Structural Basis for the Inhibition of Mammalian Membrane Adenylyl Cyclase by 2′(3′)-O-(N-Methylanthraniloyl)-guanosine 5′-Triphosphate

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Membrane-bound mammalian adenylyl cyclase (mAC) catalyzes the synthesis of intracellular cyclic AMP from ATP and is activated by stimulatory G protein α subunits (Gαs) and by forskolin (FSK). mACs are inhibited with high potency by 2′(3′)-O-(N-methylanthraniloyl) (MANT)-substituted nucleotides. In this study, the crystal structures of the complex between Gαs-GTP-S and the catalytic C1 and C2 domains from type V and type II mAC (VC1-IIIC2), bound to FSK and either MANT-GTP-Mg2+ or MANT-GTP-Mn2+ have been determined. MANT-GTP coordinates two metal ions and occupies the same position in the catalytic site as P-site inhibitors and substrate analogs. However, the orientation of the guanine ring is reversed relative to that of the adenine ring. The MANT fluorophore resides in a hydrophobic pocket at the interface between the VC1 and IIC2 domains and prevents mAC from undergoing the "open" to "closed" domain rearrangement. The Kᵢ of MANT-GTP for inhibition of VC1-IIIC2 is lower in the presence of mAC activators and lower in the presence of Mn2+ compared with Mg2+, indicating that the inhibitor binds more tightly to the catalytically most active form of the enzyme. Fluorescence resonance energy transfer-stimulated emission from the MANT fluorophore upon excitation of Trp-1020 in the MANT-binding pocket of IIC2 is also stronger in the presence of FSK. Mutational analysis of two non-conserved amino acids in the MANT-binding pocket suggests that residues outside of the binding site influence isoform selectivity toward MANT-GTP.

The nine membrane-bound isoforms of mammalian adenylyl cyclase (mAC),1 which convert ATP to the ubiquitous second messenger cAMP, respond differently to a variety of regulatory molecules (1–3). All of the mAC isoforms are activated by the stimulatory G protein α subunit (Gαs) and (except for type IX) the diterpine forskolin (FSK) and its soluble derivatives (1, 4–7). ACs require Mg2+ or Mn2+ for catalytic activity, although, in general, Mn2+ has greater affinity for mAC and is a more effective activator than the physiological ligand Mg2+ (7–9).

Adenylyl cyclases are inhibited by adenosine and certain adenosine derivatives such as 2′-5′-dideoxyadenosine and 2′-deoxyadenosine 3′-monophosphate that possess intact adenine rings and are known as “P-site” inhibitors (9–13). These compounds bind preferentially to the FSK- and Gαs-GTP-S-activated state of mAC (14). P-site inhibitors are dead-end inhibitors that bind to the catalytic site in the presence of pyrophosphate (15–17) and exhibit un- or noncompetitive inhibition in the presence of Mg2+ or Mn2+, respectively, in the direction of cAMP synthesis (18). Certain substrate analogs, such as the Rα stereoisomer of ATPαS (19) and particularly β-1′-2′-3′-dd-5′-ATP (Kᵢ = 24 nM with native mAC from rat brain) have been identified as potent competitive inhibitors (20). More recently, it has been demonstrated that nucleotide triphosphates derivatized at the ribose 2′- or 3′-exocyclic ribose ring oxygen atoms by the fluorescent MANT moiety are also highly potent inhibitors of mAC (21, 22). The unexpected inhibitory activity of MANT-GTP suggests novel routes for the design of inhibitors for mAC, which, as a significant target of G protein-coupled receptor stimulation, may be considered an appropriate target for drug development.

Crystal structures have been determined of complexes between the homologous cytosolic catalytic domains of mAC bound to FSK and GTPγS-activated Gαs (16). In these studies, the C1 and C2 domains, which constitute the N- and C-terminal halves of the catalytic core, were derived from type V and type II isoforms of AC and are thus designated VC1 and IIC2, respectively. The crystallographic studies revealed that both P-site inhibitors and substrate analogs bind, together with the metal ion co-factors, to the catalytic site located at the interface.
between C1 and C2. FSK occupies a site related by 2-fold pseudosymmetry to the catalytic site. Crystal structures have also been determined of the Gaα-activated mAC catalytic core complexed with various P-site inhibitors and substrate analogs (17, 23).

Upon binding to potent P-site inhibitors and substrate analogs such as 2′-deoxyadenosine 3′-monophosphate (K_i = 1.2 μM) (24) and β-1,2,3′-dd-5′-ATP, VC1-IIC2 undergoes a transition from an “open” to a “closed” conformation in which the α-β2, β2-β3, and α-β3 loops of C1 and the β2′-β3′ loop of C2 move toward each other, thereby closing the gap between the purine-binding pocket in the C2 domain and the triphosphate binding loop (P-loop) located primarily in the C1 domain (16). This conformational change also places the N terminus of the C1 domain α1 helix (the P-loop) in hydrogen bond contact with the β- and γ-phosphates of ATP. Therefore, the architecture of the closed conformation constitutes the functional catalytic structure that facilitates substrate binding. In contrast, weak inhibitors comparable in affinity with the substrate ATP (K_i = 0.4 mM), such as the nonhydrolyzable α,β-methylene derivative of ATP, bind to the open conformation of the enzyme (8).

In the C1 domain, two divalent metal ions are coordinated by the invariant aspartic acid residues Asp-396 and Asp-440 (type 350 mM NaCl, 0.1M MES, pH 5.6, at 16 °C. After 4–6 weeks, the crystals were soaked in reservoir solution with additional 2.5 mM MANT-GTP and 3 mM MgCl_2 or MnCl_2 for 1–2 h at room temperature and then harvested in cryoprotectant (10% (mass/volume) polyethylene glycol 8000, 30% (mass/volume) polyethylene glycol 400, 500 mM NaCl, 100 mM MES, pH 5.4, 20 mM Na° HEPS, pH 8.0, 3.3 mM dithiothreitol, and 0.2 mM MP-FSK, and 0.17 mM GTP-S°). Cryoprotected crystals were mounted in 0.1 M CaCl_2 and stored in liquid nitrogen.

**Structure Determination and Model Refinement—**Diffraction data sets were collected by the oscillation method (1°/frame, 45 s/frame) at the Advanced Photon Synchrotron SBC-CAT BM19 Beamline with an incident beam wavelength of 1.0393 Å. The images were processed with the HKL2000 package (28) (Table I). Because of anisotropy, data with I > 2σ(I) were used. The MANT-GTP complex was isomorphous with those of the VC1-IIC2FSK/MANT-GTPα°S° complex (16), and the refined coordinates of the last (Protein Data Bank code 1AZS) were used as the initial phasing model. Atomic positions and thermal parameters were refined by successive rounds of rigid body, simulated annealing, Powell minimization, and grouped B factor refinement using the CNS program suite (29). The MANT-GTP and mAC crystal structures were located in the SIGMA-A weighted [F_o - |F_c|] omit map computed with phases from the refined model. The model was iteratively improved by manual refitting into weighted |F_o - |F_c| maps using the computer graphics program O (30) and subsequent cycles of refinement with CNS. Final refinement statistics are listed in Table I. Coordinates for the MANT-GTP complex and MANT-GTP-Mg_2+° structures have been deposited in the Protein Data Bank with the codes 1TL7 and 1U0H, respectively.

**Fluorescence Spectroscopy and Data Analysis—**All of the experiments were conducted using a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier thermostatted multicell holder at 25 °C (Varian, Walnut Creek, CA). The measurements were performed in a quartz fluorescence microcuvette (Hellma, Plainview, NY). The final assay volume was 150 μl. The reaction mixtures containing a buffer consisting of 100 mM KCl, 10 mM MnCl_2, or 10 mM MgCl_2 and 25 mM Na° HEPS, pH 7.4. Steady-state emission spectra were recorded at low speed with λ_ex = 350 nm (λ_em = 370–500 nm) and λ_em = 280 nm (λ_em = 300–500 nm) with 1 μM MANT-GTP in the absence and presence of 5 μM VC1 plus 25 μM IIC2 without and with 100 μM MP-FSK unless otherwise indicated.

**AC Activity Assays—**For determination of K_M and V_max values, the assay tubes contained 40 μM to 1.5 mM ATP/Mg_2+°, 10 mM MnCl_2, or 40 μM to 1.5 mM ATP/Mg_2+° and 10 mM MnCl_2 in a buffer consisting of 100 mM KC1 and 25 mM Na° HEPS, pH 7.4. For determination of the K_M values of MANT nucleotides, the reaction mixtures contained 100 μM ATP/Mg_2+°, 10 mM MnCl_2, or 100 μM ATP/Mg_2+°, 10 mM MnCl_2, and MANT nucleotides at concentrations from 1 mM to 1 mM as appropriate to obtain saturated inhibition curves. Additionally, the assay tubes contained VC1 and IIC2 at a ratio of 1:5. For experiments with Gaα°GTP-S°, the ratio of VC1-IIC2Gaα°GTP-S° was 1:5:1.5. Depending on the specific experimental conditions examined, the absolute concentration of VC1 was varied between 3 and 100 nM to ensure both sensitive detection of product formation and linearity of substrate turnover. The reactions were conducted in the absence and presence of 100 μM FSK.

**EXPERIMENTAL PROCEDURES**

**Preparation of Proteins and Materials—**Plasmids encoding the wild type and mutant AC C1a domain from canine type V (VC1), C2a domain from rat type II (IIC2), and bovine Gaα, consisting of residues 364–580, 874–1081, and 1–396, respectively, were expressed in *Escherichia coli* BL21 (DE3) cells, and the proteins were purified and stored as described previously (27). Mutants VC1-A409P (substitution of Ala-409 with Pro) and IIC2-11006V (substitution of Ile-1006 with Val) were generated by site-directed mutagenesis (QuikChange; Stratagene). Except for IIC2, both VC1 and Gaα constructs contained a hexahistidine tag at their N termini. Gaα was activated by incubation with 10 μM GTP-S° and 2 mM MgCl_2 at 30 °C for 1 h, and the resulting Gaα°GTP-S° complex was further digested by trypsin to a smaller fragment comprising residues 39–387 (27). MANT-GTP—2′-deoxy-3′-O-(N-methylanthraniloyl)-adenosine 5′-triphosphate, MANT-ATP, and 2′(3′)-O-(N-methylanthraniloyl)-xanthosine 5′-triphosphate were obtained from Molecular Probes (Eugene, OR) or Jena Bioscience (Jena, Germany). MANT-GMIP was gift from Dr. H.-G. Genieser (Biolog Life Sciences Institute, Bremen, Germany). GTP-S° was from Roche Applied Science. Forskolin and MP-FSK were obtained from Calbiochem (La Jolla, CA). [α-32P]ATP (3,000 Ci/mmole) was from PerkinElmer Life Sciences.

**Adenylyl Cyclase Complex Formation and Crystallization with MANT-GTP—**Purified recombinant VC1, IIC2, and trypsin-treated Gaα°GTP-S° were mixed in a 1:5:1 molar ratio to form a heterotrimeric complex in the presence of excess MP-FSK and GTP-S°. This protein mixture was purified by gel filtration using tandemly arranged Superdex 75 and 200 columns (Amersham Biosciences), and fractions containing the heterotrimeric complex were collected and concentrated to 8 mg/ml in a buffer of 20 mM Na° HEPS, pH 8.0, 2 mM EDTA, 2 mM MgCl_2, 2 mM dithiothreitol, 100 mM NaCl, 25 μM MP-FSK, and 10 μM GTP-S° for crystallization.

Protein crystals were grown by the sitting drop method with a reservoir containing 7.5% (mass/volume) polyethylene glycol 8000, 0.5 M NaCl, 0.5M MES, pH 5.6, for 5–6 weeks, 2°C. Crystals were then soaked in reservoir solution with additional 2.5 mM MANT-GTP and 3 mM MgCl_2 or MnCl_2 for 1–2 h at room temperature and then harvested in cryoprotectant (10% (mass/volume) polyethylene glycol 8000, 30% (mass/volume) polyethylene glycol 400, 500 mM NaCl, 100 mM MES, pH 5.4, 20 mM Na° HEPS, pH 8.0, 3.3 mM dithiothreitol, and 0.2 mM MP-FSK, and 0.17 mM GTP-S°). Cryoprotected crystals were mounted in 0.1 M CaCl_2 and stored in liquid nitrogen.
Summary of crystallographic data collection and refinement statistics

| Parameters | MANT-GTP-Mn\(^{2+}\) | MANT-GTP-Mg\(^{2+}\) |
|------------|----------------------|----------------------|
| Cell constants (Å) | 118.2 | 133.0 |
| a | 118.2 | 133.0 |
| b | 70.4 | 70.4 |
| c | 70.4 | 70.4 |
| No. of crystals | 1 | 1 |
| \(D_{max}\) (Å) | 2.9 | 2.9 |
| Average redundancy | 3.6 (3.1\(^{a}\)) | 3.4 (1.7) |
| \(R_{sym}\) (%) | 13.9 (38.4) | 16.2 (37.8) |
| Completeness (%) | 90.7 (83.2) | 85.8 (66.2) |
| \(I/\sigma(I)\) | 7.8 (1.9) | 7.3 (1.4) |
| Resolution range for refinement (Å) | 15.2–2.9 | 15.2–2.8 |
| Total reflections used | 21911 | 22694 |
| No. of protein atoms | 5648 | 5648 |
| No. of water molecules | 37 | 18 |
| No. of ligand groups | 307 | 107 |
| rmsd bond length (Å) | 0.012 | 0.008 |
| rmsd bond angle (°) | 1.40 | 1.32 |
| \(R_{work}\) (%) | 24.55 | 25.33 |
| \(R_{free}\) (%) | 27.36 | 29.61 |
| Average B-factor (Å\(^{2}\)) | 54.4 | 45.3 |

\(^{a}\) Numbers in parentheses correspond to the statistic data from the highest resolution shell.

Following a 2-min preincubation at 30 °C, the reactions were initiated by adding 20 μl of reaction mixture containing (final) 1.0 to 10 μCi/tube [\(^{32}\)P]cAMP to 2.7 mM monooctylglycerophosphate, 0.125 IU pyruvate kinase, 1 IU myokinase, and 0.1 mM cAMP. The reactions were conducted for 10–30 min at 30 °C and were terminated by the addition of 20 μl of 2.2 M HCl. Denatured protein was sedimented by a 1-min centrifugation at 25 °C and 15,000 × g. Fifty-six μl of the supernatant fluid were applied onto disposable columns filled with 1.3 g of neutral alumina (Sigma-Aldrich, St. Louis, MO). [\(^{32}\)P]cAMP was separated from [\(^{32}\)P]ATP by elution of [\(^{32}\)P]cAMP with 4 ml of 0.1 M ammonium acetate, pH 7.0 (31). Recovery of [\(^{32}\)P]cAMP was ~80% as assessed with [\(^{33}\)P]cAMP as standard. The blank values were routinely 0.01% of the total amount of [\(^{32}\)P]cAMP added, ensuring sensitive measurement of radioactivity. The possibility that the latter is a reflection of the electron density (see Fig. S1 published online as supplementary material). Electron density for the B-site metal ion is well defined in both structures (see Fig. S2 published as supplementary material on line), but density for the A-site is weaker than that for the B-site in the Mg\(^{2+}\)-bound complex. MANT-GTP-Mn\(^{2+}\) appears better ordered than MANT-GTP-Mg\(^{2+}\). With the assumption of 100% occupancy, the average temperature factors of bound FSK and GTP-αS are 32 and 38 Å\(^2\) in the MANT-GTP-Mn\(^{2+}\) and MANT-GTP-Mg\(^{2+}\) structures, respectively. However, the average temperature factors for MANT-GTP-Mn\(^{2+}\) and MANT-GTP-Mg\(^{2+}\) are 55 and 73 Å\(^2\), respectively, indicating greater disorder or lower occupancy of MANT-GTP-Mg\(^{2+}\) in complex with VC1-IIC2.

The MANT fluorophore is bound in a cavity at the interface between the β1–α1–α2 loop of VC1 and the α4–β5′ and β7–β8′ loops of IIC2 (Fig. 1, A and B). To accommodate the MANT group within the cavity, the MANT-GTP adopts an orientation in the catalytic site that is related to that of other substrate or P-site analogs by a ~180° rotation about the purine-phosphate axis. The consequences of this unexpected binding mode on the conformation of C1-C2, recognition of the nucleotide moiety and metal ions by the enzyme and the mechanism of inhibition by MANT-GTP, are described below.

The conformation of the MANT-GTP-bound C1-C2 complex is midway between the open and closed states. As shown in Fig. 1B, in which the MANT-GTP-bound, β1–2,3′-dd-5′-ATP-bound, and unbound structures are superimposed, the β1–α1–α2 and α3–β4 loops of VC1 and β7–β8′ of IIC2 shift toward the domain interface but fail to close fully. Superposition of the MANT-GTP-bound complex with unliganded (open) and β1–2,3′-dd-5′-ATP-bound (closed) complexes yields an overall rmsd of 0.6 and 0.8 Å, respectively, for all equivalent Ca positions of the VC1-IIC2 complex. However, rmsd values for the Ca atoms in the latter interfacial structural elements are considerably larger: 1.9 Å with respect to the closed complex and 1.0 Å with respect to the open complex. For comparison, the rmsd for the same Ca atoms after superposition of open and closed complexes is 1.9 Å. The MANT fluorophore that is bound between β1–α1–α2 of VC1 and β7–β8′ of IIC2 thus acts as a wedge to prevent VC1-IIC2 from adopting a fully closed conformation.

Other than as noted above, global and local structural differences between the Mn\(^{2+}\) and Mg\(^{2+}\) complexes are small, as indicated by the rmsd between equivalent Ca positions of 0.4 Å. The largest differences are apparent in the α1 helix, β1–α1 loop, and α3–β4 loop of VC1 in the MANT-GTP-Mg\(^{2+}\) complex, which adopt a slightly more open conformation than the MANT-GTP-Mn\(^{2+}\) structure. This structural difference may reflect the higher affinity of VC1-IIC2 for MANT-GTP-Mn\(^{2+}\) (21, 22).

The MANT-GTP used in this study is presumably a racemic mixture of ribosyl 2′-O- and 3′-O-MANT derivatives. Our previous molecular modeling studies suggested that MANT nucleotides bind to mAC preferentially in the 2′-O-MANT conformation (21). However, it is clear that crystals of mAC contain only the 3′-O-enantiomer. A stereochromically reasonable model of the 2′-O-enantiomer cannot be accommodated in the experimental electron density. The possibility that the latter is a substrate for VC1-IIC2 and is thus consumed by the enzyme is unlikely because the putative reaction product MANT-cGMP shows no fluorescence increase with VC1-IIC2, and there was no time-dependent decrease in MANT-GTP fluorescence upon binding to VC1-IIC2 (data not shown; see below for discussion of MANT-GTP fluorescence).
contacts the endocyclic ring oxygen of the ribose moiety. The conformation of Asn-1025 also differs from that in complexes with MANT-GTP and thereby avoids steric conflict with the 3’-MANT-ribosyl moiety. The polar substituents of the MANT moiety are oriented toward the exterior of the binding cleft between β1–α1–α2 of VC1 and α4–β5’ of IIC2 (Figs. 1B and 3A) and form no hydrogen bonds with the protein. The nonpolar surface of the N-anthraniloyl ring is in van der Waals contact with residues in the hydrophobic pocket formed by Phe-400, Ala-404, Ala-409, Leu-412, and Leu-413 from VC1 and Val-1006, Trp-1020, Gly-1021, and Asn-1022 from IIC2 (Fig. 3A). A structure-based sequence alignment of selected mammalian AC family members (Fig. 3B) shows that the residues in the MANT-binding pocket are well conserved among type I–VIII membrane-bound mAC isoforms with the exception of type II at residues 307 (residue 409 for type V) and 1006. Several aromatic residues are part of, or close to, the MANT-binding pocket; in particular, the indole ring of Trp-1020, which is located less than 5 Å from the N-methylanthraniloyl ring of MANT-GTP.

The positions occupied by the purine ring and 5’-triphosphate substituents of MANT-GTP and the two metal ions are roughly similar to those of the corresponding moieties in P-site inhibitor-PP, or β1-1’-2’,3’-dd-5’-ATP complexes with VC1-IIC2 but differ in significant details (Fig. 1C). The β- and γ-phosphates of MANT-GTP, which coordinate the B site metal ion and the positively charged amine of Lys-1065 in IIC2, are located in approximately the same positions as the α- and β-phosphates of β1-l-2’,3’-dd-5’-ATP, respectively. Consequently, Lys-484 from VC1 cannot form an ion pair with the γ-phosphate of MANT-GTP as with β1-1’-2’,3’-dd-5’-ATP. Further, because obstruction by the MANT group prevents full closure of the catalytic site, Lys-1029 from the α4 helix of IIC2 is unable to contact the β-phosphate of MANT-GTP. The metal
of the kinetic experiments re-
sors (Table II; see also Fig. S3 published as supplementary

cannot exclude the possibility that an ordered water molecule

oxygen atoms from the
phate groups of P-site inhibitors and substrate analogs, the
phates of MANT-GTP differ from those observed with the phos-
tions between the two metal ions and the nucleotide phos-
A site appears to be considerably lower than that of Mn$^{2+}$
are omitted from the phasing model.

from each of the
MANT-GTP and the two metal ions were omitted from the phasing model.

the absence or presence of FSK and G$
protein hydrogen bonding partners (Fig. 1

is stacked upon the peptide planes of Leu-438 and Gly-439 in

respect to the purine N-9–N-1 axis) from that of adenine in

increases with enzyme activity (18). Accordingly, from a phar-
mAC activities were generally higher than under the corre-
some cases by 2–3-fold from those reported previously, but
relative values for the same inhibitors examined under various
activation conditions are similar. In the presence of Mn$^{2+}$,
mAC activities were generally higher than under the corre-
similar (Fig. 1C). As noted above, the occupancy of Mg$^{2+}$ at
A site appears to be considerably lower than that of Mn$^{2+}$. We
cannot exclude the possibility that an ordered water molecule
occupies the A site in the MANT-GTP-Mg$^{2+}$-mAC complex.

The guanine ring of MANT-GTP is located at the same po-
position occupied by the adenine rings of substrate analogs but
differs from these analogs in its orientation with respect to
protein hydrogen bonding partners (Fig. 1D). The guanine ring
is stacked upon the peptide planes of Leu-438 and Gly-439 in
the β2–β3 loop of VC1 and could form hydrogen bonds from the
guanine N-1 atom to Asp-1018, the guanine N2 atom to the
backbone carbonyl of Ile-1019, and from the guanine O-6 atom
to Lys-938. The guanine ring adopts an anti conformation ($\chi =
234^\circ$) to the ribose ring, in contrast to the glycosidic angle of
107° observed in β-1,2-3’-dd-5’-ATP and of 157° for ATP-McR
$. Thus, the orientation of the guanine ring differs by $-180^\circ$ (with
respect to the purine N-9–N-1 axis) from that of adenine in
complexes with ATP analogs. However, the guanine ring in this
reversed orientation presents a similar pattern of hydrogen
bond donors and acceptors to the enzyme, compared with ade-
dine. To accommodate the orientation of the guanine ring and
the 3’-O-ribose1-MANT group, the ribose of MANT-GTP adopts
a C2’-endo conformation (Fig. 1, C and D).

Kinetic analysis of VC1-IIC2—In previous studies we showed
that the potencies of MANT nucleotides for inhibiting various
mACs are higher in the presence of Mn$^{2+}$ than with Mg$^{2+}$ (21,
22). Those data suggested that formation of a catalytically
competent conformation by mAC might facilitate binding of
MANT nucleotides to the substrate-binding site. To further
substantiate this notion, we examined the activity of the
VC1-IIC2 protein complex in the presence of Mg$^{2+}$ or Mn$^{2+}$
in the absence or presence of FSK and G$\alpha_o$-GTP$\gamma$S or both activa-
tors (Table II; see also Fig. S3 published as supplementary
material on line). Note that for direct comparison with the
fluorescence studies (see below), the kinetic experiments re-
ported here were conducted under buffer conditions (100 mM
KCl and 25 mM Na$^+$-HEPES, pH 7.4) different from than those
used in a previous study (30 mM Tris-HCl, pH 7.4) (22). Conse-
sequently, values for kinetic constants determined here differ in
some cases by 2–3-fold from those reported previously, but
relative values for the same inhibitors examined under various
activation conditions are similar. In the presence of Mn$^{2+}$,
mAC activities were generally higher than under the corre-
sor activators (21, 22), they have in common with the noncompet-
itive (P-site) inhibitors the characteristic that inhibitor potency
increases with enzyme activity (18). Accordingly, from a phar-
mAC activities should be more sensitive to inhibition by MANT
nucleotides than basal mAC activities.

Except for two positions (residue 409 in VC1 and residue
1006 in IIC2), the amino acids that constitute the MANT
binding site are conserved among mAC isoforms (Fig. 3
neighboring structural relationships shown here were obtained
at 2.5 α. Coordinates for MANT-GTP and the two metal ions were omitted from the phasing model.
catalytic core. The effects of the two mutations in the VC1-A409P/IIC2-I1006V double mutant are less than additive (Table III).

We also found that the potency of 2′-deoxy-3′-O-(N-methylanthraniloyl)-adenosine 5′-triphosphate is between 3- and 13-fold less than that of 2′(3′)-MANT-GTP in the presence of both Mn$^{2+}$ and Mg$^{2+}$, respectively. These findings are consistent with the structural data showing possible hydrogen bond formation between the 2′-ribofuran-OF of MANT-GTP and the backbone nitrogen of Asn1025 from IIC2 (Fig. 1C). Additional studies on MANT nucleotides with different purine substitutions show that 2′(3′)-O-(N-methylanthraniloyl)-xanthosine 5′-triphosphate is a less potent inhibitor of activated mAC in the presence of either Mn$^{2+}$ or Mg$^{2+}$ than MANT-GTP, whereas MANT-ATP is a very potent inhibitor of activated mAC, and its inhibitory potency increases with the substitution of Mn$^{2+}$ for Mg$^{2+}$.

**Effects of C1/C2 and MP-FSK on Emission Spectra of MANT-GTP**—Fluorescence emission from MANT-GTP was measured in the absence and presence of VC1-IIC2 and MP-FSK either by FRET from excitation of tryptophan at 280 nm (35) (Fig. 4, A and C) or by direct excitation of MANT fluorescence at 350 nm (34) (Fig. 4, B and D). The experiments were conducted in the presence of either Mn$^{2+}$ (Fig. 4, A and B) or Mg$^{2+}$ (Fig. 4, C and D).

The addition of VC1-IIC2 in the presence of Mn$^{2+}$ increased MANT fluorescence by 70% upon excitation at 350 nm and shifted the MANT emission maximum from 450 to 420 nm (Fig. 4B). The change in fluorescence emission intensity is consistent with the transfer of the MANT group to a hydrophobic environment (34) as is the blue shift of the emission maximum toward shorter wavelengths (36, 37). The addition of MP-FSK further increased fluorescence emission of MANT-GTP to about 3-fold above the value for the unbound compound. Also in the presence of VC1-IIC2 and Mn$^{2+}$, excitation at 280 nm produces a broad emission peak at 350 nm (Fig. 4A) that is not observed in the absence of protein, and a shoulder of FRET-stimulated emission at 420 nm. Upon the addition of MP-FSK, there was a loss in fluorescence intensity at 350 nm with a concomitant increase in emission at 420 nm. These data show that binding of MP-FSK to VC1-IIC2 allows for efficient FRET between a tryptophan residue, presumably Trp-1020, in the MANT-binding site and the MANT fluorophore.

Mutation of conserved residues in mAC hydrophobic environment results in modest changes in the fluorescence emission spectrum because of binding of the MANT fluorophore. In the absence of FSK, the increase in the intensity of fluorescence emission upon direct excitation of MANT ($\lambda_{ex}$ = 350 nm) caused by binding is unchanged for VC1-A409P-IIC2, reduced by $\sim$50% for VC1-IIC2-I1006V, and reduced by $\sim$70% in the double mutant (see Fig. S4 in supplementary material published on line). Similarly, FRET-stimulated emission ($\lambda_{ex}$ = 280 nm) is markedly reduced for VC1-IIC2-I1006V and the double mutant. However, in the presence of MP-FSK, the intensity of both direct and FRET-stimulated emission from MANT is similar to that from VC1-IIC2.

In contrast to the results obtained in the presence of Mn$^{2+}$, the addition of VC1-IIC2 to MANT-GTP in the presence of Mg$^{2+}$ resulted only in a small fluorescence increase upon excitation at 350 nm (Fig. 4D). The increase in MANT-GTP fluorescence in the presence of both Mg$^{2+}$ and MP-FSK was similar to that observed in the presence of Mn$^{2+}$ alone (Fig. 4B). The blue shift in the fluorescence emission spectrum of MANT was also much less pronounced and was only observed upon the addition of MP-FSK (Fig. 4D). Finally, in the presence of Mg$^{2+}$, MP-FSK was much less efficient at promoting FRET than in the presence of Mn$^{2+}$ (Fig. 4, C versus A). Collectively, these data are indicative of a much stronger interaction between VC1-IIC2 and MP-FSK in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$ and corroborate the enzymatic and crystallographic data.

**DISCUSSION**

MANT-GTP is the most potent member of a newly described family of competitive inhibitors for mAC (21, 22). The crystallographic results described here show that MANT-GTP acts by a novel inhibitory mechanism in which the MANT substituent occupies a hydrophobic pocket at the interface between the homologous cytosolic domains that form the catalytic core of the enzyme. This pocket is not occupied by substrate and could...
the design of specific mAC inhibitors. At the same time, MANT-GTP also occupies the substrate nucleoside and triphosphate-binding sites, although by a different mode than that used by substrate analogs and P-site inhibitors.

On binding to VC1-IIC2, the MANT fluorophore of MANT-GTP stabilizes a conformation that is intermediate between the open and closed forms of the enzyme by blocking the movement of β1-α1-α2 and α3-β4 of VC1 toward β7'-β8' of IIC2. The partial collapse of these structural elements creates a hydrophobic binding site for the MANT substituent. The increase in blue-shifted MANT fluorescence that is observed upon binding of MANT-GTP to the enzyme is consistent with this mode of unification. The increase in inhibitory potency is substantially reduced (IC50 = 1,800 nM) if a bulky BODIPY group is attached to the γ-thiophosphate of GTP (21).

Mg2+ and Mn2+ both serve as co-factors for mAC catalytic activity (Table II), but the inhibitory potency of MANT-GTP and other MANT nucleotide inhibitors (with the exception of 2'-deoxy-3'-O-(N-methylanthraniloyl)-adenosine 5'-triphosphate) is 3-10-fold greater in the presence of Mn2+ compared with Mg2+. This increased inhibitory potency is paralleled by the magnitude of both intrinsic and FRET-stimulated emission from the MANT. No significant structural differences are apparent between the MANT-GTP-Mn2+ and MANT-GTP-Mg2+ complexes. However, MANT-GTP-Mg2+ has a relatively high temperature factor and overall weak electron density compared with the MANT-GTP-Mn2+. Although the resolution of the data is insufficient to allow accurate measurement of metal coordination bond lengths, it is possible that Mg2+, which possesses a smaller ionic radius than Mn2+, is more loosely tethered to coordinating groups in the enzyme.

To accommodate the fluorophore of MANT-GTP in the active site of VC1-IIC2, the nucleotide must bind in the reverse orientation to that exhibited by adenine-containing compounds. In this orientation, the guanine N-2 atom occupies the same position as the adenine N-6, and both serve as hydrogen bond donors to the carbonyl oxygen of Ile-1019. The protonated guanine N-1 atom donates a hydrogen bond to the carbonyl oxygen of Ile-1019.

### Table II

**Kinetic properties of VC1-IIC2 under various experimental conditions**

| Parameter | Man2+ | Man2+ + GTP | Man2+ + FSK | MAn2+ + FSK + GTP | MAn2+ + FSK | MAn2+ + GTP | MAn2+ + FSK + GTP | MAn2+ + FSK |
|-----------|-------|-------------|-------------|-------------------|-------------|-------------|-------------------|-------------|
| K<sub>N</sub> (µM) | 400 ± 55 | 550 ± 60 | 620 ± 130 | 430 ± 27 | 690 ± 110 | 760 ± 55 | 650 ± 130 | 850 ± 200 |
| V<sub>max</sub> (nmol/mg C1/min) | 99 ± 12 | 320 ± 50 | 3,700 ± 850 | 15,000 ± 2,800 | 2.3 ± 0.4 | 25 ± 2.9 | 25 ± 1.6 | 2,400 ± 340 |
| K<sub>IC50</sub> | 15,000 ± 2,200 | 170 ± 20 | 110 ± 16 | 12 ± 2.8 | 220,000 ± 23,000 | 860 ± 90 | 1,900 ± 130 | 110 ± 20 |
| 2'-3' MANT-GTP (µM) | 4,800 ± 800 | 900 ± 150 | 1,100 ± 200 | 210 ± 30 | 18,000 ± 1,500 | ND | ND | 300 ± 25 |
| MANT-ATP (µM) | 8,800 ± 2,500 | 610 ± 85 | 140 ± 11 | 133 ± 3.5 | 250,000 ± 18,000 | 1,200 ± 150 | 700 ± 100 | 340 ± 40 |
| MANT-XTP (µM) | 14,000 ± 1,700 | 4,300 ± 1,100 | 1,500 ± 700 | 1,100 ± 380 | ND | ND | 1,800 ± 220 | 2,900 ± 270 |

### Table III

**Inhibition of wild type and mutant VC1-IIC2 by MANT-nucleotides**

| Parameter | VC1 + IIC2 | VC1 + IIC2-11006V | VC1-A409P + IIC2 | VC1-A409P + IIC2-11006V |
|-----------|------------|-------------------|-----------------|--------------------------|
| K<sub>N</sub> (µM) | 430 ± 27 | 570 ± 74 | 530 ± 100 | 400 ± 28 |
| V<sub>max</sub> (nmol/mg C1/min) | 15,000 ± 2,800 | 11,000 ± 1,000<sup>a</sup> | 390 ND ND 1,800 | 25,000 ± 4,200<sup>a</sup> |
| K<sub>IC50</sub> | 12 ± 2.8 | 37 ± 0.7<sup>a</sup> | 27 ± 10<sup>a</sup> | 45 ± 14<sup>a</sup> |
| K<sub>IC50</sub> | 13 ± 3.5 | 84 ± 25<sup>a</sup> | 62 ± 7.8<sup>a</sup> | 100 ± 17<sup>a</sup> |
| K<sub>IC50</sub> | 1,100 ± 390 | 3,500 ± 210<sup>a</sup> | 2,900 ± 780<sup>a</sup> | 3,200 ± 500<sup>a</sup> |

<sup>a</sup> p < 0.05 for comparison of mutant combinations against wild type C1/C2 (t test).
MANT-substituted nucleotides are highly effective inhibitors of mAC and have potential for development as isofrom-specific inhibitors. The potency of MANT-GTP (IC$_{50}$ = $\sim$20 nm) is comparable with that of B-1',2',3'-dd-5'-ATP (IC$_{50}$ = $\sim$24 nm), which is among the strongest inhibitors so far identified (13, 20, 22). A comprehensive analysis of MANT-substituted nucleotides revealed that types V and VI mAC are $\sim$10-fold more selective for MANT-GTP than type II mAC (22). The difference in selectivity among these isofroms might reasonably be attributed to two residues, Ala-409 and Val-1108 in type V mAC, that are substituted by Pro and Ile, respectively, in type II mAC. These are the only residues at the MANT-binding site that are not conserved among mAC isofroms. Mutation of these residues affects the potency of MANT-GTP inhibition, consistent with their location in the inhibitor-binding pocket. As expected, MANT-GTP has less potency toward VC1-A409P-IIC2, which possesses a MANT-binding pocket similar to that of type II mAC, than toward VC1-IIC2. The reduction in the potency of the inhibitor is not reflective of a loss in the catalytic activity of the enzyme because of the mutation. Rather, the A409P mutation increases catalytic activity (Table III), consistent with partial restoration of type II-specific contacts across the domain interface. However, MANT-GTP is also less active toward VC1-IIC2-I1006V, which is expected to have a MANT-binding pocket similar to that of type V mAC. Enzyme activity is also compromised by this mutation. Thus, we are not able to elicit a “gain-of-function” response with respect to MANT-GTP inhibition by substitution of a single residue that partially restores a type V C1-C2 interface near the MANT-binding pocket. It is well to note that isofrom specificity toward MANT-GTP was demonstrated for mAC holoenzymes but was not explored for soluble domains (22). It is probable that the determinants of isofrom selectivity for MANT-GTP include residues distant from the inhibitor-binding site, as well as residues outside of the core catalytic domains (for example in C1b) that might influence interdomain contacts.

The structure of VC1-IIC2 bound to MANT-GTP has revealed a ligand-binding site that is not utilized by substrates and perhaps is therefore not highly conserved. It may be possible to design analogs of MANT-GTP to increase the strength and selectivity of binding at the MANT-binding site of mAC. Because a substantial amount of binding energy is derived from interactions at this hydrophobic site, modification or replacement of other nucleotide substituents might increase potency or specificity without compromising bioactivity. Aside from their potential as mAC inhibitors, MANT-GTP and its relatives are also convenient spectroscopic probes for mAC activation because they bind preferentially to the G$_{o}$- and FSK-bound conformational states of mAC.

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