The susceptibility of purines to form a covalent attachment with proteins upon exposure to UV irradiation was applied to adenylyl cyclase by use of [32P]2'-d-3'-AMP, a dead-end inhibitor that binds to the post-transition configuration of the enzyme. [32P]2'-d-3'-AMP was synthesized enzymatically. It and [α-32P]5'-ATP were used for direct photocross-linking to individually expressed cytosolic domains of adenylyl cyclase. Both the C1 domain of the type V isozyme (VC1) and the C2 domain of the type II isozyme (IIC2) were labeled, whether alone or combined, upon photolysis of [32P]2'-d-3'-AMP in the presence of acetone. Labeling of VC1 and IIC2 was greatly enhanced in the presence of PPi, which was almost completely suppressed by 50 μM 2',5'-dideoxy-3'-ATP, the most potent reported P-site inhibitor of adenylyl cyclases, but was partially suppressed by 1 mM 3'-IMP, a ligand that does not inhibit the enzyme via the P-site. Neither 3'-5'-cAMP nor 5'-ATP had a major effect on labeling by [32P]2'-d-3'-AMP. Direct cross-linking of VC1 with [α-32P]5'-ATP was substantially suppressed by 2',5'-dideoxy-3'-ATP and partially suppressed by 2'-d-3'-AMP, whereas cross-linking of IIC2 was less affected by the 3'-triphosphate. The data imply that either cytosolic domain can interact directly with either substrate or P-site ligand and that subunit interaction modifies the susceptibility of each domain to UV-induced covalent modification by either [α-32P]5'-ATP or [32P]2'-d-3'-AMP.

Mammalian adenylyl cyclases (ATP pyrophosphate-lyase (cycling); EC 4.6.1.1) comprise a family of proteins with similar domain organization. The enzyme contains a short and similar molecular masses of 30 and 26 kDa for VC1 and IIC2, respectively. Truncated forms of C1 and C2 have been chimerically linked or modification by either 

### EXPERIMENTAL PROCEDURES

#### MATERIALS

Materials—TdTNT buffer, alkaline phosphatase, and glycogen were from Promega. Oligonucleotide primer (pdaβ) and terminal nucleotidyltransferase were from Sigma, and [α-32P]5'-d-5'-AMP (3000 Ci/mmol) was from International Chemical and Nuclear Corp. Calf spleen phosophodiesterase (2 units/mg) was from Boehringer Mannheim. Photolysis was conducted in a Rayonet mini-reactor from Southern New England Ultraviolet Company.

VC1 and IIC2 Domains of Adenylyl Cyclase—Recombinant VC1 and IIC2 were expressed in E. coli and were purified as described (4). Purified proteins were electrophoretically homogeneous with apparent molecular masses of 30 and 26 kDa for VC1 and IIC2, respectively.

#### SYNTHESIS OF [32P]2'-d-3'-AMP—[32P]2'-d-3'-dAMP was prepared from enzymatic hydrolysis of 3P-labeled poly(dA). 3P-Labeled poly(dA) was prepared by repetitive additions of [32P]2'-d-5'-AMP moieties to the 3'-end of (pdA)35 units of terminal nucleotidyltransferase. The reaction mixture contained TdTNT buffer, 0.05 A260 units of TdTNT and 35 units of terminal nucleotidyltransferase, 1 to 2 μCi of [α-32P]2'-d-5'-AMP (3000 Ci/mmol) that had been previously lyophilized in a volume of 100 μL. After incubation at 30 °C for 4 h or overnight, the mixture was treated for 5 min with 1 unit of alkaline phosphatase to remove residual 2'-d-5'-AMP and to dephosphorylate the 5'-end of the oligonucleotide. Without this treatment, overall yields of [32P]2'-d-3'-AMP were poorer and variable. Alkaline phosphatase was inactivated by placing the reaction tube in boiling water for 5 min. 32P-Labeled

1 The abbreviations used are: 5'-AMP, adenosine 5'-triphosphate; 2'-d-3'-AMP, 2'-deoxyadenosine 3'-monophosphate; 3'-IMP, inosine 3'-monophosphate; 2',5'-d-d-3'-AMP, 2',5'-dideoxy-3'-AMP; 5'-AMP, 5'-adenosine (5'-α-methylene) triphosphate; 2'-d-3'-AIP, 2'-deoxyadenosine 3'-tetraphosphate. TdTNT buffer, buffer for terminal nucleotidyltransferase assay.
poly(dA) was isolated by precipitation with 70% ethanol in 0.3 M sodium acetate buffer, pH 5, in the presence of 1 μg of glycogen. [\( ^{32}P \)]P'2'-d-3'-AMP was released upon reaction of [\( ^{32}P \)]P'2'-d-3'-AMP with 0.2 units of calf spleen phosphodiesterase (2 units/mg) for 4 h at 30 °C in 100 μl of reaction mixture containing 50 mM sodium acetate buffer, pH 5. The sample was deproteinized by extraction with phenol/chloroform (1:1, v/v), and [\( ^{32}P \)]P'2'-d-3'-AMP was isolated by reverse phase column chromatography on an C18 Ultrasphere column (4.6 × 250 mm from Beckman). Elution was with a linear gradient of 50 mM triethylammonium bicarbonate, pH 7.5, to 50% methanol at 1 ml/min. Fractions containing [\( ^{32}P \)]P'2'-d-3'-AMP were pooled, and triethylammonium bicarbonate was removed by repetitive evaporation from methanol. The purified [\( ^{32}P \)]P'2'-d-3'-AMP gave an estimated specific activity of 1 to 2 × 10^7 cpm/mol, as determined by Cherenkov radiation.

**Adenylyl Cyclase Assay**—Adenylyl cyclase was assayed at 30 °C in a 10-min reaction in 100 μl of reaction mixture containing 50 mM HEPES buffer, pH 7.5, 1 mM MnCl₂, 100 μM forskolin, 0.5 mM [\( ^{32}P \)]5'-ATP (2 × 10^10 cpm/mol by Cherenkov radiation), and 50 mM VC₁ and 50 mM IIC₂. The reaction was started by the addition of [\( ^{32}P \)]5'-ATP and was terminated by the addition of zinc acetate and sodium carbonate. [\( ^{32}P \)]PcAMP was purified by sequential chromatography on Dowex 50 and alumina as described previously (12). Acetone was included in the assay mixture as indicated at 0.5% v/v to 10% v/v.

**Photoaffinity Labeling**—Labeling of adenylyl cyclase subunits VC₁ and IIC₂ by [\( ^{32}P \)]5'-ATP or [\( ^{32}P \)]P'2'-d-3'-AMP was achieved with a 5-min exposure to 254 nm UV light at room temperature in a Rayonet mini-reactor. The 25-μl reaction volume contained 50 mM HEPES, pH 7.5, 5 mM MnCl₂, 100 μM forskolin, 0.5% acetone, 100 μM [\( ^{32}P \)]5'-ATP (25 Ci/mmol) or [\( ^{32}P \)]P'2'-d-3'-AMP (1 to 2 × 10^10 cpm/mmol, by Cherenkov radiation), 5 μM of VC₁, and/or 5 μM of IIC₂. The reaction was initiated by the addition of VC₁ and/or IIC₂ to the rest of the reaction mixture at 0 °C. The resulting mixtures were transferred immediately to a Parafilm support in the mini-reactor with 254-nm lamps on. After irradiation, the reaction mixture was transferred to a tube containing 0.1% SDS, 0.1 M dithiothreitol, and 5% glycerol, and this was placed in a boiling water bath for 3 min. Proteins and unreacted nucleotides were separated on an 11% polyacrylamide SDS gel. Protein was visualized by silver staining as described (13). The dried gel was exposed to a phosphorimager screen for 3 to 5 h, and protein labeling was quantified by PhosphorImager and ImageQuant software (from Molecular Dynamics). Alternatively, dried gels were exposed at −65 °C to X-Omat imaging film (from Kodak) for 12 h with an intensifying screen. To quantify the incorporation of [\( ^{32}P \)]5'-AMP into proteins, bands corresponding to VC₁ and IIC₂ were cut from dried gels and counted in a liquid scintillation counter.

**RESULTS**

**ATP Photolysis and Effects of Acetone**—Simple exposure of 5'-AMP in water to high intensity UV light was not sufficient to induce photolysis (Fig. 1, panel B). No meaningful changes in the UV spectra of ATP were observed after 60 min of irradiation. However, in the presence of 0.5% (v/v) acetone, UV light induced time-dependent photoactivation of the adenine ring of 5'-AMP (Fig. 1, panel A). Half-time for photolysis of 5'-AMP was approximately 10 min, and photolysis of 2'-d-3'-AMP in acetone followed a similar time course (not shown). Consequently, in subsequent experiments, a 5-min exposure with either nucleotide was used for protein labeling. These results are consistent with the idea that energy is transferred from the UV-excited acetone molecules to the adenine ring.

Whereas low concentrations of acetone facilitated photoactivation of 5'-AMP or 2'-d-3'-AMP (above), these concentrations were essentially without effect on adenylyl cyclase (Fig. 2). Acetone at 0.5% v/v 1% (v/v) did not affect adenylyl cyclase appreciably, but, as expected, higher concentrations reacted with the enzyme. The concentration eliciting a 50% reduction in activity of purified and recombined VC₁ and IIC₂ was approximately 3% acetone. By comparison, crude enzyme extracted from rat brain by simple detergent dispersion was unaffected by concentrations of acetone as high as 10% (not shown). Consequently, in subsequent experiments on photoaffinity labeling of VC₁ and IIC₂, 0.5% acetone was used.

**Photoaffinity Labeling of Adenylyl Cyclase Domains by [\( ^{32}P \)]5'-AMP**—Irradiation of individual adenylyl cyclase cytosolic domains with UV light in the presence of [\( ^{32}P \)]5'-ATP (100 μCi) resulted in covalent modification of both VC₁ and IIC₂ (Fig. 3). [\( ^{32}P \)]PcAMP was incorporated into 5% of VC₁, as determined by excision of gel slices and counting in a scintillation counter. By this method the extent of labeling of VC₁ was found to be 3.5 times greater than that of IIC₂. The addition of 1 mM 2'-d-3'-AMP or 50 mM 2',5'-dd-3'-AMP resulted in protection of both VC₁ and IIC₂ domains from covalent labeling by [\( ^{32}P \)]5'-AMP (Fig. 3). Efficiencies of VC₁ or IIC₂ protection by 2'-d-3'-AMP and 2',5'-dd-3'-AMP were estimated by PhosphorImager techniques. Because isotope decay events (cpm) are directly proportional to arbitrary PhosphorImager units (over 5 orders of magnitude), the ratio of densities of any two bands will directly reflect the ratio of isotope incorporated into the respective proteins. Consequently, the relative protecting effect of a 3'-nucleotide ligand is simply measured by comparison of
band densities when the density with no added ligand is taken as 100% for each cytosolic domain. A summary of such data obtained from two experiments is given in Table I. It was not possible to attempt photoaffinity labeling of a mixture of VC1 and IIC2, whether exposed individually or in the form of a VC1-IIC2 complex (Fig. 4). The extent of covalent modification of IIC2 was 2%, as determined by scintillation counting of excised gels, and the ratio of $^{32}$P incorporated into VC1 and IIC2 was 0.7.

Photoaffinity labeling of VC1, IIC2, or VC1-IIC2 by $^{32}$P-[d-3]-AMP occurred in the presence of a 10-fold molar excess of either 5'-AMP (1 mM) or CAMP (1 mM), respectively, substrate and product of the cyclase reaction (Fig. 4). Although both nucleotides suppressed incorporation of $^{32}$P into VC1 by 50%, this was less evident for incorporation of $^{32}$P into IIC2 but was visually most evident when the enzyme was in the VC1-IIC2 complex (Fig. 4, lanes 7–9). By comparison, a 10-fold molar excess of 3'-IMP (1 mM) afforded partial protection against $^{32}$P incorporation into IIC2, but afforded no protection of VC1 (Fig. 5, lanes 2 and 5). 3'-IMP gave partial protection of each subunit when the VC1-IIC2 complex was irradiated in the presence of $^{32}$P-[d-3]-AMP (Fig. 5, lane 8). In striking contrast, at a concentration of only 50 $\mu$M, 2',5'-d-3'-AMP resulted in essentially complete protection of both VC1 and IIC2, individually or in complex, when labeled by 100 $\mu$M $^{32}$P-[d-3]-AMP (Fig. 5, lanes 3, 6, and 9). Data from several experiments are summarized in Table II.

The influence of Inorganic Pyrophosphate—Data from both dead-end inhibition kinetics and equilibrium binding studies with H-2'-dAdo to VC1-IIC2 in complex with Gs suggested that inorganic pyrophosphate should enhance or be required for binding of P-site ligands (14). Consequently, the effect of both adenylyl cyclase products, CAMP and PPi, alone and in combination, were evaluated for their possible effects on photoaffinity cross-linking with $^{32}$P-[d-3]-AMP (Fig. 6). As predicted, PPi (1 mM) substantially enhanced labeling of VC1, IIC2, or VC1-IIC2 by $^{32}$P-[d-3]-AMP (Table II), and CAMP afforded protection that was compatible with its effect in the absence of PPi.

DISCUSSION

Direct photocross-linking of nucleotides with proteins has been widely used for identification of nucleotide binding sites (15,16). However, protein labeling by adenosine derivatives is complicated by instability of the glycoside bond of the UV-activated nucleoside (17) and low quantum yield of purine photoactivation ($\sim 3 \times 10^{-4}$) (18). This is somewhat circumvented by the use of a chromophore to allow indirect activation of adenine by facilitating energy transfer from UV light to the adenine ring (19). Acetone has been used successfully in this capacity as a sensitizer (19), and site-specific modification of nucleotide binding sites in proteins has been demonstrated with 5'-AMP and acetone (20–22). The effect of acetone to enhance adenine nucleotide photolysis was also clearly evident here (Fig. 1). Even so, covalent modification in such experiments may not exceed a few percent of the protein, limited due either to UV-induced cleavage of the glycosidic bond of adenosine, in our case causing a loss of the reporter $^{32}$P label, or to nonspecific interactions of the labeled ligand with buffer components.

Studies of adenylyl cyclases have included enzyme kinetics,
reversibly binding ligands, and covalent modifications of wild type and mutated enzyme. Inhibition kinetics of adenylyl cyclase forward and reverse reactions are consistent with an interaction of P-site ligands with the post-transition state of the enzyme (E) as dead-end inhibitors (7, 9, 14, 23). By this mechanism, adenine nucleosides and adenosine 3'-monophosphates interact with the cAMP binding site and before metal-PPII, leaving (6, 14, 23). These analyses would place both substrate 5'-ATP and P-site ligands at a common site believed to be formed at the interface of the C1 and C2 domains during the reaction cycle (6, 14, 24, 25).

Kinetics analyses involve evaluations that include both ligand binding and enzyme conformational changes, whereas direct measurements of ligand binding assess bimolecular interactions, albeit with a set of possible enzyme configurations. Because substrate and inhibitor share a common binding site, although of different configurations, one might expect some competition between P-site ligands and substrate. However, this was not observed in equilibrium binding studies with [3H]2'-dA nor with [3H]5'-AP(CH2)4PP (6,14). 2'-d'-d'AMP neither displaced nor competed with binding. Binding of these ligands with this chimeric truncated construct of the C1C2 complex required not only the formation of an active tertiary structure but also catalysis. Because conformational states of an enzyme are in equilibrium and a certain percentage of the enzyme will be in the different configurations, reversible binding techniques may not have been able to detect associations that might be observable with labeled covalent probes.

The availability of [32P]2'-d'-d'AMP, a more potent and easily detected ligand than [3H]2'-dA, allowed these interactions to be addressed by a different technique. The data presented here clearly show that by use of direct photo cross-linking, both [α-32P]5'-ATP and [32P]2'-d'-d'AMP labeled both VC1 and IIc2 (Figs. 3–6), indicating that both ligands can interact with either VC1 or IIc2. This is consistent with the high level of sequence homology between C1 and C2 (26) in all mammalian adenylyl cyclases and is not inconsistent with the idea that the two cytosolic domains are required for catalysis and P-site-mediated inhibition (1–6). Because photoactivation induces covalent cross-linking through the adenine ring, the fact that both VC1 and IIc2 are labeled by [32P]2'-d'-d'AMP also in the catalytically competent complex of VC1-IIc2 implies contact of the adenine ring of this ligand with both VC1 and IIc2. Because cross-linking results in an irreversible linkage, it will select for and lock in those enzyme configurations with which the ligands interact. This is quite different from the results one can obtain from either enzyme kinetics or reversible binding studies. It was therefore not surprising that a measurable but weak competition between 5'-ATP and 2'-d'-d'AMP was noted. Both bind to the enzyme under similar conditions. Either will displace binding of the other, but only partially (Tables I and II), and that at 10-fold greater concentrations of the competing ligand. These observations are consistent with the steady-state kinetic behavior of the soluble enzyme (6, 9, 14) but not inconsistent with the lack of competition of 2'-d'-d'AMP in the equilibrium binding studies with [3H]5'-AP(CH2)4PP, which suggested that 2'-d'-d'AMP binds after 5'-ATP but before the return of the enzyme to its initial state. Shown here is that [32P]2'-d'-d'AMP can bind to either free enzyme (E + I → E-I) or to the post-transition state of the

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**Table II**

| Additions | Density ratio (%) |
|-----------|------------------|
| VC1       | 100              |
| IIc2      | 100              |
| VC1,IIc2  | 100              |
| VC1,IIc2  | 100              |
| VC1,IIc2  | 100              |

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**Fig. 6.** Pyrophosphate-enhanced photoaffinity labeling of VC1, IIc2, and VC1-IIc2 with [32P]2'-d'-d'AMP. VC1 and IIc2 were exposed to UV irradiation individually and in complex (VC1-IIc2) in the presence of 0.1 mM [32P]2'-d'-d'AMP and subsequently separated electrophoretically from unbound ligand as described under “Experimental Procedures.” Lanes contained 0.125 nmol of VC1 (1); 0.125 nmol of VC1 labeled with [32P]2'-d'-d'AMP in the presence of 0.1 mM 2',5'-dd-3'-ATP (2); 0.125 nmol of IIc2 labeled with [32P]2'-d'-d'AMP in the presence of 0.1 mM 2',5'-dd-3'-ATP (3); 0.125 nmol of IIc2 (4); 0.125 nmol of IIc2 irradiated in the presence of 0.1 mM 2',5'-dd-3'-ATP (5); 0.125 nmol of IIc2 irradiated in the presence of 0.1 mM 2',5'-dd-3'-ATP (6); complex of VC1-IIc2, cytosolic domains, each 0.125 nmol (7); complex of VC1-IIc2, cytosolic domains, each 0.125 nmol, labeled in presence of 0.1 mM 2',5'-dd-3'-ATP (8); and complex of VC1-IIc2 cytosolic domains, each 0.125 nmol, labeled in presence of 0.1 mM 2',5'-dd-3'-ATP (9).
Photoaffinity Labeling of Adenylyl Cyclase

enzyme in the absence of pyrophosphate (E′ + I → E′-I). PPβγ
which shifts the equilibrium of enzyme to a state that accepts
P-site ligands, enhanced labeling of the individual domains 3-
5-fold and labeling of the VCα2-IICα complex 7- to 9-fold (Table
II). This effect of PPβγ is fully consistent with the proposed model
for inhibition by P-site ligands (6, 14, 23), and it is consistent with
the effect of PPβγ to enhance binding of [5-32P]dAdo to the
VCα2-IICα complex (14). By comparison, cAMP afforded less
protection of VCα1 and IICα2 from labeling by [32P]2-d-3′-AMP
than might have been expected (Figs. 4 and 6 and Table II).
This may be explained by the low apparent affinity of cAMP for
adenylyl cyclase (Km cAMP ~16 mM in the reverse reaction).
The use of cAMP concentrations sufficient to interact with the
cytosolic domains affects access of ligand to those sites to
competing ligands (Figs. 4–6), suggests either that interaction
of the cytosolic domains affects access of ligand to those sites to
competent configuration to which the ligands have
which covalent linkages are formed or that the association
of 3′-dATP in models derived from these (27), no crystal
structures of adenine nucleosides or adenine nucleoside-3′-monophos-
phates requires the presence of PPβγ, whereas inhibition by the
adenosine-3′-triphosphorotri- or 3′-tetraphosphates (7, 28) does not.

In the VCα1-IICα complex 3′-IMP afforded only 50–60% pro-
tection of IICα2 and only 30% protection of VCα1 and afforded no
protection of VCα1 alone (Fig. 5 and Table II).

Taken together the data strongly argue that the site of co-
valent cross-linking by [32P]2-d-3′-AMP has the same charac-
teristics as that through which P-site-mediated inhibition of
native adenylyl cyclases occurs. It is selective for an intact
adenine moiety, and it recognizes the ribosyl 3′-polyphosphate
moiety (cf. Refs. 7, 8, 28, 29). Effective inhibition by or binding
of adenine nucleosides or adenine nucleoside-3′-monophosphor-
ates requires the presence of PPβγ, whereas inhibition by the
adenosine-3′-tri- or 3′-tetraphosphorotriphosphates (7, 28) does not.
P-site-directed ligand does not compete with 5′-ATP for binding with
adenylyl cyclase (9), and P-site-targeted covalent modification
occurs in the presence of 5′-ATP (11). The catalytically compo-
tent form of the enzyme exists in two conformational states,
each with which substrate, metal-5′-ATP interacts, and a post-
transition state in which product is released and with which
P-site ligands interact. It is this latter form that [32P]2-d-3′-
AMP covalently modifies and the labeling of which is enhanced
by pyrophosphate. Each cytosolic domain evidently contains
elements of a nucleotide binding site, but an effective catalytic
cleft must form only when they associate. Because the two
states of the enzyme are not in rapid equilibrium, the oppor-
tunity is provided for independent binding of ligands to sub-
strate and P-site configurations of the enzyme.

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J. Biol. Chem. 1998, 273:32416-32420.
doi: 10.1074/jbc.273.49.32416

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