Covalent Labeling of Adenyl Cyclase Cytosolic Domains with γ-Methylimidazole-2′,5′-dideoxy-[γ-32P]3′-ATP and the Mechanism for P-site-mediated Inhibition

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A truncated first cytosolic domain of type V adenyl cyclase (VC1) and a truncated second cytosolic domain of type II adenyl cyclase (IIC2) were used alone and in the readily reversible complex (VC1·IIC2) to evaluate interactions with each other and with reversible and irreversible P-site ligands. Enzyme activity was used to assess formation and dissolution of VC1·IIC2. The data suggest that binding of 2′,5′-dideoxy-3′-ATP to VC1 and IIC2 prevented formation of VC1·IIC2 and that 2′,5′-dideoxy-3′-ATP dissociation occurred slowly. To enable configuration specific cross-linking to the catalytic site, 2′,5′-dideoxyadenosine 3′-β-[γ-(1-methylimidazole)-triphosphate] (γ-MetIm-2′, 5′-dd-3′-ATP) and 2′,5′-dd-adenosine 3′-(γ-azidoanilido)-triphosphate (γ-azidoanilido-2′,5′-dd-3′-ATP) were synthesized, the former also as its γ-32P-labeled analog. γ-Azidoanilido-2′,5′-dd-3′-ATP exhibited an inhibitory potency comparable with that of 2′,5′-dd-3′-ATP. γ-MetIm-2′, 5′-dd-[γ-32P]3′-ATP labeled the individual VC1 and IIC2 domains comparably and covalently to 20% within 1 h. Formation of VC1·IIC2 resulted in reduced labeling of VC1 but enhanced labeling of IIC2. The data imply that formation of VC1·IIC2 complex affects the interaction of each domain with the 2′,5′-dd-3′-ATP, the binding of which also affects the interaction between the two cytosolic domains, leading to a pseudo-irreversible inhibition.

The proposed topology of mammalian adenyl cyclases suggests a tandem repeat of a membrane-spanning region and a cytosolic domain, with the two cytosolic domains (C1 and C2) sharing significant sequence homology (1). When the C1 domain of the type V isozyme (VC1) and the C2 domain of the type II isozyme (IIC2) are expressed separately in Escherichia coli and then recombined, a functional adenylyl cyclase is formed upon their association in solution (2). This truncated chimeric construct exhibits stimulation by Gs and Gt and then recombined, a functional adenylyl cyclase is formed.

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ammonium salt of 2',5'-dd-[g-32P]3'-ATP (200 to 400 cpm/pmol, by Cherenkov radiation) was dissolved in 100 μl of dimethyl sulfoxide. The 3'γ-phosphate of the nucleotide was activated by addition of 10 μmol of N,N'-dicyclohexylcarbodiimide and 0.5 μmol of pyridine-HCI. After a 45-min incubation period at room temperature, N,N'-dicyclohexylurea was removed by centrifugation, and 10 μl of 12.6 μl of 1-methylimidazole was added to the reaction mixture. This mixture was incubated for an additional 45 min and γ- MetIm-2',5'-dd-3'-ATP was precipitated by 500 μl of cold acetone containing 2% NaClO 4. The precipitate was washed twice with 500 μl of cold acetone, dried in a SpeedVac, dissolved in water, and used in this form for covalent labeling of adenylyl cyclase cytosolic domains.

**γAzidoanilido-2',5'-dd-3'-ATP**—Syntheses of unlabeled and γ-32P-labeled γ-azidoanilido-2',5'-dd-3'-ATP (Fig. 1) were similar to those for γ-MetIm-2',5'-dd-3'-ATP above. After activation of the γ-phosphate of unlabeled (0.1 μmol) or γ-32P-labeled 2',5'-dd-3'-ATP (200–400 cpm/pmol, by Cherenkov radiation) by 10 μmol N,N'-dicyclohexylcarbodiimide as above, the reaction mixture was treated with 0.5 μl p-azidoaniline hydrochloride titrated by triethylammonium (1/1, mol/mol). After 2 h at 22 °C the product and unreacted 2',5'-dd-3'-ATP were precipitated as described above. Product γ-azidoanilido-2',5'-dd-3'-ATP was isolated by high pressure liquid chromatography anion exchange chromatography (TosoHaas, 10 μmol DEAE-SW, 7.5 × 75 mm) developed with a 50-mI gradient of triethylammonium bicarbonate (50–500 mM, pH 8.5). Appropriate fractions were collected and triethylammonium bicarbonate was removed by repetitive evaporation from methanol.

**VC 1 and IIC 2 Domains of Adenylyl Cyclase—**Recombinant VC 1 and IIC 2 were expressed in E. coli cells and purified as described (2). Isolated proteins were electrophoretically homogeneous with apparent molecular masses of 30 and 26 kDa for VC 1 and IIC 2, respectively.

**Assay of Adenylyl Cyclase—**Adenylyl cyclase assays followed previously described procedures (19). Reactions were in a medium containing 50 mM HEPES, pH 7.5, 5 mM MnCl 2, 100 μM forskolin, 0.5 mM [α-32P]5'-ATP (10–60 cpm/pmol, by Cherenkov radiation), 1 μM VC 1, and 1 μM IIC 2, in a volume of 30 μl for 5 min at 30 °C. Reactions were started by the addition of VC 1–IIC 2 complex and were terminated by the addition of zinc acetate and sodium carbonate. [32P]AMP was purified by sequential chromatography on Dowex 50 and Al(OH) 3, and was quantified by Cherenkov radiation in a liquid scintillation spectrometer.

**Determinations of Stability of the VC 1–IIC 2 Complex—**Adenylyl cyclase activity was used as a measure of the formation of the active VC 1–IIC 2 complex. Association of the VC 1–IIC 2 complex was achieved in a reaction mixture containing 50 mM HEPES, pH 7.5, 5 mM MnCl 2, 0.1 mM forskolin, 2 μM VC 1, and 2 μM IIC 2 in a volume of 30 μl for 2 min at 22 °C. VC 1–IIC 2 complex formation was evaluated in the absence or presence of 10 μM 2',5'-dd-3'-ATP, 1 mM 5'-ATP, or in the absence of nucleotides and then after sequential treatment either with 10 μM 2',5'-dd-3'-ATP followed by 1 mM 5'-ATP, or with 1 mM 5'-ATP followed by 10 μM 2',5'-dd-3'-ATP, for 2 min at 22 °C per each ligand. Dissociation of the VC 1–IIC 2 complex was initiated by dilution of the enzyme–reaction mixture 20-fold with 50 mM HEPES, pH 7.5, 5 mM MnCl 2 and 100 μM forskolin. At various times after dilution of the enzyme–reaction mixture, 30 μl portions were added to 3 μl of 10 μl [α-32P]5'-ATP (10–20 cpm/pmol, by Cherenkov radiation). To estimate adenylyl cyclase activity before dilution, portions of the enzyme were taken directly from the complex formation mixture and were added to an adenylyl cyclase assay mixture containing 50 mM HEPES, pH 7.5, 5 mM MnCl 2, 100 μM forskolin, and 0.9 mM [α-32P]5'-ATP (10–20 cpm/pmol, by Cherenkov radiation). Adenylyl cyclase activity was then determined as above.

**Covalent Labeling of VC 1 and IIC 2 Domains with γ-MetIm-2',5'-dd-[γ-32P]3'-ATP—**Labeling of adenylyl cyclase cytosolic domains with γ-MetIm-2',5'-dd-[γ-32P]3'-ATP was performed in a reaction mixture containing 50 mM HEPES, pH 7.5, 5 mM MnCl 2, 100 μM forskolin, 10 μM γ-MetIm-2',5'-dd-[γ-32P]3'-ATP (200–400 cpm/pmol, by Cherenkov radiation), 5 μM VC 1 and 5 μM IIC 2 in a volume of 30 μl at 30 °C. The reaction was started by the addition of γ-MetIm-2',5'-dd-[γ-32P]3'-ATP to a solution of either VC 1, IIC 2, or the VC 1–IIC 2 complex previously formed for 2 min at 22 °C. When ligands were tested for their ability to protect against covalent labeling, they were added before γ-MetIm-2',5'-dd-[γ-32P]3'-ATP for 2 min at 22 °C. The covalent labeling was stopped by the addition of 0.1% SDS, 100 mM dithiothreitol, and 5% glycerol and then placed in a water bath at 65–70 °C for 3 min. Proteins and unreacted nucleotides were separated on an 11% polyacrylamide SDS gel. Labeled protein bands were quantified after a 3–5-h exposure on a PhosphorImager (Molecular Dynamics) and use of the accompanying ImageQuant software. Alternatively, bands corresponding to VC 1 or IIC 2 were cut from gels and quantified by Cherenkov radiation in a scintillation spectrometer.

**RESULTS**

**Effects of 5'-ATP and 2',5'-dd-3'-ATP on the Dissociation of Adenylyl Cyclase Cytosolic Domains—**The association of C 1 and C 2 cytosolic domains of adenylyl cyclase yields the catalytically competent VC 1–IIC 2 complex in a reversible process with an apparent K d ~1 μM (2). Because the individual VC 1 and IIC 2 domains do not exhibit meaningful catalytic activity (2), adenylyl cyclase activity could be used to estimate the amount of the functionally active VC 1–IIC 2 complex. This is demonstrated in the readily reversible nature of the active complex upon 20-fold dilution of the preformed VC 1–IIC 2 complex (Fig. 2, control plot). The dilution was from 2 μM enzyme to a concentration of each cytosolic domain that was an order of magnitude below the K d and resulted in a dissociation that occurred rapidly with >50% inactivation occurring by the time of the earliest sampling at 15 s. When VC 1 and IIC 2 were added separately to a solution already containing 10 μM 2',5'-dd-3'-ATP (plot 2) the resulting adenylyl cyclase activity was low and remained low after dilution. This suggested that the preassociation of inhibitor with enzyme prevented the formation of a functionally active VC 1–IIC 2 complex. However, when 10 μM 2',5'-dd-3'-ATP was added to a preformed VC 1–IIC 2 complex just before dilution (Fig. 2, plot 3), initially measured activity was reduced to approximately 15% of the value seen without the inhibitor (compare values at 0 min for control and plot 3). Upon dilution there may have been a small increase in activity in the 15 s sample, conceivably due to dissociation of weakly bound inhibitor, but thereafter the decrease in adenylyl cyclase activity occurred at a significantly lower rate than was seen with control enzyme in the absence of 2',5'-dd-3'-ATP. Even after 60 min the activity of the enzyme exposed to inhibitor remained higher than that of the control enzyme (Fig. 2, plot 3). A similar behavior was observed if, just before dilution, the preformed VC 1–IIC 2 complex was treated sequentially with 2',5'-dd-3'-ATP and then 5'-ATP (Fig. 2, plot 4), suggesting that even 1 mM 5'-ATP did not accelerate dissociation of the inhibitor. Thus, treatment of the VC 1–IIC 2 complex with 2',5'-dd-3'-ATP followed by 5'-ATP neither activated the enzyme nor affected the rate of subunit dissociation, when compared with enzyme treated with 2',5'-dd-3'-ATP alone (Fig. 2, plots 3 and 4).

These data suggest that the control plot reflects the rapid dissociation of the VC 1–IIC 2 complex, whereas the slow rate of activity loss observed in plot 3 might well reflect the slow rate of dissociation of 2',5'-dd-3'-ATP from the VC 1–IIC 2 enzyme complex, because activity was higher than that seen when the
The behavior of the VC1-IIC2 complex when treated with 5'-ATP (Fig. 3) was different from that above. The dissociation data of control VC1-IIC2 enzyme formed in the absence of nucleotides (Fig. 3, control plot) are the same as shown in Fig. 2, control plot. If the enzyme was treated with 5'-ATP just before dilution, though, there was a notable 2.5-fold greater initial activity (compare initial values in plots 1 and 3) and the half-time of complex dissociation was about 10 min (Fig. 3, plot 1). The apparent increase in enzyme activity was not due to an effect of carryover 5'-ATP into the assay mixture because this would represent at maximum an 11% increase in the 900 μM substrate concentration used in these assays and the $K_d$ for 5'-ATP for this chimeric construct has been reported to be in the range of 72–220 μM in assays with Mn^{2+} as cation (4). It is more likely that substrate and/or products formed during the 2-min pretreatment period stabilized the VC1-IIC2 enzyme complex or facilitated its formation. Under these reaction conditions 50% of the substrate was converted into cAMP and PP, during the treatment of the VC1-IIC2 complex with 5'-ATP for 2 min at 22 °C (not shown). This implies that the exchange of 5'-ATP, cAMP, and PP, between adenylyl cyclase and solution is a rapid process. Otherwise, saturation of the active site with nonradioactive substrate and products would have effectively restricted the formation of $[^{32}P]cAMP$ from $^{32}P$-labeled substrate in the subsequent adenylyl cyclase assay. Because these reactions were conducted with 2 μM for each VC1 and IIC2 and the $K_d$ ~1 μM, approximately half the concentration of each cytosolic domain will be bound in the VC1-IIC2 complex. The VC1-IIC2 complex was formed in the presence of inhibitor (plot 2). Alternatively, both inhibitor and substrate may stabilize the enzyme, and the shallow slopes of plots 3 and 4 may reflect enzyme inactivation due to other factors, e.g. thermal inactivation. The data (Fig. 2, plot 2) imply that binding of 2',5'-dd-3'-ATP to the individual VC1 and IIC2 domains prevents the formation of a functionally active VC1-IIC2 complex. That is, the binding of inhibitor keeps the enzyme domains apart or in an inappropriately associated complex.

FIG. 2. Effect of 2',5'-dd-3'-ATP on the dissociation of the VC1-IIC2 complex. Adenylyl cyclase cytosolic domains VC1 and IIC2 (2 μM each) were preassociated to form the VC1-IIC2 complex and then allowed to dissociate upon dilution as described under “Experimental Procedures.” The rate of cAMP formation was normalized to enzyme concentration, and activities are expressed as cAMP formed/min. Values shown are averages from either two or three separate experiments. The first (zero time) point on each curve was taken immediately before dilution and the second point (15 s) was taken as soon as possible after the 20-fold dilution. Time courses are of adenylyl cyclase activity of enzyme that had been treated as follows: Control, the VC1-IIC2 complex was formed in the absence of nucleotides; plot 2, VC1 was added to a solution of 10 μM 2',5'-dd-3'-ATP and incubated for 2 min at room temperature, then IIC2 was added and incubated for an additional 2 min before zero time sampling and then dilution; plot 3, the preformed VC1-IIC2 complex was prepared in the absence of nucleotides (with a 2-min incubation at room temperature) and then 10 μM 2',5'-dd-3'-ATP was added for an additional 2 min at room temperature before zero time sampling and dilution; plot 4, the preformed VC1-IIC2 complex was prepared in the absence of nucleotides (as for plot 3), but then before dilution was treated sequentially with 10 μM 2',5'-dd-3'-ATP and 1 μM 5'-ATP for 2 min at room temperature per each ligand. Inset, enlarged depiction of plots 2 and 3.

FIG. 3. Effect of 5'-ATP on the dissociation of the VC1-IIC2 complex. Experimental conditions were as described under “Experimental Procedures” and in the legend to Fig. 2. The rate of cAMP formation was normalized to enzyme concentration and activities are expressed as cAMP formed/min. Values shown are averages from three separate experiments. Time courses are of adenylyl cyclase activity upon the dilution of the VC1-IIC2 complex: Plot 1, formed in the absence of nucleotides and then treated before a 20-fold dilution with 1 μM 5'-ATP for 2 min at 22 °C; plot 2, formed in the absence of nucleotides, but then treated sequentially before dilution with 1 μM 5'-ATP and 10 μM 2',5'-dd-3'-ATP, 2 min at 22 °C per each ligand; control, formed in the absence of nucleotides (the same data as shown in Fig. 2, control).

FIG. 4. Inhibition of VC1-IIC2 adenylyl cyclase by 2',5'-dd-3'-ATP and γ-azidoanilido-2',5'-dd-3'-ATP. Enzyme activity was determined as described under “Experimental Procedures” with a reaction mixture containing 5 mM MnCl₂, 100 μM forskolin, 0.5 mM ATP, 1 μM VC1, and 1 μM IIC2. Reactions were started by the addition of preformed VC1-IIC2 for 5 min at 30 °C.
approximately 20% of IIC2 became labeled after a 60-min electrophoresis. The exposure was for 5 h on a PhosphorImager screen.

high substrate concentration should facilitate association of VC1 and IIC2 yielding a larger percentage of the enzyme in the catalytically active complex and result in greater measured activity.

Comparison of these effects of 5’-ATP and 2’,5’-dd-3’-ATP would suggest that binding of the inhibitor is much stronger than of substrate. This is fully consistent with the observation that IC50 values for inhibition by 2’,5’-dd-3’-ATP are three orders of magnitude lower than the Km value for metal-5’-ATP substrate, seen with native as well as recombinant adenylyl cyclase (12, 20). The data show further that 2’,5’-dd-3’-ATP and 5’-ATP do not compete for the same enzyme configuration, consistent with the noncompetitive inhibition typical of P-site ligands.

Covalent Labeling of VC1 and IIC2 with γ-MetIm-2’,5’-dd-γ-[32P]3’-ATP—To verify that 2’,5’-dd-3’-ATP could, in fact, bind to VC1 and IIC2 domains independently, and thereby alter their association to the catalytically competent VC1-IIC2 form, cross-linking ligands were synthesized and used. Substitution of 2’,5’-dd-3’-ATP at the γ-phosphate with an azidoanilido group did not meaningfully affect the potency of the 3’-nucleotide to inhibit adenylyl cyclase (Fig. 4). Although irradiation of VC1, IIC2, or VC-IIC2 with UV light at 300 nm in presence of γ-azidoanilido-2’,5’-dd-γ-[32P]3’-ATP resulted in covalent modification of both subunits (not shown), the level of labeling was less than 1%. This was measurably better incorporation of label than we noted in the earlier use of direct UV irradiation in the presence of acetone as a sensitizer (11). Because a more reactive reagent would be preferable, γ-MetIm-2’,5’-dd-3’-ATP was synthesized in both unlabeled and γ-32P-labeled forms (cf. see “Experimental Procedures”). However, due to the high rate of hydrolysis in aqueous solutions of γ-MetIm-2’,5’-dd-3’-ATP to the unsubstituted 2’,5’-dd-3’-ATP, a meaningful comparison of its inhibitory potency with either 2’,5’-dd-3’-ATP or the azidoanilido derivative was not possible. The fact that the azidoanilido group did not impair inhibitory potency, an observation that was consistent with the established tolerance of adenylyl cyclase to large substitutions at the 3’-ribosyl position (21), suggested that the comparably sized (Fig. 1) but more reactive γ-methylimidazole derivative may also be useful for covalent labeling of the enzyme.

Incubation of VC1, IIC2, or VC-IIC2 with γ-MetIm-2’,5’-dd-γ-[32P]3’-ATP resulted in covalent labeling of both cytosolic domains (Figs. 5–7). 32P-Incorporation increased with time and approximately 20% of IIC2 became labeled after a 60-min exposure to this ligand (Fig. 5). VC1 was labeled with a comparable time course (not shown) and efficacy (Figs. 6 and 7, cf. lanes 1 and 4), as calculated from PhosphorImager data. Formation of a functional VC1-IIC2 complex resulted in reduced 32P-incorporation into VC1 and enhanced 32P-incorporation into IIC2 (Figs. 6 and 7, lane 7). Quantitatively, in the VC1-IIC2 complex 32P-incorporation into VC1 decreased approximately 50% from that of VC1, labeling of IIC2 effectively doubled, and resulting in 32P-incorporation into VC1, being only 25% that into IIC2. As expected, 2’,5’-dd-3’-ATP completely blocked 32P-incorporation into VC1 and IIC2, whether alone or in the VC1-IIC2 complex (Figs. 6 and 7, lanes 3, 6, and 9), whereas there was marginal protection afforded by either 5’-ATP (Fig. 6) or 5’-AP(CH2)2PP (Fig. 7). The concentrations of 5’-ATP or 5’-AP(CH2)2PP used were 100-fold greater than that of γ-MetIm-2’,5’-dd-3’-ATP, but afforded protection of VC1 and IIC2 only 10–30% (Table I). Protection afforded by 5’-AP(CH2)2PP was
less effective with IIC2 (−10%) than with VC1 (−30%), regardless of VC1-IIC2 complex formation. In contrast, the protective effect of 5′-ATP depended on subdomain association. 5′-ATP caused ~30% reduction in labeling of the individual VC1 and IIC2 proteins, whereas formation of the VC1-IIC2 complex resulted in enhanced protection of VC1 protection (~33% with VC1 to ~75% for VC1 in the VC1-IIC2 complex) and of IIC2 (~27% with IIC2 to ~60% in the VC1-IIC2 complex). Considering that 50% of the 5′-ATP was converted to cAMP and PPi during incubation with the enzyme, it is possible that the protection afforded by 5′-ATP was actually due to the products of the enzyme reaction.

The labeling data are in agreement with those obtained by direct photo cross-linking of VC1 and IIC2 with [32P]2′-d-3′-AMP (11) and corroborate the conclusion that P-site ligands can covalently cross-link with the cytosolic domains of adenylyl cyclase, whether alone or in complex. However, the asymmetric labeling of the subunits noted here (Figs. 6 and 7) was not observed in the earlier studies (11). This difference likely lies in the fact that the earlier experiments used the nucleoside 3′-monophosphate, whereas these used the nucleoside 3′-triphosphate. First, the polyphosphate moiety may measurably influence protein-protein interactions between VC1 and IIC2; second, the site at which VC1 is labeled in the vicinity of the ligand γ-phosphate may be restricted or reconfigured by formation of the VC1-IIC2 complex, whereas the site at which IIC2 is labeled becomes more accessible. In contrast, the adenine moiety, through which direct photo cross-linking occurs, would contact both VC1 and IIC2. Moreover, it is within a domain that is shared by P-site ligand and substrate and is not significantly altered by formation of the VC1-IIC2 complex (7).

**DISCUSSION**

The data presented here demonstrate that the interaction of 2′,5′-dd-3′-ATP with the adenylyl cyclase formed from the association of the VC1 and IIC2 cytosolic domains is a process that is not in rapid equilibrium. This would explain the lack of competition between 2′,5′-dd-3′-ATP and substrate under steady state kinetic conditions. Moreover, it suggests that binding of this ligand is a pseudo-irreversible process and the non-competitive inhibition observed with it (12, 22) likely occurs by a somewhat different mechanism than does the post-transition state, dead-end inhibition previously observed with 2′,3′-AMP, which requires bound metal-PPi for inhibition (5, 8, 11). The lack of competition between substrate and 2′,5′-dd-3′-ATP was clearly evident both in the experiments on association-dissociation of VC1 and IIC2 (Figs. 2 and 3) and in the experiments involving cross-linking and labeling with γ-MetIm-2′,5′-dd-[γ-32P]3′-ATP (Figs. 5–7). Also evident was the importance of the order of addition of substrate or protecting ligand and the nucleoside 3′-triphosphate. Rates of dissociation of the preformed VC1-IIC2 complex differed depending on the nucleotides present and the order of their addition (Figs. 2 and 3). Furthermore, no protection against covalent labeling of VC1 or IIC2 by γ-MetIm-2′,5′-dd-[γ-32P]3′-ATP was afforded by 5′-ATP, 5′-AP(CH2)PP, or 2′,5′-dd-3′-ATP when the proteins were added to a mixture of the covalent affinity ligand and protecting ligand. This suggested either that γ-MetIm-2′,5′-dd-3′-ATP binds with the adenylyl cyclase subdomains faster than the other nucleotides or that it cross-links with a different site. The latter possibility was excluded by the complete protection observed when the VC1, IIC2, or the VC1-IIC2 complex were pretreated with 2′,5′-dd-3′-ATP before addition of the covalent affinity ligand (Figs. 6 and 7). The observation that the protective effect of 5′-ATP was substantially better with VC1-IIC2 than with VC1 or IIC2 individually could be due either to the presence of products (cAMP and PPi) or to an effect on the assembly of the VC1-IIC2 complex. This latter process may be promoted by substrate binding and result in stabilization of a “closed” configuration in which IIC2 effectively blocks polyphosphate binding to VC1, as contrasted with an “open” configuration in which VC1 is more accessible to γ-MetIm-2′,5′-dd-[γ-32P]3′-ATP. In general, these observations corroborate those we made earlier (11) by direct photo cross-linking of [32P]2′-d-3′-AMP with VC1, IIC2, and the VC1-IIC2 complex. Each cytosolic domain was observed to react individually with [32P]2′-d-3′-AMP, but complex formation also affected the interaction of each domain with it (11). This was consistent with the enzyme being distributed between two conformational states that are not in rapid equilibrium, one binding 5′-ATP and the other binding 2′-3′-AMP and PPi.

No structural data are available for adenylyl cyclases in complex with any adenine nucleoside 3′-triphosphate. The data presented here showed that both VC1 and IIC2 domains interact with 2′,5′-dd-3′-ATP. This interaction affects the association of the major cytosolic domains and the resulting conformation of the enzyme; the formation of a functionally active VC1-IIC2 complex is prevented. From comparisons with structural data obtained with 2′-3′-AMP and PPi (7) and with analogs of 5′-ATP (22), it would be expected that the polyphosphate group of 2′,5′-dd-3′-ATP would interact with both VC1 and IIC2 domains. The terminal β-and γ-phosphates should interact with the C1 domain through divalent cation and with the C2 domain through Arg-1029 and/or Lys-1065 (7, 22), amino acids susceptible to covalent interaction with γ-MetIm-2′,5′-dd-3′-ATP. Labeling of VC1 and IIC2 domains by the 32P-labeled derivative occurred with similar efficiency, but after stimulation by Mg2+ and forskolin and the formation of VC1-IIC2, the IIC2 domain was labeled preferentially. Furthermore, treatment of VC1 with 2′,5′-dd-3′-ATP prevented formation of catalytically competent VC1-IIC2. If one assumes that the dissociated open state of VC1 and IIC2 accurately models the inactive state of native holoenzyme, binding of 2′,5′-dd-3′-ATP would prevent the conformational changes promoted by enzyme interactions with activated G α and/or forskolin. The 2′,5′-dd-3′-ATP would hold the enzyme in an inactive configuration. This is conceptually different from conclusions drawn from earlier experiments with 2′-3′-AMP and 2′,5′-dd-adenosine, inhibition by either of which relies on product PPi, being in the catalytic site. That

**TABLE I**

Effects of 5′-ATP, 5′-AP(CH2)PP, and 2′,5′-dd-3′-ATP on covalent labeling of VC1 and IIC2 by γ-MetIm-2′,5′-dd-[γ-32P]3′-ATP

Data are from PhosphorImager images presented in Figs. 6 and 7 as quantified with ImageQuant software as described under “Experimental Procedures.” Values are volumes (area × intensity) observed with nucleotide relative to volumes observed with no additions and are presented as percentages.

| Additions | VC1 | IIC2 | VC1, VC1-IIC2 | IIC2, VC1-IIC2 |
|-----------|-----|------|---------------|---------------|
| None      | 100 | 100  | 100           | 100           |
| 5′-ATP    | 67  | 73   | 26            | 40            |
| 5′-AP(CH2)PP | 72  | 93   | 74            | 88            |
| 2′,5′-dd-3′-ATP | <2 | <2   | <2            | <2            |
is, inhibition was preferentially of stimulated enzyme. The requirement of enzyme activity to generate the pyrophosphate necessary for inhibition is obviated by 2'-d-3'-ATP or 2',5'-dd-3'-ATP, because these ligands by their nature already contain the PPi group and can inhibit basal as well as stimulated forms of the enzyme.

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