Interactions of Forskolin and ATP with the Cytosolic Domains of Mammalian Adenylyl Cyclase*

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Fragments of the two cytoplasmic domains of mammalian adenylyl cyclases can be synthesized independently (and abundantly) as soluble proteins; Gsα and forskolin-stimulated enzymatic activity is restored upon their mixture. We have utilized this system to characterize the interactions of adenylyl cyclase with forskolin and its substrate, ATP. In the presence of Gsα, adenylyl cyclase is activated in response to occupation of only one forskolin-binding site. A single binding site for forskolin was identified by equilibrium dialysis; its KD (0.1 μM) corresponds to the EC50 for enzyme activation. The affinity of forskolin for adenylyl cyclase is greatly reduced in the absence of Gsα (~40 μM). Binding of forskolin to the individual cytoplasmic domains of the enzyme was not detected. A single binding site for the ATP analog, αβ-methylene ATP (Ap(CH2)pp), was also detected by equilibrium dialysis. Such binding was not observed with the individual domains. Binding of Ap(CH2)pp was unaffected by P-site inhibitors of adenylyl cyclase. A modified P-loop sequence located near the carboxyl terminus of adenylyl cyclase has been implicated in ATP binding. Mutation of the conserved, non-glycine residues within this region caused no significant changes in the Km for ATP or the Kd for Ap(CH2)pp. It thus seems unlikely that this region is part of the active site. However, a mutation in the C1 domain (E518A) causes a 10-fold decrease in the binding affinity for Ap(CH2)pp. This residue and the active site of the enzyme may lie at the interface between the two cytosolic domains.

Mammalian adenylyl cyclases have a characteristic membrane-bound topology, consisting of a short, intracellular amino terminus, followed by a set of six transmembrane spans, a large (~40 kDa) cytoplasmic domain (designated C1), a second set of six transmembrane spans, and a carboxyl-terminal cytoplasmic domain (C2), which is homologous to C1 (1, 2). However, very little direct structural information has been available until recently, and there is no knowledge of the number and nature of catalytic sites, as well as those for a plethora of activators and inhibitors. The elimination of several of the proteins’ domains, including the amino terminus and the transmembrane spans, has permitted expression of a soluble version of mammalian adenylyl cyclase (3, 4). This molecule displays many of the interesting regulatory features that characterize these enzymes: synergistic interactions between activators (e.g. between Gsα and forskolin) and inhibition by P-site inhibitors.

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1 The abbreviations used are: Gsα, the α subunit of the G protein that stimulates adenylyl cyclase; G protein, heterotrimeric guanine nucleotide-binding protein; GTPγS, guanosine 5’-[γ-thio]triphosphate; Ap(CH2)pp, αβ-methylene adenosine 5’-triphosphate; 2’-deoxy-3’AMP, 2’-deoxyadenosine 3’-monophosphate; 8-N,ATP, 8-azido-adenosine triphosphate.

Remarkably, these important regulatory properties and highly efficient catalysis are both displayed by a mixture of two independently synthesized ~25-kDa fragments of adenylyl cyclase, one each from C1 and C2. Both of these small proteins can be synthesized and purified in quantities sufficient to pose basic biochemical and structural questions (5–7).

All mammalian adenylyl cyclases are activated by forskolin, a naturally occurring diterpene (8). Forskolin binds to several intrinsic membrane proteins, including adenylyl cyclases, glucose transporters, voltage-gated K+ channels, ligand-gated ion channels, and P glycoproteins (9). It was believed that this hydrophobic molecule bound in or near the membrane spans, as it appears to do with P glycoproteins and glucose transporters (10, 11). However, construction and expression of the soluble mammalian adenylyl cyclase mentioned above permitted unequivocal demonstration that the capacity of this enzyme to be activated by forskolin resides within its cytoplasmic domain. Although there is no evidence that a forskolin-like molecule plays a physiological role in regulation of adenylyl cyclase activity, investigation of its interactions with the enzyme will contribute to comprehension of mechanisms of stimulation and synergistic regulation of catalytic activity. To date, we know that forskolin increases the apparent affinity of the C1 and C2 domains of adenylyl cyclase for each other and promotes much more efficient catalysis as a result of this protein-protein interaction; the same can be said of Gsα (5, 6).

Mystery also shrouds the ATP-binding site(s) of adenylyl cyclase. Devoid of any traditional P-loop or Walker motifs (12), the number and location of catalytic sites in the molecule are unknown, although its tandemly duplicated structure provides grounds for speculation. To date, we have demonstrated, not surprisingly, that only one molecule of ATP is consumed during the synthesis of one molecule of cyclic AMP and one molecule of pyrophosphate (4). In addition, all mammalian adenylyl cyclases are inhibited by adenosine and certain analogs thereof (13–15). The mechanism and location of this inhibitory site (the so-called P site) and its relationship to the substrate-binding site are also unknown.

We are now able to characterize the interactions of both forskolin and a nonsubstrate analog of ATP (Ap(CH2)pp) with the cytosolic, catalytic domains of mammalian adenylyl cyclase in a reasonably rigorous and quantitative manner. The results of these studies are described below, as are analyses of mutants that provide insight into the location of the catalytic site.

EXPERIMENTAL PROCEDURES

Materials—[α-32P]ATP (800 Ci/mmol), [12-3H]forskolin (31 Ci/mmol), and Ap(CH2)pp (20 Ci/mmol) were purchased from NEN Life Science Products. 

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Products. All tritiated compounds were lyophilized regularly to remove [3H]H2O. Unlabeled Ap(Ch2)pp was purchased from ICN.

Protein Purification—Recombinant Gs, IIC2, and VC1(591)Flag were purified from Escherichia coli as described (5, 7, 16). Recombinant Gs was activated by incubation with 50 mM NaHepes (pH 8.0), 10 mM MgSO4, 1 mM EDTA, 2 mM dithiothreitol, and 400 μM GTP-S; at 30 °C for 30 min. Free GTP-S was removed by gel filtration. GTP-S-activated Gs was utilized in all experiments, and the active protein concentration was determined by quantification of GTP-S binding. Preparations of Gs, utilized for titrations (Figs. 1 and 2) were 90% active. Gs was present in excess for other experiments and was typically 75% active.

Mutagenesis of IIC2 and VC1—IIC2 was subcloned into the pAlter-1 mutagenesis vector (Promega) by digestion of pQE60-IIC2H6 (5) with the restriction enzymes EcoRI and HindIII. VC1 was subcloned by digestion of pQE60-HVC1(364–591)Flag (7) with NcoI and HindIII and utilizing the vector, pAlter-IC1IIC2L3, which contained an NcoI site at the extreme 5′ end of the IIC2L3 clone (4). Mutations of IIC2 residues Tyr-1054, Arg-1058, and Lys-1065 to alanine were created using the oligonucleotides 5′-GCAGCGCTTGGGCACGCAGTCTACATGTC, 5′-CAGGTGTACCATGCCAGTATCATCAGTACATCATCAGTTG, and 5′-GGTATCATCAATGGGCAGTACCTAGGCGAACAG. Amino acids are designated according to their position in the full-length, membrane-bound adenylyl cyclases. The mutations R1059A and Y1064A of IIC2 were introduced to the expression plasmid pQE60-IIC2H6 using the restriction sites BsrGI and HindIII. Mutant K1065A was cloned with the restriction enzymes BstXI and HindIII. The mutation E518A of VC1 was placed in its appropriate expression vector with the restriction enzymes NcoI and HindIII. Each mutant cDNA was sequenced and expressed in E. coli strain BL21(DE3).

Adenylyl Cyclase Activity—Assays were performed as described (17) for 10–15 min at 30 °C in a final volume of 100 μl, unless stated otherwise. The final concentrations of free MgCl2 and MnCl2 were 10 and 1.8 mM, respectively. Activities are expressed per milligrams of the limiting adenylyl cyclase domain in the assay (VC1 or IIC2). For determination of kinetic constants, MgATP or MnATP was varied from 20 μM to 2.56 mM with a fixed excess of Mg2+ or Mn2+. Titrations of Gs or forskolin were performed using a high but limiting concentration of one cyclase domain (1.5–2 μM) and an excess of the second (2.9–3 μM). These assays were carried out at 4 °C (to slow catalysis at high protein concentrations) in a final volume of 50 μl. Reactions in which Gs was titrated contained 100 μM soluble form of forskolin, 6-O-[3′-(piperidino)propionyl]forskolin (Calbiochem), and serial dilutions of Gs from 31 nM to 6 μM (final concentration). Reactions in which forskolin was titrated contained 100 μM Gs and serial dilutions of forskolin as indicated, yielding a final concentration of 0.4% ethanol in the assay. Proteins and forskolin were first incubated for 6 min on ice before initiation of the reaction with 10 μM [α-32P]ATP. Reactions were terminated after 5 min with 850 μl of stop solution containing 0.25% sodium dodecyl sulfate, 5 mM ATP, and 0.175 mM cyclic AMP. [32P]Cyclic AMP was isolated by sequential chromatography on Dowex-50 and Al2O3. [32P]Cyclic AMP was added to monitor recovery of [32P]cAMP during purification.

Equilibrium Dialysis—Equilibrium dialysis chambers were purchased from Hoeffer. Chambers (60 μl) were separated by dialysis membrane with a cutoff of 14 kDa. To examine forskolin binding, each chamber contained 20 mM NaHepes (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol, and the indicated amount of [3H]forskolin (0.05–5 μM). One chamber contained all three proteins required for binding: 2 μM VC1, 4 μM IIC2, and 6 μM Gs;GTP-S; the opposite chamber contained either buffer alone or only 5 μM IIC2 or 4 μM VC1 and 6 μM Gs;GTP-S to minimize the effects of any nonspecific binding. Similar results were obtained with both methods. For measurements in the absence of GTP-S-Gs, one chamber contained 30 μM VC1 and 120 μM IIC2, while the opposite chamber contained either buffer alone or 120 μM IIC2. Samples were removed after dialysis for 24 h at 4 °C with rotation; duplicate 15-μl aliquots from each chamber were analyzed by liquid scintillation spectrometry.

Equilibrium dialysis reactions with the ligand Ap(Ch2)pp contained 20 mM NaHepes (pH 8.0), 0.65 mM MnCl2, 0.1 mM ascorbate, and the indicated amount of [3H]Ap(Ch2)pp (1–40 μM). One chamber included 30 μM each of VC1, IIC2, and GTP-S-Gs. Samples were processed as just described.

RESULTS

Titrations of VC1/IIC2 with Gs and Forskolin—To determine the number of binding sites for forskolin and substrate in a complex of VC1 and IIC2, it is essential to know the fraction of protein in each of the two preparations that is active, i.e., capable of forming a catalytically productive complex with its partner. We know that Gs, VC1, and IIC2 form a complex containing one molecule of each protein (7), and the fraction of purified Gs that is first readily measured by assessing radioactive guanine nucleotide binding. We thus titrated mixtures of VC1 and IIC2 with Gs or forskolin, observing the increase in activity caused by small incremental additions of activator. To obtain precise information, these experiments must be performed at concentrations well above the Kd for the interaction of the two domains of the enzyme and for the interaction of the activator with the enzyme. Thus, high concentrations of forskolin were included for titrations with Gs and vice versa. Experiments were performed with either a very high concentration of VC1 in excess of a high concentration of IIC2 (1.5 μM) or with a very high concentration of IIC2 in excess of a high concentration of VC1 (2 μM) (Fig. 2). Titrations with Gs (with either IIC2 (Fig. 1A) or VC1 (Fig. 2A) limiting) indicated that 80 and 75% of the protein in the preparations of IIC2 and VC1, respectively, was functional. Titrations with forskolin, performed with the same protein mixtures and at the same time, demonstrated that essentially identical amounts of forskolin and Gs were required to achieve maximal activity, indicating that only one molecule of forskolin is necessary for maximal enzyme activation (Figs. 1B and 2B).

Equilibrium Dialysis with Forskolin—We have quantified forskolin binding by equilibrium dialysis. Binding of the diterpene to either VC1 (40 μM) or IIC2 (120 μM) alone is not observed over the range of concentrations that could be explored (in the presence or absence of Gs). Successful experiments were performed at limiting concentrations of one of the cyclase...
domains and excess concentrations of the second to ensure maximal formation of VC1/IIC2 heterodimers (Fig. 3, A and B). The concentrations of the adenylyl cyclase domains required for association in the presence of Gs are relatively low (~2–4 μM). Regardless of which domain was limiting, it is clear that 1 molecule of forskolin binds to a C1/C2 heterodimer. The $K_r$ for forskolin binding, 0.09 ± 0.03 μM (n = 6), is very similar to the EC50 for activation of the enzyme (0.1 μM) (7) (data not shown).

We were not successful in attempts to detect a second binding site for forskolin using much higher protein concentrations than those used in Fig. 3. We estimate that we would have observed a second site if its $K_r$ was below 60 μM.

Quantification of forskolin binding in the absence of Gs is problematic. The EC50 for activation of the enzyme is much greater (~15 μM), and, more importantly, the apparent $K_r$ of C1 for C2 is 10 μM or greater in the absence of activators. The observed binding is shown in Fig. 4. The estimated $K_r$ (40 μM ± 20 μM, n = 6) compares reasonably (but not precisely) with the EC50 for activation of VC1/IIC2 in the absence of Gs, 15 μM. However, saturation was not achieved because of the insolubility of forskolin. In addition, one cyclase domain (C2) was present at very high concentrations (120 μM) to drive the interactions, and the proteins are less stable in the absence of additional activators.

**Inhibition of Adenylyl Cyclase by Ap(CH2)pp**—The ATP analog Ap(CH2)pp is a competitive inhibitor of adenylyl cyclase and is not utilized by the enzyme as a substrate. The IC50 for Ap(CH2)pp is dependent on the divalent cation utilized. In the presence of Gs, and Mg2+, the $K_r$ for Ap(CH2)pp is equal to the $K_m$ for ATP (320 μM). However, in the presence of Mn2+, the $K_r$ for Ap(CH2)pp is 15 μM, roughly 10-fold lower than the $K_m$ for ATP under these conditions (Fig. 5, Table I). Similar phenomena are observed when forskolin is the activator (Table I). These observations do not appear to be due to simple structural differences between ATP and Ap(CH2)pp, since the $K_r$ for Ap(CH2)pp is the same as the $K_m$ for ATP in the presence of Mg2+.

**Equilibrium Dialysis with Ap(CH2)pp**—Analysis of binding of [3H]Ap(CH2)pp to adenylyl cyclase by equilibrium dialysis in the presence of Gs and Mn2+ revealed a single binding site per C1/C2 heterodimer with a $K_r$ of 0.5 μM (n = 3), similar to the value of $K_r$ under these conditions (15 μM) (Fig. 6). There is no detectable binding of [3H]Ap(CH2)pp to either VC1 or IIC2 alone (data not shown).

Adenylyl cyclases are inhibited by direct interactions with adenosine analogs known as P (purine)-site inhibitors. This phenomenon is curious kinetically, since inhibition of VC1/IIC2 is uncompetitive with respect to MgATP and noncompetitive with respect to MnATP. A potent P-site inhibitor, 2′-deoxy-3'-
FIG. 4. Equilibrium dialysis measurements of forskolin binding in the absence of Gs. IIC2 (120 μM) and VC1 (30 μM) were incubated with 2.5–100 μM [3H]forskolin for 24 h at 4 °C and allowed to come to equilibrium with buffer containing [3H]forskolin or buffer containing 120 μM IIC2 plus [3H]forskolin in the opposite chamber (see “Experimental Procedures”). The estimated Kf was calculated by fit of a single hyperbola to the data. These results are representative of six experiments.

FIG. 5. Competitive inhibition of adenylyl cyclase by the substrate analog Ap(CH2)pp in the presence of Gαs and Mn2+. Assays (0.7 nM VC1, 1 μM IIC2) were performed in the presence of 1.8 mM free MnCl2, 400 μM activated Gs, 0.04–2.5 mM ATP, and no inhibitor (●) or 12.5 μM (■), 25 μM (▲), or 50 μM (▼) Ap(CH2)pp. Values for Kf and Vmax were determined by Eadie-Hofstee analysis, and replots (inset) of KfVmax (x intercept) versus the corresponding concentration of Ap(CH2)pp were used to determine the Kf for inhibition by Ap(CH2)pp.

AMP, was tested for its capacity to disrupt binding of Ap(CH2)pp. Although the Kf for 2′-deoxy-3′-AMP is 7 μM in the presence of Gαs and Mn2+, binding of [3H]Ap(CH2)pp was unaffected by 400 μM 2′-deoxy-3′-AMP (Fig. 6). This prototypical P-site inhibitor clearly does not compete for binding of ATP to the free enzyme or disrupt ATP binding in any capacity.

Mutations That Alter ATP Binding—Droste et al. (18) identified a 25-amino acid peptide near the carboxyl terminus of type I adenylyl cyclase (residues 1027–1051) that interacted with the photoaffinity probe [γ-32P]S-N3ATP. We have mutated each of the conserved, non-glycine residues within this region of IIC2 to alanine and measured the resultant effects on the Kf for ATP, Vmax, and Ki for Ap(CH2)pp in the presence of VC1 (Table II). All of the mutant proteins displayed synergistic activation in the presence of Gαs and forskolin, suggesting that there was no gross disruption of structure. The yields for two of the mutant proteins, K1065A and Y1054A, were poor, and they displayed greatly reduced activity. However, changes in Kf and Ki values were modest in the presence of Mg2+, and these values were normal with Mn2+. The yield of mutant R1059A was normal, as was its Vmax. Interestingly, its Kf for ATP and Ki for Ap(CH2)pp were elevated 10- and 3-fold, respectively, with Mg2+, but these values were identical to those for the wild type protein in the presence of Mn2+. Equilibrium dialysis studies with this protein demonstrate a normal Kf for Ap(CH2)pp binding in the presence of Gαs and Mn2+. The only other conserved residue in this sequence is Lys-1067, which is analogous to Glu-518 of VC1 and provided the opportunity to see if its affinity for substrate analog Ap(CH2)pp was determined as shown in Fig. 5 (see “Experimental Procedures”). The concentrations of proteins utilized in the absence of an activator were 13–73 nM VC1 and 5 μM IIC2.

![Graph](https://via.placeholder.com/150)

**Forskolin and ATP Binding to Adenylyl Cyclase**

**TABLE I**

| Activator | Mg²⁺ | Mn²⁺ |
|-----------|------|------|
| Kf (μM)   | Vmax (μmol/min/mg) | Kf (μM)   | Vmax (μmol/min/mg) |
| Gαs       | 1030 | 6.6  | 220 | 6.8  |
| Forskolin | 315  | 63   | 140 | 80   |

The Ki for Ap(CH2)pp was determined as shown in Fig. 5 (see “Experimental Procedures”). The estimated Kf was calculated by fit of a single hyperbola to the data. These results are representative of three experiments.
and forskolin, and its affinity for Gs, is altered by no more than 2-fold. The apparent affinity of this mutant C1 domain for IIC2 is also only slightly altered (Fig. 7). This is an important point, since the newly described crystal structure of a IIC2 homodimer suggests that this residue lies at the interface between the two domains (20).

**DISCUSSION**

The interaction of forskolin with soluble complexes of the cytosolic domains of adenylyl cyclase has been demonstrated by two independent methods. In the presence of Gs, only one forskolin molecule is required to activate the enzyme (Figs. 1 and 2) and only one binding site can be detected (Fig. 3); the Kd for this site corresponds to the EC50 for activation. Lower affinity sites were not detected at high concentrations of enzyme. In the absence of Gs, forskolin binds to a low affinity site with a Kd of approximately 40 µM, a value that is somewhat higher than the EC50 for enzyme activation under these conditions (15 µM). Although these latter experiments were technically difficult, this discrepancy might suggest that the binding curve (Fig. 4) reflects a mixture of contributions from nonidentical sites. If true, the hypothetical second (lowest affinity) site has no apparent effect on enzymatic activity.

Others have suggested the possibility of two binding sites for forskolin. Using membrane-bound type I adenylyl cyclase, Sutkowski et al. (21) identified two apparent binding sites for an isothiocyanate derivative of the diterpene. The high affinity (300 nM) site appeared to be silent, in that covalent binding of the isothiocyanate derivative of the diterpene. The high affinity site had no apparent effect on enzymatic activity. If true, the hypothetical second (lowest affinity) site that is occupied only in the presence of Gs, and a separate, low affinity site that is occupied only in its absence; occupation of either site activates the enzyme. The high affinity site observed by Sutkowski et al. (21), which is not linked to changes in enzymatic activity, might be characteristic of enzymes that do not display synergistic activation by forskolin. This site may be quite distinct from those that regulate catalysis and/or may reflect binding properties of the complete, membrane-bound enzyme. The low affinity site on type I adenylyl cyclase resembles that observed on the soluble VC1-IIC2 complex in the absence of Gs.

The homologous nature of the C1 and C2 domains suggests the possibility of two nucleotide-binding sites, as occur in P glycophorins (22) and cystic fibrosis transmembrane conductance regulator (23). Alternatively, a single ATP-binding site may be wholly contained within one domain or shared by the two domains at their interface. If there is a multiplicity of catalytic sites, the pure hyperbolic nature of plots of reaction velocity versus ATP concentration demands their near identity and a lack of interaction between them. Using equilibrium dialysis and a non-substrate ATP analog, Ap(CH2)pp, we have identified a single ATP-binding site. The affinity of nucleotide for this site is greatly influenced by the metal cofactor, as shown by the large difference in the Km for Ap(CH2)pp in the presence of Mg2+ and Mn2+. Gs, and forskolin do not have substantial effects on the Km for Ap(CH2)pp, suggesting that these activators do not influence substrate binding significantly. In the case of MnATP, the Km for ATP is only partially determined by substrate binding, as indicated by the difference between the Km for MnATP and the Km for ATP, this difference is not apparent when Mg2+ replaces Mn2+.

All mammalian adenylyl cyclases are inhibited directly by adenosine analogs known as P-site inhibitors (24), which display several unique features. Stimulated forms of the enzyme are substantially more sensitive to P-site inhibition than are nonactivated forms (13–15, 25). Inhibition is dependent on

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**TABLE II**

 Mutagenesis of putative ATP-binding sites

| Mutation       | Gs/Mg2+ | Gs/Mn2+ |
|----------------|---------|---------|
|                | Kd/µM   | Vmax/µM | Km/µM | Vmax/µM | Km/µM | Vmax/µM | Km/µM |
| Wild type      | 320     | 60      | 340   | 140    | 80    | 15     |
| Gs-Y1054A      | 750     | 4      | 720   | 110    | 28    | 6      |
| Gs-E1058A      | 2000    | 60     | 1000  | 150    | 160   | 11     |
| Gs-K1065A      | 1200    | 1     | 490   | 120    | 6    | 11     |
| VC1-E518A      | 3200    | 0.7    | 2000  | 1900   | 200   | 190    |

The activities of these proteins have been adjusted to reflect the amount of active enzyme in the preparations, based on forskolin binding by equilibrium dialysis.

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**FIG. 7. Interaction of wild type and E518A VC1, with IIC2.** The indicated concentrations of IIC2 were assayed in the presence of 0.7 mM VC1 (●) or VC1, E518A (○, □) for 10 min in the presence of 0.4 µM Gs (●, ○) or 100 µM forskolin plus 0.4 µM Gs (●, ■). The concentration of ATP was 2 mM and activity was assessed with 3.8 mM Mn2+ rather than Mg2+. Activities are expressed per mg of VC1.
metal and is characteristically uncompetitive or noncompetitive with respect to metal-ATP, depending on the activator and the metal (26). A large excess of a potent P-site inhibitor had no effect on either the amount of Ap(CH₂)pp bound or its affinity, as measured by equilibrium dialysis (Fig. 6). This result provides important clues to the mechanism of P-site inhibition, suggesting that the inhibitor must either bind at an intermediate point along the reaction coordinate or to an entirely separate site on the enzyme that does not mediate its inhibitory effects via alterations of substrate binding. Florio (27) and Florio and Ross (25) suggested the possibility of dead-end inhibition through the formation of an adenylyl cyclase-phospho-phosphate-inhibitor complex that would include both phosphate and a P-site inhibitor at the active site. Johnson and Shoshani (26) have proposed a distinct binding site for P-site inhibitors in which both substrate and inhibitor are bound simultaneously (26). Both models are supported by a great deal of kinetic data, and there is no current means to distinguish between the two.

Adenylyl cyclase lacks the conventional motif GXXXGKS, characteristic of nucleotide-binding sites, called the phosphate-binding or P-loop (12). However, an alternative sequence, G(X₉₋₁₋₂)KG(X₉₋₁₋₂)L(X₉₋₁₋₂)S/T, is conserved in mammalian, bacterial, and yeast adenylyl cyclases (28). A 25-amino acid peptide, located near the carboxyl terminus of type I adenylyl cyclase and overlapping with the modified consensus site, was identified as the labeling site for the photoactivatable ATP analog, [γ-³²P]-N₃ATP (18). We have mutated each of the conserved, non-glycine amino acid residues within the peptide region; none of these proteins exhibits any appreciable difference in its Kₘ for ATP or its Kₘ for Ap(CH₂)pp. Mutation of the key lysine residue in the P-loop of p2¹αβ or adenylyl kinase leads to a reduction in substrate binding of 100- or 20-fold, respectively, along with variable decreases in Kᵥ (29, 30). It thus seems unlikely that Lys-1065 (or Lys-1067, examined in Ref. 19) plays a role in substrate binding. Although no change in binding was observed, there was an appreciable change in Vₘₐₓ for 2 of these mutants (Y1054A and K1065A). Levin and Reed (31) identified this region as necessary for regulation of activity by protein kinase C. Mutation of this regulatory region may thus change catalytic activity.

Since neither isolated cytosolic domain of adenylyl cyclase can bind Ap(CH₂)pp, the interface of these two molecules is a logical spot for the active site. However, identification of this site has been difficult, and few Kₘ mutants of adenylyl cyclase have been identified. Of the 19 conserved residues that have been mutated to produce adenylyl cyclases that retain catalytic activity (Refs. 19 and 20, and this study), only 2 show any significant change in Kₘ when assayed in the presence of Mn²⁺. One of these (E518A of type V) is located in the C₁ domain and has a 10-fold reduction in its Kₘ for ATP. We have shown that this reduction in Kₘ is reflected in a reduction of substrate binding. Assuming a similar fold for both C₁ and C₂, Glu-518 is located near the interface of the two domains (20). The altered residue in the only other Kₘ mutant of adenylyl cyclase that has been identified (K923A in type I) and several other residues that are essential for catalysis are located in the C₂ domain but also at the interface between C₁ and C₂ (19, 20). Thus, the nucleotide-binding motif of adenylyl cyclase appears to differ greatly from the traditional motifs of G proteins, kinases, and other ATP-requiring enzymes. We presume that residues lining the interface between the two domains contribute to binding and catalysis. This arrangement may permit optimal linkage of small changes in conformation to large changes in catalytic activity.

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