Mutations Uncover a Role for Two Magnesium Ions in the Catalytic
Mechanism of Adenylyl Cyclase*

(Received for publication, March 30, 1998, and in revised form, May 5, 1998)

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The recent determination of the crystal structure of adenylyl cyclase has elucidated many structural features that determine the regulatory properties of the enzyme. In addition, the characterization of adenylyl cyclase by mutagenic techniques and the identification of the binding site for P-site inhibitors has led to modeling studies that describe the ATP-binding site. Despite these advances, the catalytic mechanism of adenylyl cyclase remains uncertain, especially with respect to the role that magnesium ions may play in this process. We have identified four mutant mammalian adenylyl cyclases defective in their metal dependence, allowing us to further characterize the function of metal ions in the catalytic mechanism of this enzyme. The wild-type adenylyl cyclase shows a biphasic Mg2+ dose-response curve in which the high-affinity component displays cooperativity (Hill coefficient of 1.4). Two mutations (C441R and Y442H) reduce the affinity of the adenylyl cyclase for Mg2+ dramatically without affecting the binding of MgATP, suggesting that there is a metal requirement in addition to the ATP-bound Mg2+. The results of this study thus demonstrate multiple metal requirements of adenylyl cyclase and support the existence of a Mg2+ ion essential for catalysis and distinct from the ATP-bound ion. We propose that adenylyl cyclase employs a catalytic mechanism analogous to that of DNA polymerase, in which two key magnesium ions facilitate the nucleophilic attack of the 3'-hydroxyl group and the subsequent elimination of pyrophosphate.

Intracellular levels of cAMP are primarily regulated at the level of its synthesis by adenylyl cyclase. cAMP, in turn, regulates a wide variety of cellular processes such as protein phosphorylation levels, gene expression, and ion channel conductance. Currently, nine isoforms of mammalian adenylyl cyclase have been identified by molecular cloning techniques (reviewed in Refs. 1–3), and they display a common deduced topology composed of a short cytoplasmic amino terminus followed by a region of six transmembrane domains (M1) and a large cytoplasmic loop (C1). This motif is then repeated with a second transmembrane region (M2) and a large cytoplasmic carboxyl terminus (C2). Sequence comparison has revealed that each cytoplasmic loop contains subdomains (denoted C1a and C2a) that are highly conserved among all adenylyl cyclase isoforms and that also display great homology to each other. The C1a and C2a domains can be expressed separately, and catalytic activity is reconstituted when they are mixed in vitro, although each domain by itself shows no activity (4, 5).

Many of the structural motifs of adenylyl cyclase responsible for catalytic activity and for the recognition of regulatory molecules are presently being uncovered. A region in the C2 domain of type II adenylyl cyclase (residues 956–982), for example, has been implicated in the binding of the G protein Gβγ subunits (6), whereas another region in the C1 domain of type I adenylyl cyclase (residues 495–522) appears to be involved in the regulation by calmodulin (7, 8). The binding sites for forskolin and Gsα, the two common activators of adenylyl cyclase, have recently been characterized by a combination of mutagenic techniques (9, 10) and the crystallographic analyses of the cytoplasmic domains of adenylyl cyclase (11, 12). The crystal structures of adenylyl cyclase also provided the basis for modeling the ATP-binding site of adenylyl cyclase (12, 13); however, these structures do not include the MgATP substrate, and several questions remain about the precise catalytic mechanism of this enzyme.

The structure of adenylyl cyclase was observed to be remarkably similar to that of DNA polymerase (14), despite low sequence homologies. This similarity is not surprising because both enzymes catalyze analogous chemical reactions, involving the in-line attack of a ribose 3'-hydroxyl on the α-phosphate of a nucleotide triphosphate, resulting in the release of a pyrophosphate group. DNA polymerase employs two key catalytic aspartate residues that bind two essential magnesium ions (15–17). One Mg2+ ion is associated with the nucleotide triphosphate, whereas the second one coordinates the 3'-hydroxyl, thus facilitating the nucleophilic attack. The positions of these aspartate residues are spatially conserved in the structure of adenylyl cyclase, where they were shown to bind to at least one Mg2+ ion (12), suggesting that the catalytic mechanism employed by both enzymes is also conserved. Modeling studies on the active site of adenylyl cyclase have predicted a single ATP-bound magnesium ion (12, 13) and suggest that the 3'-hydroxyl is deprotonated by an acidic residue. Although the crystal structure of adenylyl cyclase shows only a single Mg2+ ion (12), an alternative model in which a second Mg2+ ion promotes this deprotonation was acknowledged. Thus, the exact role and number of metal ions in the adenylyl cyclase-catalyzed reaction remain to be elucidated.

We have employed a genetic selection system for the identification of mutant adenylyl cyclases defective in their regulatory properties (10). This system relies on the expression of mammalian adenylyl cyclase in yeast (Saccharomyces cerevisiae) and makes use of the requirement of cAMP for growth in yeast (18). In this manner, we identified four mutant mamma-

* This work was supported by United States Public Health Service Grant GM53645 and by the Burroughs Wellcome Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: G protein, heterotrimeric guanine nucleotide-binding regulatory protein; Gαs, the α subunit of the G protein that stimulates adenylyl cyclase; GTPγS, guanosine 5'-O-(3-thiotriphosphate); ATPγS, adenosine 5'-O-(1-thiotriphosphate).
bian adenylyl cyclases displaying specific defects in their metal dependence. These mutations occur in highly conserved residues that are located in proximity to each other and to the two aspartate residues that coordinate magnesium. Characterization of the metal dependence of these mutants has shown that two of the mutations (C441R and Y442H) cause severe catalytic defects when assayed in the presence of magnesium, without compromising the ability of adenylyl cyclase to bind MgATP. Our data provide evidence for the existence of multiple magnesium-binding sites and strongly support a two-metal mechanism in which one metal ion is complexed with ATP, whereas the second plays a primarily catalytic role. Such a mechanism is remarkably similar to that identified for DNA polymerase, and we propose that this catalytic mechanism may be shared by a diverse family of enzymes involved in phosphoryl transfer reactions.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmid Construction, and Mutant Selection**—Yeast strain TC41-1 (MATa, leu2-3, leu2-112, ura3-52, his3, his4, cam1, cam2, cam3, cyr1 Δ::URA3) was a generous gift of Warren Heideman (University of Wisconsin, Madison, WI). The construction of an isogenic derivative of this strain expressing rat Gsα and the construction of the plasmid pADHprACVLeu encoding the dog type V adenylyl cyclase (20) were previously described (10). Randomly mutated libraries of plasmid pADHprACVLeu were generated by error-generating polymerase chain reaction mutagenic techniques as described elsewhere (10). Both the selection of type V adenylyl cyclase mutants and the retesting of these mutants in yeast were performed as described previously (10).

**DNA Sequencing**—Sequencing of the adenylyl cyclase mutants was performed using Thermal Sequenase (Amersham Pharmacia Biotech) following the procedures supplied by the manufacturer. In some cases, automated sequencing was performed using an Applied Biosystems sequencer.

**Sf9 Cell Culture and Preparation of Cell Membranes**—The procedure for the culture of Sf9 cells and the amplification of recombinant baculovirus have been outlined by Summers and Smith (21). Generation of recombinant baculovirus encoding both the wild-type and mutant type V adenylyl cyclases was performed using the Bac-to-BAC system (Life Technologies, Inc.) as described previously (10). Sf9 membranes containing individual adenylyl cyclase isoforms were prepared as described (22).

**Purification of G Protein Subunits**—Recombinant Gsα was synthesized in bacteria and purified as described by Lee et al. (23). Protein concentrations were estimated by staining with Amido Black (24). The a subunits were activated by incubation with GTPγS as described previously (25).

**Adenylyl Cyclase Assay**—Adenylyl cyclase activity was measured using the procedure of Smigel (26). All assays were performed for 10 min at 30 °C in a final volume of 100 μl containing 20 μg of membrane protein. The concentration of free metal ions was calculated with WinMaxC software that accounts for the buffering effects of 500 mM ATP and 400 μM EDTA in the reaction mixture.

**Adenylyl Cyclase Purification and Immunoblotting**—Wild-type and mutant type V adenylyl cyclase proteins were purified from Sf9 membranes using published procedures (22). Samples were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted as described (27) using a primary rabbit antibody specific for type V/VI adenylyl cyclase (28) as described previously (10).

**Data Analysis**—Data were analyzed using the GraphPad Prizm program to determine EC50 values and Hill coefficients of the dose-response curves.

**RESULTS AND DISCUSSION**

We have previously reported the development of a yeast genetic selection system for the identification of mutant adenylyl cyclases defective in their regulatory properties (10). Briefly, the system relies on the expression of mammalian type V adenylyl cyclase in a strain of yeast with a deletion in the essential CYR1 gene locus that encodes the yeast adenylyl cyclase (18); this strain is non-viable unless cAMP is added to the growth medium. Alternatively, the yeast cells are able to grow when the exogenously expressed mammalian adenylyl cyclase is activated by either forskolin or Gsα. Forskolin and Gsα activate type V adenylyl cyclase synergistically in vitro, and the simultaneous activation of the exogenous adenylyl cyclase in yeast by both activators results in a synergistic increase in intracellular cAMP levels. However, despite these high levels of cAMP, the yeast cells fail to grow, presumably due to toxic effects of cAMP at high concentrations. We have previously reported how this growth phenotype was used to identify mutant adenylyl cyclases defective in their regulatory properties by Gsα (10).

We have employed the yeast selection system to identify adenylyl cyclase mutants with defects in their catalytic activity. This involved expressing a library of randomly mutagenized adenylyl cyclase constructs in the CYR1-deleted yeast strain and selecting for transformants that grow in the presence of both forskolin and Gsα, but that have little or no growth in the presence of either activator alone (data not shown).
We reasoned that the mutations in some of these constructs may result in reduced catalytic activities while retaining sufficient activity in the presence of synergistic activation by Gs\textsubscript{a} and forskolin to support growth.

To determine whether the biochemical properties of the mutants could account for the altered growth phenotypes of the yeast, we introduced the mutant constructs into recombinant baculovirus and overexpressed them in Sf9 cells; membranes from these cells were then used as a source of adenylyl cyclase for biochemical studies. Initial characterization involved measuring the activity of the wild-type and mutant enzymes in response to various activators in the presence or absence of Mn\textsuperscript{2+}. For wild-type type V adenylyl cyclase, the addition of Mn\textsuperscript{2+} to adenylyl cyclase resulted in an enhanced basal and forskolin-stimulated catalytic activity, in agreement with previously published studies (29), with little or no effect on the Gs\textsubscript{a}-stimulated enzymatic activity (Fig. 1A). Four mutants (F423L, R434S, C441R, and Y442H) were identified that have common defects in their catalytic activity, displaying low activities in the presence of Mg\textsuperscript{2+}, while showing normal activities when Mn\textsuperscript{2+} was added (behavior exemplified by the C441R mutant) (Fig. 1B). This suggests that these mutants are not catalytically compromised, but instead display specific defects in their metal dependence.

Recent advances in the structural characterization of adenylyl cyclase have suggested different roles for metal ions in the catalytic mechanism of adenylyl cyclase. The determination of the crystal structure of the catalytic core of adenylyl cyclase has led to models predicting a single Mg\textsuperscript{2+} ion present in the MgATP substrate complex (12, 13). These structural data, however, did not elucidate how the 3'-hydroxyl is deprotonated, and the possibility that a second Mg\textsuperscript{2+} ion could assume this role was not excluded (12). The structure of the adenylyl cyclase catalytic core was shown to be highly similar to that of the DNA polymerase palm domain (14). DNA polymerase employs two magnesium ions as part of its catalytic mechanism; one is associated with the \(\beta\)- and \(\gamma\)-phosphates of the incoming nucleotide triphosphate, whereas the other coordinates to and activates the attacking ribose 3'-hydroxyl of the terminal nucleotide (reviewed by Steitz (17)). The homology of the structures of

\(2 \times 10^7\) yeast transformants were obtained following transformation of yeast with a library of randomly mutated adenylyl cyclases (library complexity = \(4 \times 10^5\)). 52 colonies were isolated for their ability to grow in the presence of simultaneous forskolin and G\textsubscript{a} activation.
Metal Dependence of Mammalian Adenylyl Cyclase

Table I

| Mutant       | Relative expression* | Mg2+ EC50 b | Mn2+ EC50 b | MgATP Kc, c | MnATP Kc, c | ATPoS Kc, d |
|--------------|----------------------|-------------|-------------|-------------|-------------|-------------|
| WT           | % of WT              | µM          | µM          | µM          | µM          | µM          |
| F423L        | 100 ± 0              | 400         | 40          | 78 ± 4      | 46 ± 1      | 36 ± 5      |
| R434S        | 103 ± 6              | 3000        | 54          | 689 ± 71    | 74 ± 24     | 165 ± 7     |
| C441R        | 96 ± 11              | 5400        | 47          | 1280 ± 11   | 109 ± 12    | 812 ± 60    |
| Y442H        | 209 ± 20             | 30,000      | 430         | 176 ± 6     | 73 ± 2      | 63 ± 3      |
|              | 215 ± 22             | 36,000      | 580         | 190 ± 11    | 52 ± 8      | 60 ± 11     |

* Expression levels of active type V adenylyl cyclase mutants in S99 membrane preparations were determined by Western blotting and are reported as the percent of the wild-type expression level. As previously described (10), relative expression levels were calculated by determining the activity of the membrane preparations and dividing this value by the specific activity of the wild-type and mutant enzymes. First, purified wild-type or mutant adenylyl cyclase protein (equivalent activities) were quantitated by Western blot analysis to calculate the specific activities of the mutants relative to the wild-type enzyme. Relative specific activity = (catalytic activity loaded on Western blot/optical density of cyclase band as % of wild type). Next, expression levels were calculated by dividing the activity of S99 membrane preparations by the specific activity of the wild-type and mutant enzymes. Relative expression level = (catalytic activity of S99 membranes/relative specific activity of mutant cyclases).

b The EC50 values of adenylyl cyclase for magnesium and manganese were calculated by fitting dose-response curves (see Fig. 2) using GraphPad Prizm software.

c Kc values for MgATP and MnATP were calculated from dose-response curves performed in the presence of either 20 mM MgCl2 or 3 mM MnCl2 using GraphPad Prizm software. The plots comprise 14 points with ATP concentrations ranging from 0.25 to 2000 µM.

d Kc values for ATPoS were calculated by performing Dixon plots on ATPoS dose-response curves that were performed at two ATP concentrations (30 and 180 µM for the wild type, C441R, and Y442H and 1000 and 3000 µM for F423L and R434S) in the presence of 20 mM MgCl2. Each plot comprised 10 points ranging from 0 to 2000 µM ATPoS, and Kc values were determined as described elsewhere (53). Data are representative of at least three experiments.

WT, wild type.

adenylyl cyclase and DNA polymerase and the similarities in the chemical reactions they catalyze have led to the prediction that these enzymes share the same catalytic mechanism.

To elucidate the role of metal ions in the catalytic mechanism of adenylyl cyclase, we characterized the metal dependence of the wild-type and mutant enzymes by measuring enzymatic activity at varying levels of Mg2+ or Mn2+ (Fig. 2). As shown in Fig. 2A, the forskolin-stimulated wild-type adenylyl cyclase displayed a biphasic dose-response curve with a high-affinity component (EC50 ~ 400 µM) and a low-affinity component that only became apparent at non-physiological concentrations of free Mg2+ (>10 mM). In contrast, we only observed a single magnesium component for the Gαs-stimulated adenylyl cyclase (EC50 ~ 300 µM), and concentrations of Mg2+ above 2 mM actually inhibited this activity (data not shown).

All four adenylyl cyclase mutants displayed a rightward shift in their Mg2+ dose-response curves (Fig. 2, A and B), indicating a reduced affinity for the metal ion. The mutants can be grouped according to the severity of this shift, with the F423L and R434S mutants (group I) showing more moderate effects (10–20-fold increase in the EC50 for Mg2+) (Fig. 2A and Table I) and the C441R and Y442H mutants (group II) displaying more severe shifts (~100-fold increase in the EC50 for Mg2+) (Fig. 2B and Table I) in their Mg2+ dependence. All mutants displayed dramatically reduced activities when the dose-response curve was repeated in the presence of Gαs (data not shown), consistent with the observed inhibition of Gαs-stimulated activity at the high concentrations of Mg2+ required to observe forskolin-stimulated activity.

Next, we measured the activity of the wild-type and mutant adenylyl cyclases at varying concentrations of Mn2+ (Fig. 2, C and D) to evaluate the strong Mn2+ dependence of these mutants. In this case, the wild-type enzyme displayed a dose-response curve with an EC50 of 40 µM, and manganese inhibited at lower concentrations (>1 mM) than magnesium. The group I mutants with moderate Mg2+ defects (F423L and R434S) showed a Mn2+ dependence that was almost identical to that of the wild-type enzyme (Fig. 2C), with EC50 values that did not differ substantially from the wild-type value (Table I). The group II mutants (C441R and Y442H) showed an increase in the EC50 for Mn2+ (10-fold) (Table I), which paralleled the reduction in their affinity for Mg2+, although the latter effect was more severe (100-fold increase in Mg2+ EC50 versus 10-fold shift in Mn2+ EC50). Taken together, these data suggest that the disrupted metal-binding site(s) of these mutants are better able to accommodate manganese than magnesium.

The dose-response curves we observed in Fig. 2 could be due to the formation of the metal-ATP substrate during the titration with magnesium or manganese. However, the Mg2+ dependence of the wild-type adenylyl cyclase did not appear to be determined solely by the formation of MgATP because the EC50 of the Mg2+ dose-response curve (400 µM) was significantly higher than the Kc of ATP for Mg2+ (64 µM). In addition, we observed little or no catalytic activity at concentrations of Mg2+ sufficient to produce substrate (MgATP) at up to three times the Kc value of adenylyl cyclase for MgATP (80 µM) (Table I). These results are consistent with previous reports documenting that Mg2+ concentrations in excess of ATP are required for catalytic activity in cyc7 cells of S49 murine lymphoma (30). Closer examination of the high-affinity component revealed non-hyperbolic behavior, with a Hill coefficient of 1.4, indicating multiple Mg2+ sites (at least two) that display cooperative binding. Our data on the wild-type enzyme therefore suggest a role for a second Mg2+ ion in the catalytic mechanism of adenylyl cyclase in addition to the one present in the MgATP substrate complex.

If adenylyl cyclase utilizes only a single magnesium ion, present in the MgATP substrate complex, reducing the affinity of adenylyl cyclase for this Mg2+ should also lower the enzyme’s affinity for MgATP. To evaluate this prediction, we measured the Km values of the wild-type and mutant adenylyl cyclases for MgATP. As depicted in Table I, the mutants can once again be placed into two groups based on the severity of the shift in their Km values. In this case, however, the severity of the shift in the Km for MgATP was opposite from that seen in the EC50 for Mg2+. The group I mutants (F423L and R434S) displayed moderate increases both in the EC50 for Mg2+ and in the Km for MgATP, whereas the group II mutants (C441R and Y442H) displayed a dramatic increase in the EC50 for Mg2+, but showed only a minor increase in their Km for MgATP. In contrast, none of the mutants displayed significant changes in their Km for MnATP (Table I), consistent with the small effect these mutations have on enzymatic activity in the presence of Mn2+.

To confirm that the changes in Km correspond to defects in the binding of MgATP, we measured the K of these cyclase mutants for the competitive inhibitor ATPoS in the presence of Mg2+. We found that the group I mutants (F423L and R434S)
showed a large increase in the $K_i$ for ATP$aS$, whereas the group II mutants (C441R and Y442H) displayed only a moderate reduction in their affinity for ATP$aS$, paralleling the changes in $K_m$ values for MgATP (Table I). Because the $K_i$ values for the inhibitor are primarily dependent on the binding affinity of the enzyme for this compound, these data indicate that the binding of MgATP to the group II mutants is relatively unaffected, whereas the group I mutants have a reduced affinity for MgATP.

The large increase in the $K_m$ for MgATP and the $K_i$ for ATP$aS$ of F423L and R434S suggests that the Mg$^{2+}$ defect of these mutants may be primarily reducing the affinity of adenylyl cyclase for MgATP, and we did indeed observe normal activities when the MgATP concentrations were raised to 3 mM (data not shown). This interpretation also explains the normal activity of the mutants in the presence of Mn$^{2+}$ because the $K_m$ for MnATP is not affected by the mutations (Table I). The dramatic reduction in the affinity of the C441R and Y442H mutants for Mg$^{2+}$, however, does not appear to reduce the affinity of adenylyl cyclase for MgATP, suggesting that this defect affects the binding of a Mg$^{2+}$ ion that is essential for catalysis, but not bound to ATP. It is possible that this additional metal requirement is due to an allosteric effect, but the similarities between adenylyl cyclase and DNA polymerase suggest that the second Mg$^{2+}$ ion fulfills the same function as the ion coordinating the attacking 3'-hydroxyl in DNA polymerase.

We next examined the position of the mutated residues in the structure of adenylyl cyclase. All four mutations occur in the C1a cytoplasmic domain of adenylyl cyclase that is highly conserved among all isoforms of adenylyl cyclase as well as the catalytic domains of guanylyl cyclase. Sequence comparison of the nine isoforms of mammalian adenylyl cyclase and the Droso- phila rutabaga gene product showed that the four mutated residues are absolutely conserved among these enzymes. Interestingly, the group II mutants are immediately adjacent to Asp-440, which is equivalent to one of two key catalytic Asp residues that bind Mg$^{2+}$ in DNA polymerase (14). The function of these Asp residues appears to be at least partially conserved in adenylyl cyclase because Asp-440 and Asp-396 were also shown to coordinate to Mg$^{2+}$ in the adenylyl cyclase crystal structure (12).

The crystal structure of adenylyl cyclase demonstrates that all four mutated residues, as well as the two Mg$^{2+}$-binding aspartate residues (Asp-440 and Asp-396), occur in the same region of adenylyl cyclase (Fig. 3). ATP and the Mg$^{2+}$ ion coordinating to the 3'-hydroxyl are not part of the crystal structure and are depicted in their approximate positions based on the binding sites for pyrophosphate and the P-site inhibitor 2'-deoxy-3'-adenosine monophosphate (12). Asp-440 is located at the top of a $\beta$-turn formed by the $\beta$2 and $\beta$3 strands, whereas Cys-441 and Tyr-442 are immediately adjacent on the $\beta$3 strand. It seems likely that mutations in these residues will result in conformational changes of the $\beta$-turn, thus disrupting the orientation of Asp-440 and consequently reducing the affinity of the metal-binding pocket for Mg$^{2+}$. Phe-423 and Arg-434 are more distant from these aspartate residues and are buried beneath the adenylyl cyclase active site. Arg-434 forms part of the $\beta$2 strand, whereas Phe-423 is located on the preceding $\alpha$2 helix. The side chains of these residues lie in close proximity to that of Tyr-442, which points away from the catalytic site.

The direct mutagenesis of either catalytic Asp residue in type I adenylyl cyclase was shown to result in inactive enzymes (13, 31) and thus does not elucidate the function of these residues in catalysis. Our functional data, however, suggest that the group II mutations (C441R and Y442H) reduce the affinity of adenylyl cyclase for metal ions by disrupting the orientation of Asp-
440, confirming the essential role that this residue plays in binding Mg\(^{2+}\). The group I mutants (F423L and R434S), on the other hand, are more distant from the catalytic Asp residues and may affect the Mg\(^{2+}\)-binding pocket via more global conformational changes. The Mg\(^{2+}\) defect of these mutants is primarily reflected in a reduced affinity for MgATP, and thus, it seems likely that the mutations may be reducing the affinity of the aspartic acid residues for the ATP-bound Mg\(^{2+}\). The residue equivalent to Arg-434 was previously targeted for mutagenesis in type I adenylyl cyclase (R348A in type I adenylyl cyclase) (31). In contrast to our data, the mutant shows normal activity and displays no change in the \(K_m\) for MgATP, indicating that different amino acid substitutions at this position can lead to very different phenotypes. These mutagenic studies (31) also targeted Glu-518 and Arg-484 (Glu-432 and Arg-398 numbering in type I adenylyl cyclase), producing mutants that also display a strong manganese dependence. The recent availability of detailed structural information reveals that these residues are in close contact to Asp-396 (type V numbering), indicating that the effect of these mutations may be due to a disruption of the orientation of this aspartate residue. These results therefore support the role of Asp-396 in coordinating metal ions, as proposed by the crystallographic analysis of adenylyl cyclase (12).

The characterization of DNA polymerase by mutagenic techniques has also focused on residues adjacent to the key catalytic aspartic acid (Asp-1004 in DNA polymerase α) equivalent to Asp-440 in type V adenylyl cyclase. Mutating either residue adjacent to Asp-1004 in human DNA polymerase α (32) results in a phenotype remarkably similar to that observed for the C441R and Y442H mutants. In both cases, the mutant enzymes display low activity in the presence of Mg\(^{2+}\), but normal activity in the presence of Mn\(^{2+}\). In addition, the mutations in DNA polymerase α do not alter the \(K_m\) of the enzyme for the MgdNTPs, indicating that the binding of nucleotides remains relatively unaffected. Mutations of the catalytic aspartate residue (D1004A) results in a non-functional DNA polymerase, emphasizing the crucial role of this residue in catalysis and paralleling the effects of this mutation in adenylyl cyclase (31). These striking similarities are likely the result of a shared catalytic mechanism between adenylyl cyclase and DNA polymerase.

Our data on the metal dependence of the wild-type and mutant adenylyl cyclases suggest a two-metal catalytic mechanism similar to that of DNA polymerase (Fig. 4). According to this model, the enzyme utilizes two Mg\(^{2+}\) ions, one associated with the β- and γ-phosphates of ATP (denoted site B in Fig. 4) and one catalytic Mg\(^{2+}\) ion primarily associated with the enzyme (denoted site A). In analogy to DNA polymerase, we predict that the Mg\(^{2+}\) at site A will coordinate to the 3'-hydroxyl of ATP, promoting the nucleophilic attack of this group on the α-phosphate, whereas the Mg\(^{2+}\) ion at site B assists in the leaving of the pyrophosphate group and may also facilitate the cleavage of the bond between the α- and β-phosphates by its electron-withdrawing effect. Both metal ions are also responsible for stabilizing the negative charge associated with the pentavalent transition state of this chemical reaction. We propose that this model describes a highly conserved catalytic mechanism for a diverse group of enzymes, including DNA and RNA polymerases as well as guanylyl and adenylyl cyclases, that catalyze analogous phosphoryl transfer reactions.

Acknowledgments—We thank Dr. R. Green for the rabbit antibody specific for type VNI adenylyl cyclase; J. Spanbauer for technical assistance; Dr. E. Fauman for help with molecular modeling analysis of the adenylyl cyclase structure and figures; and Drs. R. Neubig and M. Marletta for help with data analysis, thoughtful discussion, and critical reading of the manuscript.

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