Exchange of Substrate and Inhibitor Specificities between Adenylyl and Guanylyl Cyclases*

(Received for publication, February 27, 1998)

Roger K. Sunahara‡‡, Annie Beuve‡, John J. G. Tesmer¶, Stephen R. Sprang¶¶, David L. Garbers§§, and Alfred G. Gilman‡‡‡

From the ‡Department of Pharmacology, the ¶Howard Hughes Medical Institute, and the §§Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235

The active sites of guanylyl and adenylyl cyclases are closely related. The crystal structure of adenylyl cyclase and modeling studies suggest that specificity for ATP or GTP is dictated in part by a few amino acid residues, invariant in each family, that interact with the purine ring of the substrate. By exchanging these residues between guanylyl cyclase and adenylyl cyclase, we can completely change the nucleotide specificity of guanylyl cyclase and convert adenylyl cyclase into a nonselective purine nucleotide cyclase. The activities of these mutant enzymes remain fully responsive to their respective stimulators, sodium nitroprusside and GTP.

Adenylyl cyclases and guanylyl cyclases synthesize the intracellular second messengers cAMP and cGMP, respectively, in response to a variety of regulatory signals. Mammalian adenylyl cyclases are intrinsic plasma membrane proteins that contain a duplicated module consisting of a hexahedral transmembrane region followed by a roughly 40-kDa cytosolic domain (1). Highly conserved and homologous sequences within the two cytosolic domains contribute to the active site of the enzyme (2). We have previously produced a soluble and catalytically active form of adenylyl cyclase that consists of the C1α domain from type V adenylyl cyclase and the C2 domain from the type II enzyme (3). This VC1/IIC2 heterodimer retains the regulatory features that are shared by membrane-bound adenylyl cyclases, including activation by the G protein subunit Gαi2 or the diterpene forskolin, as well as inhibition by so-called P-site inhibitors such as 2’d3’AMP (3, 4). We have also solved the x-ray crystal structure of this soluble enzyme complexed with both activators and inhibitors (5).

Guanylyl cyclases exist as both soluble and membrane-bound species. Both types of the enzyme contain cytoplasmic domains similar to those of the adenylyl cyclases (6). Membrane-bound guanylyl cyclases are homodimers, whereas the soluble enzymes contain α and β subunits, both of which are required for catalysis (7, 8). Each subunit contains a carboxyl-terminal domain that is homologous to the C1 and C2 domains of adenylyl cyclase; α most closely resembles C1 while β more closely resembles C2. Binding of nitric oxide (NO) to a prosthetic heme group (possibly located within the amino-terminal domain of β (9)) causes marked activation of soluble guanylyl cyclases.

Adenylyl cyclases and guanylyl cyclases show high specificity for their respective substrates; this is obviously essential for the coexistence and fidelity of signaling pathways, since virtually all cells contain both enzymes. The crystal structure of adenylyl cyclase with bound 2’d3’AMP reveals the amino acid residues that interact with the adenine ring of the inhibitor (5). Although P-site inhibitors are not competitive with respect to substrate, substantial evidence indicates that these compounds do bind at the active site (5, 10). Lyn-938 and Asp-1018, both within the C2 domain of adenylyl cyclase, are invariant residues that form hydrogen bonds with the N-1 and N-6 atoms of the adenine ring of 2’d3’AMP and are thus modeled to do so with ATP (see Fig. 1A). Their equivalents in the guanylyl cyclase family, also invariant, are Glu and Cys, respectively, which could interact with the N-2 and O-6 atoms of a guanine ring (see Fig. 1B and C). Modeling studies based on the crystal structure of the adenylyl cyclase IIC2 homodimer (11, 12) also predict that an invariant Arg residue may be important for substrate recognition by guanylyl cyclase; its equivalent in adenylyl cyclase is Gln (see Fig. 1). We describe herein the effects of exchanging these three residues in various combinations between the two purine nucleotide cyclases. We used the soluble, recombinant VC1/IIC2 form of adenylyl cyclase described above and soluble, recombinant αβ1 guanylyl cyclase from rat (13).

MATERIALS AND METHODS

Mutagenesis—cDNAs encoding the α and β subunits of rat guanylyl cyclase (13), cloned into the mammalian expression vector pCMV5 (14), served as the templates for site-directed mutagenesis (Quickchange, Stratagene). Templates for adenylyl cyclase were pQ6E60-H6-VC (364–591)Flag (3) and pQ6E60-IIC2-H2 (15). Sequences of synthetic mutagenic sense and antisense primers are available on request.

Expression of and Analysis of Guanylyl Cyclase (α and β) in COS-7 Cells—COS-7 cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (100 units/ml). Cells were transfected with SuperFectTM reagent using the protocol of the supplier (Qiagen). To screen guanylyl cyclase mutants in intact cells, COS-7 cells were plated in 12-well plates and transfected at 60% confluency with 1.5 μg each of plasmids encoding wild-type or mutant
The model for ATP was based on the structure of G\(\alpha\)-VC1, IIC2, complexed with 2\(\delta\)S AMP (see “Materials and Methods”). Putative hydrogen bonds are shown as dotted lines. Protein carbons are gray, nitrogens are blue, oxygens are red, phosphorus atoms are green, and sulfurs are yellow. Magnesium is shown as a metallic sphere. Carbons in ATP are copper, B, stick model of the guanine binding pocket of soluble guanylyl cyclase.

The coloring scheme is the same as that for panel A except that carbon atoms of residues that belong to the \(\alpha\) subunit and their labels are colored mauve. Carbon atoms belonging to the \(\beta\) subunit are gray. Panels A and B were created with MOLSCRIPT (26) and rendered with POVRAY (27). C, structure-based alignment of highly conserved amino acid sequences that contribute to the purine binding pocket of selected adenylyl cyclases and guanylyl cyclases.

The alignment is restricted to sequences that correspond to the regions targeted by mutagenesis in adenylyl cyclases and guanylyl cyclases. The alignment is restricted to acid sequences that contribute to the purine binding pocket of selected C, POVRAY (27).

Metallic sphere

Putative hydrogen bonds are shown as red, phosphorus atoms are green, and sulfurs are yellow. Magnesium is shown as a metallic sphere.

Cyclic nucleotide phosphodiesterases. This medium (360 mM KCl, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) was then collected by centrifugation at 100,000 × g for 20 min at 4 °C.

**FIG. 1.** Tertiary and primary structures of the purine binding pockets of adenylyl guanylyl cyclase. A, stick model of the adenine binding pocket in adenylyl cyclase. The model for ATP was based on the structure of G\(\alpha\)-VC1, IIC2, complexed with 2\(\delta\)S AMP (see “Materials and Methods”). Putative hydrogen bonds are shown as dotted lines. Protein carbons are gray, nitrogens are blue, oxygens are red, phosphorus atoms are green, and sulfurs are yellow. Magnesium is shown as a metallic sphere. Carbons in ATP are copper, B, stick model of the guanine binding pocket of soluble guanylyl cyclase. The coloring scheme is the same as that for panel A except that carbon atoms of residues that belong to the \(\alpha\) subunit and their labels are colored mauve. Carbon atoms belonging to the \(\beta\) subunit are gray. Panels A and B were created with MOLSCRIPT (26) and rendered with POVRAY (27). C, structure-based alignment of highly conserved amino acid sequences that contribute to the purine binding pocket of selected adenylyl cyclases and guanylyl cyclases. The alignment is restricted to sequences that correspond to the regions targeted by mutagenesis in this study. The G\(\alpha\) and C\(_\beta\) domains of adenylyl cyclases and the \(\alpha\) and \(\beta\) subunits of soluble guanylyl cyclases are all homologous and thus are aligned together. The \(\alpha\) subunit of soluble guanylyl cyclase is more closely related to the C\(_\beta\) domain of adenylyl cyclase, whereas \(\beta\) is more closely related to the G\(\alpha\) domain. The top three sequences in the alignment correspond to G\(\alpha\) or \(\alpha\) domains, and the lower three correspond to C\(_\beta\) or \(\beta\) domains. Guanylyl cyclase A (GCA) is a membrane-bound, homodimeric enzyme (28). Secondary structures are drawn above the alignment as per the structure of G\(\alpha\)-VC1, IIC2 (5). Arrows represent \(\beta\) strands, and coils depict \(\alpha\) helices. Colored amino acids correspond to residues modified by mutagenesis in this study; green amino acids are those that are invariant in adenylyl cyclases and red amino acids are those invariant in guanylyl cyclases.

\(\alpha\), and \(\beta\) subunits. After 40 h, cells were placed on ice and washed once with phosphate-buffered saline and once with DMEM containing 50 mM NaHepes (pH 7.4) and 1 mM 1-methyl-3-isobutylxanthine (an inhibitor of cyclic nucleotide phosphodiesterases). This medium (360 mM) was then added to each well, and plates were warmed for 5 min at 37 °C prior to exposure for 15 min to 40 µl of DMEM with or without 1 mM sodium nitroprusside. The incubation was terminated by addition of 40 µl of 33% perchloric acid, and the amount of cGMP or cAMP in the perchlorate extract was measured by radioimmunoassay (16).

To prepare COS-7 cell cytosol for assay of guanylyl cyclase activity, cells (100-mm dish) were transfected with 10 µg of plasmid encoding each wild-type or mutant subunit. After 40 h, cells were washed twice with 5 ml of phosphate-buffered saline and sonicated in 0.5 ml of homogenization buffer (50 mM Tris-HCl, pH 8.0 at 4 °C, 150 mM NaCl, 2 mM diithiothreitol, and protease inhibitors). The cytosolic fraction was collected by centrifugation at 100,000 × g for 20 min at 4 °C.

**FIG. 2.** Intact cells screen for mutants of soluble guanylyl cyclase that exhibit adenylyl cyclase activity. The top panel illustrates the effect of sodium nitroprusside (SNP) on cellular concentrations of cGMP in COS-7 cells transfected either with no DNA, with soluble guanylyl cyclase (a-wt/\(\beta\)-wt), or with different combinations of wild-type and mutant subunits. The bottom panel documents accumulation of cAMP in the same cells. Data shown are representative of three experiments.

**Exchange of Substrate Specificity of Nucleotide Cyclases**

**Materials and Methods**

To prepare COS-7 cell cytosol for assay of guanylyl cyclase activity, cells (100-mm dish) were transfected with 10 µg of plasmid encoding each wild-type or mutant subunit. After 40 h, cells were washed twice with 5 ml of phosphate-buffered saline and sonicated in 0.5 ml of homogenization buffer (50 mM Tris-HCl, pH 8.0 at 4 °C, 150 mM NaCl, 2 mM diithiothreitol, and protease inhibitors). The cytosolic fraction was collected by centrifugation at 100,000 × g for 20 min at 4 °C.

**Purification of Adenylyl Cyclase Fragments and G\(\alpha\)---Wild-type and mutant VC1, and IIC2 fragments of adenylyl cyclase were synthesized in Escherichia coli, and the proteins were purified by metal chelate (Talon, CLONTECH or Ni-NTA, Qiagen) and ion exchange (MonoQ, Amersham Pharmacia Biotech) chromatography (3, 15). Trypsinized GTP-P\(\gamma\)S-G\(\alpha\) was purified as described (17). Tryptic digestion of GTP-P\(\gamma\)S-G\(\alpha\) ensures inactivation of any GDP-G\(\alpha\) in the preparation and thus reduces the possibility of GTP hydrolysis by this protein.

**Enzyme Assays**

The adenylyl cyclase activity of adenylyl cyclase and guanylyl cyclase proteins was measured for 10–15 min at 30 °C in a final volume of 100 µl as described (18). Unless stated, the final concentrations of trypsinized GTP-P\(\gamma\)S-G\(\alpha\), ATP, and sodium nitroprusside were 400 nM, 1 mM, and 100 µM, respectively. An ATP regeneration system was not utilized when purified proteins were assayed (wild-type and mutant adenylyl cyclase preparations).

Guanylyl cyclase activity was determined as described (16). A GTP regeneration system was not utilized for purified preparations of adenylyl cyclases. Assays were performed in a volume of 100 µl for 15 min at 30 °C. The concentration of GTP was 100 µM for wild-type guanylyl cyclases, 500 µM for mutant guanylyl cyclases, and 1 mM for adenylyl cyclases, unless otherwise stated.

**Determination of K\(_m\) and V\(_{max}\)**

Determination of K\(_m\) and V\(_{max}\) were performed with substrate concentrations ranging from 5 to 3000 µM. All assays were performed in duplicate, and each experiment was repeated 2–4 times.

**SDS/PAGE and Western Blot Analysis**

Protein concentrations...
were determined as described (19). For purified adenylyl cyclases, 1 mg of protein was resolved on a 15% SDS-PAGE gel and visualized with Coomassie Blue. Cytosolic fractions (20 mg) containing guanylyl cyclases were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose. Transferred proteins were probed with antibodies specific for the a1 (F563) or b1 (A794) subunits of guanylyl cyclase and detected by chemiluminescence.

Modeling of Enzyme-Substrate Complexes—The model of ATP bound to VC1zIIC2 was based on the crystal structure of the GsazVC1zIIC2 complex containing bound 29d3AMP and pyrophosphate (5). The purine bases of ATP and 29d3AMP were superimposed, and the b and g phosphates of ATP were placed close to the phosphates of pyrophosphate. The a-phosphate and ribose of ATP was positioned such that the 39-hydroxyl of ATP could displace pyrophosphate by in-line attack. The ribose is in the 39-endo conformation. The structure was then subjected to harmonically restrained molecular dynamics with simulated annealing starting at 2500K (20) and conventional conjugate gradient refinement in crystallography and NMR systems 0.3 (21).

The model of GTP bound to soluble guanylyl cyclase was created by manually replacing and optimally fitting the side chain residues in the crystal structure of the VC1zIIC2 complex with their equivalents in guanylyl cyclase using the program O, Version 6.1 (22). ATP from the model of adenylyl cyclase was changed to GTP, and the structure was refined as described above.

RESULTS AND DISCUSSION

The crystal structure of GsazVC1zIIC2 containing bound 2d3AMP suggests that the purine ring of ATP binds in a hydrophobic pocket containing three elements that confer specificity for adenosine: Lys-938, Asp-1018, and the backbone carbonyl of residue 1019 (Fig. 1A). The a1-atom of Lys-938 interacts with the N-1 nitrogen of ATP and forms a salt bridge with Asp-1018. The backbone carbonyl of residue 1019 forms hydrogen bonds to the N-6 amino group of ATP. Lys-938 and Asp-1018 are invariant among all adenylyl cyclases, and alanine scanning mutagenesis...

were determined as described (19). For purified adenylyl cyclases, 1 mg of protein was resolved on a 15% SDS-PAGE gel and visualized with Coomassie Blue. Cytosolic fractions (20 mg) containing guanylyl cyclases were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose. Transferred proteins were probed with antibodies specific for the a1 (F563) or b1 (A794) subunits of guanylyl cyclase and detected by chemiluminescence.

Modeling of Enzyme-Substrate Complexes—The model of ATP bound to VC1zIIC2 was based on the crystal structure of the GsazVC1zIIC2 complex containing bound 29d3AMP and pyrophosphate (5). The purine bases of ATP and 29d3AMP were superimposed, and the b and g phosphates of ATP were placed close to the phosphates of pyrophosphate. The a-phosphate and ribose of ATP was positioned such that the 39-hydroxyl of ATP could displace pyrophosphate by in-line attack. The ribose is in the 39-endo conformation. The structure was then subjected to harmonically restrained molecular dynamics with simulated annealing starting at 2500K (20) and conventional conjugate gradient refinement in crystallography and NMR systems 0.3 (21).

The model of GTP bound to soluble guanylyl cyclase was created by manually replacing and optimally fitting the side chain residues in the crystal structure of the VC1zIIC2 complex with their equivalents in guanylyl cyclase using the program O, Version 6.1 (22). ATP from the model of adenylyl cyclase was changed to GTP, and the structure was refined as described above.

RESULTS AND DISCUSSION

The crystal structure of GsazVC1zIIC2 containing bound 2d3AMP suggests that the purine ring of ATP binds in a hydrophobic pocket containing three elements that confer specificity for adenosine: Lys-938, Asp-1018, and the backbone carbonyl of residue 1019 (Fig. 1A). The a1-atom of Lys-938 interacts with the N-1 nitrogen of ATP and forms a salt bridge with Asp-1018. Both the acid side chain of Asp-1018 and the backbone carbonyl of residue 1019 form hydrogen bonds to the N-6 amino group of ATP. Lys-938 and Asp-1018 are invariant among all adenylyl cyclases, and alanine scanning mutagenesis...
Exchange of Substrate Specificity of Nucleotide Cyclases

We have mutated residues αR592, βE473, and βC541 of guanylyl cyclase to their equivalents in adenylyl cyclase, singly and in combinations, to determine their contributions to substrate specificity. The three single mutants, the three double mutants, and the triple mutant were tested for their capacity to support cyclic nucleotide synthesis in vivo, and the best candidates were further characterized in cellular extracts in vitro. Likewise, Gln-503 in the C$_2$ domain of type V adenylyl cyclase and residues Lys-938 and Asp-1018 of the C$_2$ domain of type II adenylyl cyclase were mutated to their equivalents in guanylyl cyclase and characterized as purified proteins in a reconstituted system in vitro. Note that major differences in the specific activities between adenylyl cyclase and guanylyl cyclase proteins are due in large part to the fact that guanylyl cyclases were assayed in cytosolic extracts, whereas adenylyl cyclases were assayed as purified proteins.

In Vivo Screen of Guanylyl Cyclase Mutants—Guanylyl cyclase mutants were screened in transfected COS-7 cells to detect alterations in nucleotide specificity (Fig. 2; Table I). As expected, cells transfected with wild-type guanylyl cyclase (α-wt/β-wt) accumulated high concentrations of cGMP following exposure to sodium nitroprusside (Fig. 2, top panel). Of the seven mutant combinations tested, only COS-7 cells transfected with the double mutant αR592Q/βC591D (α-Q/β-D) retained the capacity to accumulate substantial concentrations of cGMP. However, we do not know if properly folded protein accumulated in all cases.

Significant adenylyl cyclase activity was detected for only one of the single mutants, βC541D (α-wt/β-D) (Fig. 2, bottom panel). When double mutants were tested, cells expressing both βE473K/βC541D (α-wt/β-KD) and α-Q/β-D accumulated cAMP; α-Q/β-D thus appears to be a nonselective purine nucleoside cyclase. The greatest accumulation of cAMP was observed in cells bearing the triple mutant, αR592Q/βE473K/βC541D (α-Q/β-KD). Mutants α-Q/β-KD and α-wt/β-KD were further characterized in vitro. Mutants α-wt/β-D and α-Q/β-D failed to demonstrate reproducible adenylyl cyclase activity in vitro, apparently because of instability of the β-D subunit (data not shown). The βC541D mutation introduces an acidic side chain next to the glutamic acid residue at position 473, which may destabilize the β subunit and the interface between α and β.

The counterparts of Lys-938 and Asp-1018 in guanylyl cyclase are also invariant within their respective families. In the structure of Gs, Glu and Cys, respectively, might alter the purine specificity of the enzyme; similarly, mutation of βE473 and βC541 in guanylyl cyclase to Lys and Asp, respectively, might enable the enzyme to synthesize cAMP. A model of the active site of guanylyl cyclase is shown in Fig. 1B.

Gln-503 in the C$_1$ domain of adenylyl cyclase and αR592 in guanylyl cyclase are also invariant within their respective families. In the structure of Gs, Gln forms van der Waals contacts across the heterodimer interface and with the side chain of Lys-938 (Fig. 1A). However, in guanylyl cyclase, αR592 may also form a salt bridge to βE473 and thereby stabilize and properly orient the glutamic acid side chain (Fig. 1B). αR592 was also implicated in GTP binding by modeling studies based on the structure of the IIC$_2$ homodimer (11).

When double mutants were tested, cells expressing both βE473K/βC541D (α-wt/β-KD) and α-Q/β-D accumulated cAMP; α-Q/β-D thus appears to be a nonselective purine nucleoside cyclase. The greatest accumulation of cAMP was observed in cells bearing the triple mutant, αR592Q/βE473K/βC541D (α-Q/β-KD). Mutants α-Q/β-KD and α-wt/β-KD were further characterized in vitro. Mutants α-wt/β-D and α-Q/β-D failed to demonstrate reproducible adenylyl cyclase activity in vitro, apparently because of instability of the β-D subunit (data not shown). The βC541D mutation introduces an acidic side chain next to the glutamic acid residue at position 473, which may destabilize the β subunit and the interface between α and β.

**Kinetic Analysis of Guanylyl Cyclase Proteins α-Q/β-KD and α-wt/β-KD—α-Q and β-KD subunits were expressed in cytosolic fractions of COS-7 cells at levels comparable with those of the wild-type proteins (Fig. 3A). In agreement with the in vivo screen, significant nitroprusside-stimulated adenylyl cyclase activity was observed in the cytosolic fractions for both α-Q/β-KD and α-wt/β-KD, but not native guanylyl cyclase (Fig. 3B). The low adenylyl cyclase activity observed for α-wt/β-KD in vitro, compared with its apparent activity in vivo (Fig. 2, bottom panel), is due in part to its poor Km for ATP (see Fig. 4B and Table I); a fraction of the protein may also be inactive. No significant synthesis of cGMP was detected in cytosolic fractions from cells transfected with the two mutant proteins.**

**Fig. 4. Kinetic analysis of guanylyl cyclase mutants.** A, cyclic GMP (●) or cAMP (○) synthesis was measured in response to increasing concentrations of sodium nitroprusside (SNP) for α-wt/β-wt (■) or α-Q/β-KD (●) guanylyl cyclases, respectively. Enzymatic activity was measured at 100 μM GTP (α-wt/β-wt) or 500 μM GTP (α-Q/β-KD). B, synthesis of cAMP by native guanylyl cyclase is shown in the inset. Guanylyl cyclases are also invariant within their respective families. In the structure of Gs, Glu and Cys, respectively, might alter the purine specificity of the enzyme; similarly, mutation of βE473 and βC541 in guanylyl cyclase to Lys and Asp, respectively, might enable the enzyme to synthesize cAMP. A model of the active site of guanylyl cyclase is shown in Fig. 1B.

Gln-503 in the C$_1$ domain of adenylyl cyclase and αR592 in guanylyl cyclase are also invariant within their respective families. In the structure of Gs, Glu and Cys, respectively, might alter the purine specificity of the enzyme; similarly, mutation of βE473 and βC541 in guanylyl cyclase to Lys and Asp, respectively, might enable the enzyme to synthesize cAMP. A model of the active site of guanylyl cyclase is shown in Fig. 1B.

Gln-503 in the C$_1$ domain of adenylyl cyclase and αR592 in guanylyl cyclase are also invariant within their respective families. In the structure of Gs, Glu and Cys, respectively, might alter the purine specificity of the enzyme; similarly, mutation of βE473 and βC541 in guanylyl cyclase to Lys and Asp, respectively, might enable the enzyme to synthesize cAMP. A model of the active site of guanylyl cyclase is shown in Fig. 1B.


have similar catalytic efficiencies with their respective substrates. Thus, the α-Q/β-KD triple mutant represents a complete change of nucleotide specificity from GTP to ATP with preservation of both sensitivity to nitroprusside and catalytic efficiency.

In Vitro Analysis of Purified Mutants of Adenylyl Cyclase—The native IIC2 domain of adenylyl cyclase (C2-wt), the single mutants K938E (C2-E) and D1018C (C2-C), and the double mutant K938E/D1018C (C2-EC) were synthesized in E. coli, purified to homogeneity, and mixed with either purified native VC1 (C1-wt) or the mutant Q503R (C1-R) prior to assay. The yields of C2-E and C2-C were approximately 1 and 3% of the values achieved with native IIC2 (data not shown). Only complexes containing C1-wt or C1-R and C2-EC are capable of synthesizing significant amounts of cGMP (Fig. 5B). The double mutant C1-wt/C2-EC yielded the highest ratio of guanylyl cyclase to adenylyl cyclase specific activity, but this was only 0.32 (Table I, Figs. 5 and 6). Adenylyl cyclase activity is reduced for some mutants (10-fold or more) but is not eliminated in any of them. The EC_{50} for Gsα-stimulated cGMP synthesis by C1-wt/C2-EC is approximately 200 nM (Fig. 6A), the same as that for cyclic AMP synthesis by the wild-type protein (Fig. 6A). At 400 nM effective concentrations of Gsα, C1-wt/C2-EC has a \( K_m \) for GTP of 450 μM and a \( V_{max} \) of 0.67 μmol/min/mg. With ATP as the substrate, the corresponding values are 630 μM and 2.1 μmol/min/mg, respectively (Table I). Although the \( K_m \) for ATP for the double mutant is similar to that of wild-type enzyme, \( V_{max} \) is reduced about 15-fold. Thus, C1-wt/C2-EC is a nonselective purine nucleotide cyclase with less catalytic efficiency for either ATP or GTP than that displayed by wild-type adenylyl cyclase with ATP.

The C1-R/C2-EC triple mutant of adenylyl cyclase mirrors the guanylyl cyclase mutant (α-R/β-KD) where nucleotide specificity was effectively switched. C1-R/C2-EC has significant guanylyl cyclase activity (Fig. 5B) but retains most of the adenylyl cyclase activity characteristic of the wild-type protein. Either specificity for adenine in adenylyl cyclase is not entirely dictated by the targeted residues or the mutations somehow open the binding pocket, permitting purine nucleotides to bind nonselectively.

Effects of P-site Inhibitors—2′d3′AMP inhibits adenylyl cyclase with an EC_{50} of approximately 30 μM (Fig. 7D). Inhibition is uncompetitive or noncompetitive with respect to ATP (depending on assay conditions), and P-site inhibitors act by binding to and stabilizing the complex of the enzyme with one of its products, pyrophosphate (10). An intact adenine ring is required for effective inhibition. Kinetic analysis, binding studies (10), and the crystal structure of adenylyl cyclase complexed with 2′d3′AMP and pyrophosphate (5) strongly suggest that the inhibitor occupies the same purine binding pocket as does ATP. Nevertheless, some have hypothesized two binding sites for adenine nucleotides on adenylyl cyclase (24), and these hypotheses are fueled by the pseudo-symmetrical structure of the enzyme.

If the triple mutant of guanylyl cyclase is indeed an enzyme with fully altered nucleotide specificity for substrate and if P-site inhibitors truly act at the active site, we expect a similar change of specificity for the inhibitors. Wild-type guanylyl cyclase is effectively inhibited by 2′d3′GMP; the adenylyl cyclase activity of the triple mutant of guanylyl cyclase is not (Fig. 7A). Wild-type guanylyl cyclase is not inhibited by 2′d3′AMP; the guanylyl cyclase activity of the triple mutant of guanylyl cyclase is so inhibited (Fig. 7B). Inhibition of this activity by 2′d3′AMP is not competitive with respect to ATP (Fig. 7C). Thus, 2′d3′AMP is a P-site inhibitor of both wild-type adenylyl cyclase and a guanylyl cyclase mutated to acquire specificity for ATP as substrate. 2′d3′GMP is a P-site inhibitor of wild-type guanylyl cyclase but not of the mutant enzyme.

Despite its ability to use ATP or GTP as substrate, Gα-stimulated C1-wt/C2-EC is not strongly inhibited by either
2'd3'AMP (Fig. 7D) or 2'd3'GMP (data not shown). This result is presumably explained by poor affinity of the mutant for 2'd3'AMP and its substantially reduced V_max. Since P-site inhibitors bind to the enzyme-product complex, their apparent potency as inhibitors is dependent on the activity state of the enzyme. This result is also consistent with the phenotype of the K938A mutant of type I adenylyl cyclase, where the apparent affinity of P-site inhibitors was increased to a much greater extent than was the K_m for ATP (23). Together, these data indicate that P-site inhibitors have a greater reliance on Lys-938 for binding and inhibition (as measured by K_i) than does ATP for binding and activity (as evaluated by its K_m). Collectively, these data also further justify the now strong conclusion that P-site inhibitors bind at the active site of nucleotide cyclases and that adenylyl cyclase has but one binding site for adenine nucleotides.

Structural Implications for Recognition of GTP by Guanylyl Cyclases—Although the guanylyl cyclases studied herein that harbor single mutations apparently demonstrate little capacity to synthesize cGMP in vivo (Fig. 2, top panel), it is difficult to assess the true importance of each residue for GTP binding. It is not known if properly folded protein was present in all cases. The mutant b-D subunit, which has two adjacent negatively charged residues (bE473 and bD541), is clearly unstable. In addition, the residues were replaced by their invariant homologs in the adenylyl cyclase family rather than by alanine, which would have minimized steric conflicts. Further complicating interpretation is the fact that we have created net uncompensated buried charge in the purine ring binding pocket in all of the mutant proteins except a-Q/b-KD. This probably destabilizes and/or disrupts the structure of the purine binding pocket (the extreme cases are a-Q/b-D and a-wt/b-K).

However, the data do suggest that aR592, bE473, and bC541 are all important for GTP binding and recognition. Our modeling predicts aR592 to be a second shell ligand for the guanine
ring (Fig. 1B), and its mutation to glutamine should not perturb the binding pocket grossly although it does leave uncompensated negative charge buried within the protein. However, α-Q/β-wt has no detectable adenylyl cyclase or guanylyl cyclase activity. This result is consistent with the model that αR592 stabilizes charge and orients βE473, which in turn interacts with the N-2 amino group of GTP. βE473 may be the most important residue for recognition of GTP since mutant guanylyl cyclases or adenylyl cyclases that lack glutamic acid at the position equivalent to βE473 have no significant guanylyl cyclase activity. In addition, the only guanylyl cyclase mutant that retains guanylyl cyclase activity is α-Q/β-D. The βC541S mutant of soluble guanylyl cyclase has been characterized previously and has greatly reduced catalytic efficiency despite the conservative nature of the substitution (25). Finally, in the adenylyl cyclase mutants tested here, glutamate and cysteine must both be present to observe any guanylyl cyclase activity.

The triple mutant α-Q/β-KD has significantly more adenylyl cyclase activity in vitro than does α-wt/β-KD even though Gln-503 in adenylyl cyclase is not predicted to play a major role in specificity for that enzyme. This discrepancy is most easily explained by the deleterious effect of an uncompensated positive charge in α-wt/β-KD and the close proximity of lysine at position βE473 to arginine at position α592, which may render this particular mutant largely inactive.

Structural Implications for Recognition of ATP by Adenylyl Cyclases—As noted above, the only adenylyl cyclase mutants that were able to synthesize significant amounts of cGMP are those that have a C2-EC subunit. More cGMP synthesis was observed when C2-EC was paired with C1-wt than with C1-R. In C1-wt/C2-EC, adenylyl cyclase activity has been reduced more than 10-fold, whereas the guanylyl cyclase activity has been elevated from essentially undetectable levels to one-third to more than 10-fold, whereas the guanylyl cyclase activity has been elevated from essentially undetectable levels to one-third of the mutant's adenylyl cyclase activity (Fig. 5B). Because the triple mutant in guanylyl cyclase, α-Q/β-KD, displays a full change of nucleotide specificity, it is somewhat surprising that the analogous triple mutant of adenylyl cyclase does not. This result indicates that nucleotide specificity in adenylyl cyclase is dictated in part by elements other than the amino acid residues mutated in this study. Of the mutated residues, Asp-1018 appears to be the most important for specificity of ATP binding because the βC541D mutation alone in guanylyl cyclase is sufficient to permit cAMP synthesis, whereas the addition of the βE473K mutation does not significantly enhance this activity.

There are two other factors that may allow adenylyl cyclase to bind adenine in preference to guanine. The first of these is the backbone carbonyl of residue 1019, which forms a hydrogen bond with the N-6 amino group of 2′,3′AMP (5) and presumably ATP (Fig. 1A). This group cannot be altered readily. In the model of guanylyl cyclase (Fig. 1B), slight rotations of this carbonyl and the guanine ring permit binding in the pocket without steric overlap between the carbonyl oxygen and the O-6 atom of guanine. There are several conservative changes in the hydrophobic core of guanylyl cyclase compared with adenylyl cyclase at and near position 1019 that may alter the conformation of the backbone in this region and allow the O-6 atom of guanine to bind, unimpeded by the backbone carbonyl.

The second factor is pocket size. Guanine, with its extra N-2 substituent, is a bigger purine than adenine. There are two residues in adenylyl cyclase, Leu-438 in VC1 and Ile-940 in IIIC2, that may preclude guanine from binding properly because of their potential steric overlap with the N-2 amino group of the ring. These residues are substituted by isoleucine and valine, respectively, in soluble guanylyl cyclase (Fig. 1C). Hence, mutation of Leu-438 and Ile-940 to their equivalents in guanylyl cyclase might allow the bulkier purine ring of GTP to bind in a more optimal fashion, perhaps with its O-6 atom oriented away from the backbone carbonyl of 1019. Even so, the same backbone carbonyl could still serve as a hydrogen bond acceptor to ATP, and this mutant enzyme might still be a nonselective purine nucleotide cyclase.

In summary, we demonstrate that nucleotide specificity in guanylyl cyclases is dictated by three invariant residues at positions equivalent to α592, βE473, and β541. A cyclase that utilizes ATP and not GTP can be created by changing the identity of these residues to their equivalents in adenylyl cyclase. Similar maneuvers resulted in only partial conversion of adenylyl cyclase to a guanylyl cyclase because specificity appears to be additionally dictated by elements other than those that we manipulated by site-directed mutagenesis. In both adenylyl cyclase and guanylyl cyclase, the residues complementary to the exocyclic amines of the purine nucleotide substrate appear most crucial for alteration or abolition of specificity. P-site inhibitors, in contrast to ATP, appear to rely more on residues at positions equivalent to 938 and 1018 of adenylyl cyclase for binding and/or inhibition. This result is consistent with both prior mutagenesis (23) and the hypothesis that P-site inhibitors bind to a different conformational state of the enzyme than does the substrate ATP (10).

Acknowledgments—We thank Julie Collins and Debbie Miller for superb technical assistance. We also thank Lynda Doolittle for DNA sequencing, Janet Friisen for help with radioimmunoassays, and Lothar Easer for assistance in preparing figures.

REFERENCES

1. Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) Annu. Rev. Pharmac. Toxicol. 36, 461-480
2. Tang, W.-J., and Gilman, A. G. (1995) Science 268, 1769-1772
3. Sunahara, R. K., Dessauer, C. W., Whisnant, R. E., Kleuss, C., and Gilman, A. G. (1997) J. Biol. Chem. 272, 22265-22271
4. Dessauer, C. W., Slurly, T. T., and Gilman, A. G. (1997) J. Biol. Chem. 272, 22272-22277
5. Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Science 278, 1907-1916
6. Garbers, D. L., Koelling, D., and Schultz, G. (1994) Mol. Biol. Cell 5, 1-5
7. Harteneck, C., Koelsing, D., Soling, A., Schultz, G., and Bohme, E. (1990) FEBS Lett. 272, 221-225
8. Buechler, W. A., Nakane, M., and Murad, F. (1991) Biochem. Biophys. Res. Commun. 174, 351-357
9. Wedel, B., Humberi, P., Harteneck, C., Foerster, J., Malkewitz, J., Bohme, E., Schultz, G., and Koelling, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2592-2596
10. Dessauer, C. W., and Gilman, A. G. (1997) J. Biol. Chem. 272, 27787-27795
11. Liu, Y., Rusho, A. E., Rao, V. D., and Hurley, J. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13414-13419
12. Zhang, G., Liu, Y., Rusho, A. E., and Hurley, J. H. (1997) Nature 386, 247-253
13. Nakane, M., Arai, K., Saheki, S., Kuno, T., Buechler, W., and Murad, F. (1990) J. Biol. Chem. 265, 16841-16845
14. Yuen, P. S. T., Doolittle, L. K., and Garbers, D. L. (1994) J. Biol. Chem. 269, 791-793
15. Whisnant, R. E., Gilman, A. G., and Dessauer, C. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6621-6625
16. Domino, S. E., Tubb, D. J., and Garbers, D. L. (1991) Meth. Enzymol. 195, 345-355
17. Tausvig, R., Tang, W.-J., Hepler, J. R., and Gilman, A. G. (1994) J. Biol. Chem. 269, 6095-6100
18. Simonel, M. D. (1986) J. Biol. Chem. 261, 1976-1982
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
20. Brunger, A. T., Krukowski, A., and Erickson, J. W. (1990) Acta Crystallogr. Sec. A 46, 585-593
21. Brunger, A. T., Adams, P. D., Clore, G. M., Gross, P., Gaus-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sec. D 54, 1194–1198
22. Tang, W.-J., Stanzel, M., and Gilman, A. G. (1995) Biochemistry 34, 14565–14572
23. Johnson, R. A., and Shoshani, I. (1990) J. Biol. Chem. 265, 11595-11600
24. Friebe, A., Wedel, B., Harteneck, C., Foerster, J., Schultz, G., and Koelling, D. (1997) Biochemistry 36, 1134–1138
25. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946-950
26. Young, C. (1997) Persistence of Vision Ray Trace, Version 3.02
27. Chinkers, M., Garbers, D. L., Chang, M.-S., Lowe, D. G., Chin, H., Goeddel, D. V., and Schulz, S. (1989) Nature 338, 78-83