″)-IIIa) have a histidine residue (His-201) instead of an asparagine, which coordinates to one of the magnesium ions (Fig. 3, A and B). The activation segment is partially conserved, with the DFG motif present in all the APH enzymes, and the aspartate residue (Asp-218) involved in magnesium binding (Fig. 3B). However, the aminoglycoside kinases do not possess the long PK-like activation segment and are not subject to regulation by phosphorylation (12). Instead, the APHs have an open cleft in roughly the same location that is the site of aminoglycoside substrate binding (34, 44, 56). The structures of the nucleotide-bound complexes of six APH enzymes are known, five with ATP or an analog bound, APH(2″)-IIa (34), APH(3‴)-Ia (PDB code 3R78), APH(3‴)-IIIa (42), APH(9)-Ia (39), and Rv3168 (40), and now the GDP complex of APH(2″)-IIIa, and in all cases, irrespective of the identity of the nucleotide (adenine or guanine), the triphosphate moiety is bound in the same location and by a highly conserved set of structural determinants.

Our recent kinetic studies demonstrated that unlike the majority of the ePKs, all four members of bacterial aminoglycoside-2′″ phosphotransferase subfamily are capable of utilizing GTP as the phosphate donor (25). Two of these enzymes are able to use both nucleotides, with APH(2″)-IIa having a slight preference for ATP over GTP, whereas the other, APH(2″)-IVA, has similar relative affinities for these NTPs (Table 3). The two other members of the family, APH(2″)-Ia and APH(2″)-IIIa, are unique in their ability to use GTP exclusively, their respective relative affinities for GTP 250- and 400-fold greater than that for ATP (Table 3). Moreover, the relative affinity of APH(2″)-Ia and APH(2″)-IIIa for GTP is significantly higher than the relative affinity for ATP of various ATP-specific APHs and ePKs. The crystal structure of the Mg₂GDP complex of APH(2″)-IIIa represents the first structure of one of these GTP-specific aminoglycoside kinases and offers insights into how this nucleotide can be accommodated in a binding site thought to be fine-tuned to recognize only ATP. By superimposing recognition templates for both adenine and guanine on hinge peptide, this family of enzymes has gained the ability to recognize both ATP and GTP to the point where the APH(2″)-Ia and APH(2″)-IIIa aminoglycoside kinases have evolved to exclusively bind GTP and use it as the source of phosphate in phosphoryl transfer reactions. Comparative analyses of the known APH(2″) enzyme structures shows that the guanine specificity template is identical in all three enzymes. Although the guanine-specific template is positioned slightly farther toward the outside edge of the nucleotide binding cleft relative to the ATP-specific template, the conformation of the hinge allows for the productive binding of either GTP or ATP, such that triphosphate binding is essentially identical irrespective of the identity of the nucleotide. Moreover, our analyses also show that a guanine specificity template also exists on the hinge region of enzymes from other APH families, although these enzymes have not been tested for the ability to utilize GTP, with the one exception. It has been reported recently that APH(4)-Ia has the ability to use both ATP and GTP (38), and it is highly likely that the same template involved the L3 residue and the L3-L5 water binding pocket will be used to accommodate the guanine ring, although confirmation of this must await further structural information.

Analysis of the NSM of all PKs, which have nucleotide bound and whose structures are available in the Protein Data Bank, demonstrates that, with the exception of CK2, which has a similar NSM and a conserved guanine-specificity template comparable with the APHs, all other PKs show a marked difference in their NSMs. Perhaps the most important difference between the APHs and the PKs is in the relative position of the binding sites for the nucleotide base and the triphosphate (the G-loop). When APH(2′″)-IIIa and CAMPK are superimposed based on strands β1 and β2 and the G-loop, the CAMPK-like NSM is substantially translated toward the core subdomain, placing its adenine-specific template ~3 Å from the APH adenine-specific template and almost directly on top of the APH(2′″)-IIIa guanine specificity template. The L3 residue is an additional 3 Å farther away (Fig. 5B). For guanine to make viable hydrogen-bonding interactions with the L3 residue in a CAMPK-like NSM, the triphosphate moiety of the GTP would be forced to move away from the G-loop, thus giving rise to an potentially unproductive complex. Because it may not be possible to satisfy the hydrogen bonding requirements of both specificity elements (the G-loop and the guanine-specific template) simultaneously, the affinity for GTP might be expected to be low in the majority of the PKs. Although individually the two nucleotide binding elements (the G-loop and the L1-L3 portion of the NSM) are similar in conformation in the PKs and the APHs, it is the spatial disposition of these two elements that is critical in determining which nucleotide is able to bind in an active configuration. Not only must the nucleotide base be anchored firmly to the NSM, but the triphosphate group must interact with the G-loop in such a way that the γ-phosphate is presented to the incoming substrate. This is equally true in the PKs and the APHs. The PKs have clearly evolved a nucleotide binding site specifically tuned to accept ATP in a productive manner, and given the structural repositioning of the NSM in these enzymes, it seems unlikely that GTP would be a viable substrate for most PKs. This clearly seems to be the case given the preponderance of evidence for ATP being the canonical phosphate donor in these enzymes and the comparative rarity of GTP utilization by kinases.

Detailed analysis of the available structures of APHs along with bacterial and eukaryotic protein kinases indicates that templates for the binding of GTP are both structurally and spatially conserved within the aminoglycoside phosphotransferase superfamily but not in the PKs. At present there are no clinically available inhibitors of APHs aimed at either the ATP or the aminoglycoside substrate binding sites. Because the APH enzymes seem to be quite promiscuous with respect to their aminoglycoside substrate profiles (44), the design of a general inhibitor that could act against a large number of the APHs seems impracticable. In addition, the prospects for designing inhibitors targeted at the ATP site are also poor due to the structural similarities between the ATP binding templates of
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APHs and ePKs, as such inhibitors would be expected to be highly toxic as they could potentially inhibit a wide array of human protein kinases. The discovery that there exist consecutive templates for selective adenine and guanine binding within the hinge region of the APH enzymes, which show a much greater variability in ePKs, provides a rationale for the design of selective inhibitors of the APHs. Because the structural templates for GTP and ATP binding by aminoglycoside kinases overlap to some extent, small molecules targeting the GTP specificity template of APHs are also expected to block access to the ATP binding template of these enzymes, ensuring efficient inhibition. Moreover, inhibitors targeting GTP binding in the APHs are expected to exhibit low toxicity as they would not bind promiscuously to the majority of human ePKs and affect their activity. Furthermore, because the mode of guanine binding in the APHs differs markedly from other human GTP-dependent enzymes including the small GTPases and the G proteins, cross-reactivity with these enzymes would also be minimal. Such inhibitors could then be co-administered with existing aminoglycosides, and this would be a major breakthrough in the treatment of life-threatening infections caused by multidrug-resistant microorganisms.

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