Molecular Basis for P-Site Inhibition of Adenylyl Cyclase†‡


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ABSTRACT: P-site inhibitors are adenosine and adenine nucleotide analogues that inhibit adenylyl cyclase, the effector enzyme that catalyzes the synthesis of cyclic AMP from ATP. Some of these inhibitors may represent physiological regulators of adenylyl cyclase, and the most potent may ultimately serve as useful therapeutic agents. Described here are crystal structures of the catalytic core of adenylyl cyclase complexed with two such P-site inhibitors, 2′-deoxyadenosine 3′-monophosphate (2′-d-3′-AMP) and 2′,5′-dideoxyadenosine 3′-triphosphate (2′,5′-dd-3′-ATP). Both inhibitors bind in the active site yet exhibit non- or uncompetitive patterns of inhibition. While most P-site inhibitors require pyrophosphate (PPi) as a coinhibitor, 2′,5′-dd-3′-ATP is a potent inhibitor by itself. The crystal structure reveals that this inhibitor exhibits two binding modes: one with the nucleoside moiety bound to the nucleobase binding pocket of the enzyme and the other with the β and γ phosphates bound to the pyrophosphate site of the 2′-d-3′-AMP:PPi complex. A single metal binding site is observed in the complex with 2′-d-3′-AMP, whereas two are observed in the complex with 2′,5′-dd-3′-ATP. Even though P-site inhibitors are typically 10 times more potent in the presence of Mn2+, the electron density maps reveal no inherent preference of either metal site for Mn2+ over Mg2+. 2′,5′-dd-3′-ATP binds to the catalytic core of adenylyl cyclase with a $K_d$ of 2.4 µM in the presence of Mg2+ and 0.2 µM in the presence of Mn2+. Pyrophosphate does not compete with 2′,5′-dd-3′-ATP and enhances inhibition.

Adenylyl cyclase is an integral membrane protein composed of alternating pairs of membrane and cytoplasmic domains. The two major conserved cytoplasmic domains, C1a and C2a, form the catalytic core of the enzyme with the active site at their interface (1, 2). The catalytic core also contains the primary binding sites for activators of adenylyl cyclase such as G$_\alpha$ and the diterpene forskolin. Early studies demonstrated that adenosine and certain adenosine derivatives could inhibit the production of cAMP in intact cells (3). Such analogues required the presence of an intact adenine ring (4), and their locus of action has been designated as the “P”-site. The locus for P-site inhibition was eventually established to be on adenylyl cyclase itself (5–9) and, more specifically, localized to the catalytic cleft of the enzyme in studies of purified C1a and C2a domains (10). P-site inhibitors occur naturally in vivo, and as such may represent physiological regulators of adenylyl cyclase (11, 12).

Kinetic and structural analysis of P-site inhibition demonstrate that the reaction coordinate of the chemical transformation catalyzed by adenylyl cyclase is complex and involves several conformational states (13). P-site inhibitors are not competitive with activators such as Mn2+, forskolin or G$_\alpha$, and they are much more potent inhibitors of the activated than the basal enzyme (8). Inhibition with respect to ATP is typically un- or noncompetitive in the presence of Mg2+ or Mn2+, respectively (13), but is competitive with respect to cAMP for the reverse reaction and requires the product PPi (14). P-site inhibitors are now believed to form a dead-end complex with adenylyl cyclase by occupying the site that previously accommodated the second product,

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† Abbreviations: G$_\alpha$, the stimulatory G protein $\alpha$ subunit; cAMP, adenosine-3′,5′-cyclic monophosphate; 2′-d-Ado, 2′-deoxyadenosine; 2′-d-3′-AMP, 2′-deoxyadenosine 3′-monophosphate; 2′,5′-d-d3′-AMP, 2′,5′-dideoxyadenosine 3′-diphosphate; 2′,5′-dd-3′-ATP, 2′,5′-dideoxyadenosine 3′-triphosphate; 2′, 5′-dd-3′-A4P, 2′,5′-dideoxyadenosine 3′-tetraphosphate; PPi, pyrophosphate; ATP, $5\prime$-adenosine monophosphate; GTP, guanosine $5\prime$-triphosphate; ATP/S, adenine $5\prime$-(a-thio)triphosphate; ATP/S (R$_a$), adenine $5\prime$-(a-thio)triphosphate, R$_a$ enantomer; MES, 2-(N-morpholino)-ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

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cAMP, before PPi is released (8, 14). Indeed, high-resolution crystal structures show that the P-site inhibitor 2'-d-3'-AMP-PPi binds to the active site of the enzyme in a manner analogous to that of ATP or, perhaps more accurately, cAMP-PPi (2). Recently, adenosine-3'-polyphosphates have been developed as a new class of P-site inhibitors with enhanced potency (12, 15). Extrapolating from the structure of 2'-d-3'-AMP-PPi, in complex with adenylyl cyclase, it has been proposed that the \( \gamma \) and \( \beta \) phosphates of adenosine nucleoside-3'-triphosphates occupy the PPi, binding site (16, 17).

Reported here are biochemical and crystallographic studies aimed at understanding the mechanism of action and metal dependence of P-site inhibitors, in particular the adenosine nucleoside-3'-polyphosphates. The biochemical studies examine the mode of inhibition, and the binding constants of 2',5'-dd-3'-ATP for the catalytic domains of adenylyl cyclase in the presence of either Mg\(^{2+}\) or Mn\(^{2+}\); assess the requirement for PPi and look for competition with adenosine and ATP analogues. Six high-resolution crystal structures are reported: those of adenylyl cyclase with 2'-d-3'-AMP-PPi, or 2',5'-dd-3'-ATP, each with three different combinations of metal ions. One structure for each P-site inhibited complex is described in detail.

**EXPERIMENTAL PROCEDURES**

**Materials.** 2',5'-dd-3'-ADP and 2',5'-dd-3'-ATP were synthesized as described previously (12, 15). 2',5'-dd-[\( \gamma^3\)P]-3'-ATP was synthesized by substrate level phosphorylation from 2',5'-dd-3'-ADP and 32P.2 Carrier-free 32P, (orthophosphoric acid, typically 10 mCi in \( \sim 67 \) \( \mu \)L of water) was from New England Nuclear Corp.

**Preparation of Proteins.** Canine type V C1a domain, rat type II C2a domain, and bovine G\( \alpha \) (short form) were synthesized in *Escherichia coli* and purified as described previously (2, 18, 19). The C1a construct includes an amino-terminal hexahistidine tag. The amino acids included in each construct are as follows: C1a, residues 364–591 for the biochemical analyses and residues 364–580 for the crystallographic analyses; C2a, residues 874–1081; G\( \alpha \), residues 1–396 (numbering reflects the sequence for the long form of G\( \alpha \)). G\( \alpha \) was activated by incubation with GTP\( \gamma \)S and Mg\(^{2+}\) at 35\(^\circ\)C for 3 h as described (20) to yield GTP\( \gamma \)S–G\( \alpha \). For the crystallographic studies, GTP\( \gamma \)S–G\( \alpha \) was treated with trypsin as described (19); the resulting fragment spans residues 39–389.

**Adenylyl Cyclase Assays.** Synthesis of cAMP was measured for 10 min at 30 \( ^\circ \)C in a final volume of 100 \( \mu \)L as previously described (10). Inhibition kinetics were determined on enzyme assayed with concentration of divalent cation fixed in excess of the ATP concentration as described previously (14, 21).

**Equilibrium Dialysis.** Equilibrium dialysis was performed essentially as described (22). Each chamber contained 20 mM NaHEPES (pH 8.0), 5 mM MgCl\(_2\), 2 mM dithiothreitol, 50 mM NaCl, 2',5'-dd-[\( \gamma^3\)P]-3'-ATP (0.1–12.8 \( \mu \)M), and other additions as indicated. One chamber contained 4 \( \mu \)M active C1a(S591), C2a, and GTP\( \gamma \)S–G\( \alpha \). The opposite chamber contained buffer in lieu of proteins. Samples were removed after dialysis for 24 h at 4 \( ^\circ \)C with liquid scintillation spectrometry.

**Crystallization and Formation of P-Site Complexes.** C1a, C2a, and trypsin-treated G\( \alpha \) were mixed to form a ternary complex in the presence of forskolin (C1a/C2a/G\( \alpha \)) and then purified by gel filtration. Crystals were grown as described previously (2). The crystals were then soaked for various lengths of time at room temperature in harvesting solution (10% PEG 8000, 30% PEG 400, 100 mM MES pH 5.6, 20 mM NaHEPES, pH 8.0, 3.33 mM dithiothreitol, 500 mM NaCl, 200 \( \mu \)M 7-deacetyl-\( \gamma \)-(N-methylpiperezino)-\( \gamma \)-butyryl-forskolin, 166 \( \mu \)M GTP\( \gamma \)S) that also contained various combinations of inhibitors and metal ions. For the 2',5'-dd-3'-AMP-PPi, Mg\(^{2+}\) complex, crystals were soaked in harvesting solution that in addition contained 5.5 mM MgCl\(_2\), 2.9 mM 2',5'-dd-3'-AMP, and 2.9 mM NaPPi, for 1.75 h; for 2',5'-dd-3'-AMP-PPi, Mn\(^{2+}\), 500 \( \mu \)M MgCl\(_2\), 250 \( \mu \)M MnCl\(_2\), 2.9 mM 2',5'-dd-3'-AMP, and 2.9 mM NaPPi, for 1.5 h; for 2',5'-dd-3'-AMP-PPi, Zn\(^{2+}\), 500 \( \mu \)M MgCl\(_2\), 500 \( \mu \)M ZnCl\(_2\), 2.9 mM 2',5'-dd-3'-AMP, and 2.9 mM NaPPi, for 1.5 h; for 2',5'-dd-3'-ATP-Mg\(^{2+}\), 5 mM MgCl\(_2\) and 1 mM 2',5'-dd-3'-ATP for 1 h; for 2',5'-dd-3'-ATP-Mn\(^{2+}\), 250 \( \mu \)M MnCl\(_2\) and 0.5 mM 2',5'-dd-3'-ATP for 1.5 h; for 2',5'-dd-3'-ATP-Zn\(^{2+}\), 250 \( \mu \)M ZnCl\(_2\) and 1.0 mM 2',5'-dd-3'-ATP for 1.5 h. Mg\(^{2+}\) was present in these last two soaks only in residual amounts, as crystals were grown in the presence of MgCl\(_2\).

**Structure Determinations.** Diffraction maxima from each complex were collected on a 2 k × 2 k Quantum 4 CCD area detector at beam line FL at the Cornell High Energy Synchrotron Source (CHESS). The wavelength of the incident beam was 0.921 Å. The data were integrated and reduced using MOSFLM and SCALA of the CCP4 programming suite (23). Data collection statistics are shown in Table 1. Initial phases were generated by molecular replacement using the previously reported 2.8 Å resolution structure of adenylyl cyclase in complex with 2'-d-3'-AMP-PPi, with the inhibitor coordinates omitted from the model (2). After one round of rigid body refinement, Powell minimization and simulated annealing with the program CNS (24), a SIGMAA-weighted \( |F_o| - |F_c| \) omit map was generated to locate and fit the inhibitor and metals in each structure. At this point, three difference Fourier maps were calculated for each P-site inhibitor: \( |F_o| \) − \( |F_c| \), \( |F_o| \) − \( |F_{o \alpha}| \) and \( |F_{o \alpha}| \) where \( |F_{o \alpha}| \) and \( |F_{o |\alpha}| \) are the observed structure factor amplitudes for crystals of the Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) complexes, respectively. Because these maps revealed no significant differences among the three data sets, only the data sets judged to be the best for each inhibitor, based on the quality of the data (merging R factor and \( \langle \sigma^2|q| \rangle \) and electron density map, (2',5'-dd-3'-AMP-PPi, Zn\(^{2+}\) and 2',5'-dd-3'-ATP-Mn\(^{2+}\)) were used in subsequent refinement of the respective models by alternating rounds of Powell minimization, individual B-factor refinement, and model building. In each of these models, both metal A and metal B were refined as Mg\(^{2+}\). Anisotropic overall B-factors and a bulk solvent mask were used throughout refinement. For the 2',5'-dd-3'-ATP-Mn\(^{2+}\) data set, the inhibitor electron density was modeled as a molecule of 2',5'-dd-3'-AMP and a triphosphate group. The \( \beta \) phosphate of 2',5'-dd-3'-AMP and the \( \alpha \) phosphate of the triphosphate occupy the same position in this model. The occupancies of these two groups

\(^2\)R. A. Johnson, unpublished observations.
were set to 50%, and the van der Waals interaction terms between them were omitted during refinement. No residues from either model exhibited main chain conformations corresponding to disallowed regions of the Ramachandran plot. Final refinement statistics are given in Table 1. Coordinates for the 2',3'-AMP-PP, Mg2+ and 2',3'-d-3'-AMP complexes have been deposited in the Protein Data Bank with the codes 1CS4 and 1CUL, respectively.

RESULTS

Crystal Structures of 2',3'-AMP-PP, Complexes. The structure of a complex of C1a-C2a-G μα with 2',3'-AMP-PP-Mg2+ at 2.8 Å resolution was previously reported (2). The inhibitor binds in the interface between the C1a and C2a domains and interacts with conserved residues known to be involved in catalysis, ATP binding, and P-site inhibition (25). It was hypothesized that ATP binds in a similar fashion, and a model of ATP was docked into the active site such that its purine ring superimposed with that of 2',3'-AMP, and its β and γ phosphates superimposed with those of PPγ. This model was later corroborated by site-directed mutagenesis (26, 27) and further refined by the recent structures of C1a-C2a-G μα complexed with the competitive ATP analogues, β1,2',3'-dideoxyadenosine 5'-triphosphate and ATPαS (R₇) (28). In these structures, it was conclusively shown that two metal ions, called A and B, bind in the active site of adenylyl cyclase and that these metal sites have a preference for Zn2+ and Mn2+, respectively, over the presumed physiological metal Mg2+. Zn2+ is an inhibitor of adenylyl cyclase with an IC₅₀ of 15 μM, whereas Mn2+ is an activator that typically increases the activity of C1a-C2a-G μα 10-fold. Metals A and B play important roles in binding ATP and in catalysis. On the basis of modeling studies (28), metal A coordinates and shields the developing negative charge of the leaving group (PPγ). Both metals are expected to stabilize the pentavalent transition state of the reaction by coordinating the pro-R oxygen of the α-phosphate.

Only metal B was observed in previous structure determinations of adenylyl cyclase complexed with 2',3'-AMP-PP. A second metal might be expected to be present because adenylyl cyclase and certain other enzymes that catalyze phosphoryl transfer use a two metal ion mechanism (29, 30). Furthermore, the 3'-phosphate of 2',3'-AMP is in close proximity to PPγ and two aspartic acid side chains and a metal cation seems necessary to complement the excess negative charge. It is possible that the lower resolution (2.8 Å) of the previous structure determination was insufficient to reveal metal A. To examine this issue in more detail, we have determined higher resolution structures of this inhibitor bound to adenylyl cyclase in the presence of three different metal ions. Because Zn2+ preferentially binds in the metal A site when adenylyl cyclase is complexed with ATP analogues (28), Zn2+ was used in one of the structure determinations in an attempt to enhance electron density at site A. The structure of the complex was also determined in the presence of Mn2+. Enhanced density at site B would help explain the lower Kd and increased potency of P-site inhibitors in the presence of this activating cation.

However, metal B is likewise the only metal observed in the three structures of the 2',3'-AMP-PP complex reported here. There are no significant electron density peaks in a difference Fourier map generated between the 2',3'-AMP-PP; Zn2+ and 2',3'-AMP-PP; Mg2+ data sets, and there is no electron density or room in the active site for metal A. There are also no significant electron density peaks in the difference Fourier map between the 2',3'-AMP-PP; Mn2+ and 2',3'-AMP-PP; Mg2+ data sets. This seems to indicate that Mn2+ does not replace Mg2+ at site B in the complex with this inhibitor. Because Mn2+ is more electron dense than Mg2+, any mixture of Mn2+ + Mg2+ at site B should
have resulted in a positive peak in the Mn$^{2+} - \text{Mg}^{2+}$ difference map. The observed lack of difference density indicates that the mole fraction of Mn$^{2+}$ is low at this site. However, the metal ion specificity observed in complexes with competitive inhibitors still leads us to expect that Mn$^{2+}$ activates adenylyl cyclase for P-site inhibition by binding at the metal B site and coordinating PP$\_i$, more tightly than would Mg$^{2+}$. Mn$^{2+}$ could also enhance inhibition by accelerating production of PP$\_i$, the coinhibitor, in addition to lowering the $K_i$ of the inhibitor. We may be unable to observe binding of Mn$^{2+}$ at the metal B site because the C$_{1a}$-C$_{2a}$-G$\alpha\_\alpha$ complex used for our experiments was isolated in the presence of forskolin, and Mn$^{2+}$ has negligible effect on the potency of P-site inhibition under these conditions (see below).

**Biochemical Studies of 2',5'-dd-3'-ATP with Soluble Adenylyl Cyclase.** 2',5'-dd-3'-ATP is one of the most potent P-site inhibitors known with an IC$_{50}$ of 40 nM against native adenylyl cyclase from rat brain in the presence of Mn$^{2+}$ (12, 15). Its mode of inhibition is either noncompetitive or uncompetitive, depending on the conditions of enzyme activation, and 2',5'-dd-[\(\gamma\)-32P]-3'-ATP has been demonstrated to bind directly to native adenylyl cyclases (31).\(^2\) It is important to assess the ability of this ligand to bind and inhibit the recombinant C$_{1a}$ and C$_{2a}$ proteins used for our crystallographic studies and to assess competition with ATP analogues, PP$\_i$, or cAMP.

Inhibition of the G$\alpha\_\alpha$-stimulated activity of the C$_{1a}$ and C$_{2a}$ cytoplasmic domains of adenylyl cyclase by 2',5'-dd-3'-ATP was noncompetitive with respect to Mg$^{2+}$-ATP and exhibited an apparent $K_i$ of 4 \(\mu\)M (Figure 2). The 100-fold decrease in affinity of 2',5'-dd-3'-ATP for the C$_{1a}$-C$_{2a}$ complex relative to that for native enzyme might be attributed to the absence in the latter of a covalent linkage between domains, and of other missing polypeptide segments that may promote interactions between domains in the native enzyme. This kinetic behavior is nevertheless consistent with that demonstrated previously with native and recombinant wild-type enzyme (15). Native and recombinant wild-type adenylyl cyclases exhibit an apparent high affinity for 2',5'-dd-3'-ATP [IC$_{50}$ 40–280 nM (12, 15, 32)], which can be measured by a filtration binding assay using 2',5'-dd-[\(\gamma\)-32P]-3'-ATP (31).\(^2\) However, because of the comparatively high $K_d$ of the C$_{1a}$-C$_{2a}$ complex [$K_d \approx 1 \mu\text{M}$ (14)] and the accordingly higher $K_i$ for inhibition of these domains, equilibrium dialysis was used to quantify binding of 2',5'-dd-[\(\gamma\)-32P]-3'-ATP to the C$_{1a}$-C$_{2a}$ complex in the presence of G$\alpha\_\alpha$ and Mg$^{2+}$ (Figure 3). Binding of 2',5'-dd-[\(\gamma\)-32P]-3'-ATP with the C$_{1a}$-C$_{2a}$ complex in the absence of PP$\_i$ was consistent with a noncompetitive pattern of inhibition. Inclusion of forskolin increased inhibitor binding (Figure 3), which was similar to results previously described for $[^{3}H]$2',5'-dideoxyadenosine 3',5'-adenylyl-(1-methylimidazole)-triphosphate, suggested that the inhibitor interacts with individual C$_{1a}$ and C$_{2a}$ domains (17). However, no binding of 2',5'-dd-[\(\gamma\)-32P]-3'-ATP could be detected by equilibrium dialysis using the individual domains, despite the inclusion of Mg$^{2+}$, Mn$^{2+}$ or Mg$^{2+}$-PP$\_i$.

For enzyme activated with G$\alpha\_\alpha$, binding of 2',5'-dd-[\(\gamma\)-32P]-3'-ATP was approximately 2-fold greater in the presence of Mn$^{2+}$, when it with Mg$^{2+}$ (Figures 3 and 4). However, for enzyme activated with G$\alpha\_\alpha$ and forskolin, Mn$^{2+}$ had a much smaller effect. The $K_d$ for binding 2',5'-dd-[\(\gamma\)-32P]-3'-ATP was significantly lower in the presence of Mn$^{2+}$ (0.2 \(\mu\)M versus 2.4 \(\mu\)M with Mg$^{2+}$), as determined by Scatchard analysis with only a single molecule of inhibitor binding per molecule of adenylyl cyclase (Figure 4).

Competition between 2',5'-dd-3'-ATP and ATP analogues, P-site inhibitors and cAMP are consistent with the binding of 2',5'-dd-3'-ATP at the active site. In contrast, PP$\_i$ enhances binding of the inhibitor (Figures 3 and 5). Consistent with the requirement for PP$\_i$ for inhibition of adenylyl cyclases by adenine nucleosides and adenine nucleoside monophosphates, inhibition of 2',5'-dd-[\(\gamma\)-32P]-3'-ATP binding by 2'-3'-AMP was also enhanced by PP$\_i$ (Figure 5). Further, a Dixon plot of 1/velocity versus the concentration of PP$\_i$, at different fixed concentrations of 2',5'-dd-3'-ATP yields a family of intersecting lines above the [PP$\_i$] axis (Figure 6). The increase in binding noted with PP$\_i$, has consistently been demonstrated in experiments with the C$_{1a}$-C$_{2a}$ complex, as shown here, as well as with native and recombinant wild-type enzyme (31).\(^2\) These observations suggest paradoxically that both 2',5'-dd-3'-ATP and PP$\_i$ bind adenylyl cyclase simultaneously.

**Crystal Structures of 2',5'-dd-3'-ATP Complexes.** As for the three complexes of 2'-3'-AMP-PP$\_i$, no significant peaks appeared in the difference Fourier maps computed with the data set for the 2',5'-dd-3'-ATP-Mg$^{2+}$ complex with respect to that for either 2',5'-dd-3'-ATP-Mn$^{2+}$ or 2',5'-dd-3'-ATP-Zn$^{2+}$. This result appears contrary to the observation that Mn$^{2+}$ enhanced P-site inhibition by 2',5'-dd-3'-ATP (Figures 3 and 4). However, because forskolin was included in the formation of the ternary complex of C$_{1a}$-C$_{2a}$-G$\alpha\_\alpha$, we may be unable to observe binding to the metal B site for Mg$^{2+}$ and that for Mn$^{2+}$ (Figure 3). The absence of any noticeable difference peaks for Zn$^{2+}$ again indicates that the metal A site, if extant, has no apparent preference for either Zn$^{2+}$ or Mn$^{2+}$. Mn$^{2+}$ apparently predominates at both sites, despite the fact that it was not specifically included in the soaks for Zn$^{2+}$ or Mn$^{2+}$. Alternatively, Zn$^{2+}$ or Mn$^{2+}$ may be bound at such a low mole fraction that they are indistinguishable from a fully occupied Mg$^{2+}$.

The electron density omit map for 2',5'-dd-3'-ATP-Mn$^{2+}$ bound to adenylyl cyclase is shown in Figure 1B. A single model of the inhibitor could not be fit into the electron density with reasonable stereochemistry. Therefore, the inhibitor was modeled as the structurally overlapping fragments 2',5'-dd-3'-AMP and triphosphate. The 3' phosphate of 2',5'-dd-3'-AMP and the $\alpha$-phosphate of triphosphate both correspond to the $\alpha$-phosphate of 2',5'-dd-3'-ATP and are partially overlapping. The ligand therefore appears to exhibit static disorder such that it contacts the enzyme either via its triphosphate tail or its nucleoside moiety, but neither at the same time. After refinement of this model for the 2',5'-dd-3'-ATP-Mn$^{2+}$ complex, the average temperature factors of forskolin and GTP$\_S$ (which is bound in the active site of G$\alpha\_\alpha$) are 33 and 32 Å$^2$, respectively. Both are assumed to bind at 100% occupancy. The average temperature factor of the 2',5'-dd-3'-AMP fragment is 37 Å$^2$, suggesting nearly
complete occupancy. Hence, when binding by its nucleoside moiety, the triphosphate tail of 2′,5′-dd-3′-ATP is poorly ordered and probably lies along the same general path as the triphosphate fragment. The average temperature factor for triphosphate is 89 Å², indicating much greater disorder or much lower occupancy.

The fact that 2′,5′-dd-3′-ATP exhibits static disorder upon binding to adenylyl cyclase might reflect the inability of the crystalline enzyme, composed of truncated domains from two adenylyl cyclase isozymes, to attain a “fully closed” conformation. A partially open active site would thus not allow the nucleoside and pyrophosphate moieties of the inhibitor
to bind simultaneously (their respective sites are 1–2 Å too far apart in the refined structure). However, the kinetic data supports the observed static disorder. First, PPi does not compete with, but rather augments binding of 2′,5′-dd-3′-ATP (Figure 3,5). This is consistent with the observed electron density, which suggests that the triphosphate of 2′,5′-dd-3′-ATP binds with low affinity and, therefore, could easily be displaced by PPi. Second, the greater potency of 2′,5′-dd-3′-A4P (IC50 ≈ 7.4 nM) compared with the corresponding 3′-triphasphate (IC50 ≈ 40 nM) (33) is consistent with an active site large enough to accommodate a tetraphosphate. In the active site of the 2′,5′-dd-3′-ATP complex, there is room for a fourth phosphate. This would not be possible if the active site were compressed such that it could accommodate 2′,5′-dd-3′-ATP in a single binding mode.

After refinement of the model for the statically disordered 2′,5′-dd-3′-ATP complex, a weak (1–2 standard deviations above the mean value of the map) peak was observed in the difference Fourier map close to the position of metal A seen in complexes with ATP analogues. The peak was modeled as Mg2+ and is located 2 Å from the oxygen substituents of both the 3′-phosphate of 2′,5′-dd-3′-AMP fragment and the triphosphate α-phosphate. The metal is also coordinated by D396 and D440 (Figure 1B). Thus, the number of observed ligands is less than four. Poorly ordered water molecules could serve as additional ligands.

**Figure 2:** Noncompetitive inhibition of Gα-stimulated adenylyl cyclase activity by 2′,5′-dd-3′-ATP. Assays [0.7 nM C1α(591), 1 μM C2α, 0.4 μM GTPγS–Gα] were performed in the presence of 10 mM free MgCl2, 0.02–2.56 mM MgATP, and either 0 (●), 1 (■), 3 (▲), or 6 (▼) μM 2′,5′-dd-3′-ATP. The apparent Kᵢ (4 μM) was determined by Lineweaver-Burk analysis.

**Figure 3:** Binding of 2′,5′-dd-[γ-32P]-3′-ATP to adenylyl cyclase. The individual proteins (10 μM) C1α(591), C2α, or GTPγS–Gα were incubated with 1.5 μM 2′,5′-dd-[γ-32P]-3′-ATP for 24 h at 4°C. Equilibrium dialysis was performed in the presence of either 4 mM MgCl2, 2 mM MnCl2, or 4 mM MgCl2 and 2 mM PPi. Binding of 2′,5′-dd-[γ-32P]-3′-ATP was also measured in the presence of all three proteins [2 μM C1α(591), C2α, and GTPγS–Gα] in the presence or absence of 100 μM forskolin. Results shown are representative of two experiments.

**Figure 4:** Binding of 2′,5′-dd-[γ-32P]-3′-ATP to adenylyl cyclase in the presence of Mg2+ or Mn2+. C1α, C2α, and GTPγS–Gα were incubated with 0.1–12.8 μM 2′,5′-dd-[γ-32P]-3′-ATP for 24 h as described under Experimental Procedures. (A) equilibrium dialysis reactions contained 4 μM of each protein and 5 mM MgCl2. (B) reactions contained 2 μM of each protein and 2.5 mM MnCl2. Dissociation constants were calculated by Scatchard analysis (insets). The relative amount of bound 2′,5′-dd-3′-ATP (R) represents the total concentration of bound ligand divided by the total concentration of active adenylyl cyclase. These results are representative of three experiments.
FIGURE 5: Competition for the binding of 2′,5′-d-[γ-32P]-3′-ATP to adenylyl cyclase. C₅₉(591), C₅₉, and GTPγS–Gₛα (4 μM) were incubated with 0.08 μM 2′,5′-d-[γ-32P]-3′-ATP in the presence of 5 mM MgCl₂. Competition for binding by equilibrium dialysis was performed with 6 mM Mg²⁺-AP(CH₂)₃PP, 25 mM cAMP, 2 mM Mg²⁺-PP, 400 μM 2′-d-3′-AMP, or 2′-d-3′-AMP (400 μM) and Mg²⁺-PP (2 mM). Results shown are representative of three experiments.

DISCUSSION

The crystal structures reported here allow speculation on the molecular basis of the relative potency of P-site inhibitors. All P-site inhibitors require an intact adenine ring, reflecting the fact that their purine rings use the same binding pocket as the substrate ATP. The removal of 2′ and 5′ hydroxyl groups from P-site ligands enhances their potency by 7-200-fold, depending on other modifications (4, 12, 15). These deletions remove potential collisions of the 2′-hydroxyl with nearby protein superstructure. A 2′-hydroxyl group in the structure of the 2′-d-3′-AMP·PP₃ complex (Figure 1A) would collide with the side chain of D440 and the backbone carbonyl of residue 438. This may be reflected in the reduced potency of 3′-AMP (IC₅₀ ≈ 9 μM) compared with 2′-d-3′-AMP (IC₅₀ ≈ 2.7 μM) reported for native enzyme (4). Ribose rings with less substitution are inherently more flexible than their fully substituted counterparts. Therefore, 2′-deoxy and 2′,5′-dideoxy ribose groups and especially acyclic adenine derivatives (34) would allow the inhibitor to adopt conformations that better conform to the active site. Such flexibility may be especially important when the inhibitor has a bulky 3′-substitution as does 2′,5′-dd-3′-ATP.

Comparison of the 2′-d-3′-AMP·PP₃ structure with that of 2′,5′-dd-3′-ATP yields further insights (Figure 1C). Compounds with ribose rings lacking 5′-hydroxyl groups inhibit 2-7 times more efficaciously than their substituted counterparts. From the structures reported here, the removal of a 5′-oxygen allows the ribose ring to slip further back into the nucleoside binding pocket, where the 5′-methylene groups packs against helix α₄ of Cₛ₉. This interaction is apparently more favorable than maintenance of a hydrogen bond between the 5′-hydroxyl and the side chain of Thr401, as occurs in the 2′-d-3′-AMP·PP₃ structure. Adenine-nucleoside 3′-polyphosphates are the most potent P-site ligands, with the successive addition of phosphate groups to the 3′-position adding substantially to potency: 2′,5′-dd-3′-AMP (IC₅₀ ≈ 460 nM), 2′,5′-dd-3′-ATP (IC₅₀ ≈ 40 nM); 2′,5′-dd-3′-A₄P (IC₅₀ ≈ 7.4 nM) with native enzyme (12, 15, 33). These potencies are consistent with each successive phosphate contributing ~2-4 kcal of binding energy. On the basis of the 2′,5′-dd-3′-ATP structure reported here, 3′-polyphosphate inhibitors are statically disordered in the active site with the 3′-polyphosphate being most poorly ordered. However, 3′-polyphosphates could enhance inhibition in a number of other ways besides contribution of binding energy. First, the polyphosphate group can interact with and neutralize the positive charges in the active site. Second, the polyphosphate could stabilize the enzyme in a closed conformation required for tight binding of the adenosine moiety of the inhibitor. Finally, 3′-polyphosphate moieties can be thought of as covalently linked PP₃ “tags”. P-site inhibitors that possess such tags will have an entropic advantage, arising from approximation of functional moieties, over inhibitors that require binding of two separate molecules (e.g., 2′-d-3′-AMP·PP₃) for effective inhibition.

The α-phosphate group of 2′,5′-dd-3′-ATP occupies a different position from that in 2′-d-3′-AMP (Figure 1C). As a consequence, the α-phosphate of the latter inhibitor occludes metal ion site A. Therefore, P-site inhibitors with 3′-polyphosphates could also be more potent because they bind concurrently with two metal ions. The extra metal helps to neutralize excess negative charge in the active site such as that found in the 2′-d-3′-AMP·PP₃ complex. The weak electron density observed for metal A in the 2′,5′-dd-3′-ATP complex with that 2′,5′-dd-3′-ATP structure reported

FIGURE 6: Dixon plot of PP₃ and 2′,5′-dd-3′-ATP inhibition of adenylyl cyclase activity. Assays [4 nM C₅₉(591) and 1 μM Cₛ₉] were performed in the presence of 10 mM free MgCl₂, 1 mM ATP, and 400 nM activated Gₛα. The indicated concentrations of Mg–PP₃ and 2′,5′-dd-3′-ATP were added and the reaction was incubated at 30 °C for 10 min.
of 2',5'-dd-[γ-32P]-3'-ATP suggests that this may occur. In this scenario, PPi would displace the poorly ordered β and γ phosphates of 2',5'-dd-3'-ATP. Alternatively, synergistic activity of PPi could be due to hysteretic behavior of the enzyme, such that the active conformation promoted by PPi might persist after that ligand dissociates and thereby favor subsequent binding of 2',5'-dd-3'-ATP.

In contrast, 2',5'-dd-3'-ATP in the presence of Mn²⁺ could be classified as an inactivator of adenylyl cyclase. If the binding of 2',5'-dd-3'-ATP-Mn²⁺ traps a fraction of adenylyl cyclase in a dead-end complex, an apparent noncompetitive pattern of inhibition will result because the effective total enzyme concentration (and hence \( v_{\text{max}} \)) is reduced while \( K_m \) is unchanged.

It seems clear from its uncompetitive mode of inhibition, its requirement for PPi, and its ability to inhibit the reverse reaction of adenylyl cyclase competitively that the typical P-site inhibitor is essentially a cAMP analogue that binds to the enzyme before the second product PPi, is released (14). By comparison, P-site inhibitors with 3'-polyphosphates appear to function as covalently linked analogues of cAMP and PPi, whose greater affinity arises in part from their inherent entropic benefits. By superimposing the structure of cAMP onto that of 2'-d-3'-AMP (Figure 1D), we can generate a reasonable model for the enzyme-substrate complex (28). Unlike P-site inhibitors, which adopt 2'-endo, 3'-exo ribose conformations, cAMP is constrained in the 3'-endo, 2'-exo conformation. To facilitate nucleophilic attack of the 3'-hydroxyl upon the 5'-phosphate, ATP must also adopt a 3'-endo conformation in the activated complex, and its polyphosphate backbone must adopt a conformation that brings O3' within van der Waals contact distance of the 5'-phosphate. This may engender some steric strain. The 3'-polyphosphate backbone of 2'-d-3'-ATP and the 3'-monophosphate of 2'-d-3'-AMP in conjunction with PPi are able to occupy the active site without internal strain and therefore are efficacious inhibitors. These models, developed with truncated chimeric constructs, provide an excellent starting point for the rational development of conformation-selective drugs that are specific for native adenylyl and guanylyl cyclases.

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**REFERENCES**