# Physiological Inhibitors of the Catalytic Subunit of cAMP-Dependent Protein Kinase: Effect of MgATP on Protein–Protein Interactions†

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ABSTRACT: The catalytic (C) subunit of cAMP-dependent protein kinase interacts with two classes of inhibitors. The regulatory (R) subunits, types I and II, associate to form an inactive holoenzyme complex that is activated in response to cAMP. The C-subunit is also inhibited by small heat-stable protein kinase inhibitors (PKI's). Inhibition by both PKI and $R^I$-subunit requires the synergistic high-affinity binding of MgATP. The stabilizing effect of ATP was quantitated by using analytical gel chromatography. Both the type I holoenzyme and the C·PKI complex in the presence of MgATP show apparent $K_d$'s for subunit association that are below 0.1 nM, while in the absence of MgATP the apparent $K_d$'s are 125 nM and 2.3 $\mu$M, respectively, for the two complexes. In the absence of MgATP both complexes also can be dissociated readily and, hence, activated by salt-induced dissociation. Under physiological salt concentrations, salt-induced dissociation would be substantial in the absence of the high-affinity binding of MgATP. In both complexes, the ATPase activity of the free C-subunit is abolished. The off rates for MgATP also indicate that the type I holoenzyme is more stable than the C·PKI complex. The off rate ($t_{1/2}$) for MgATP from the C·PKI complex is 17 min, while the off rate for the type I holoenzyme is 11.7 h. When the C·PKI complex is incubated with $R^I$-subunit in the presence or absence of MgATP, the C-subunit preferentially reassociates with the $R^I$-subunit, forming holoenzyme. In contrast, free PKI cannot compete for the C-subunit when it is part of a holoenzyme complex. These *in vitro* results suggest that the formation of the C·PKI complex may not be a dead-end pathway. Instead, PKI could function as a shuttle mechanism for eventually returning the C-subunit to a holoenzyme complex.

The protein kinases are typically tightly regulated enzymes that are activated in response to very specific signaling pathways. In the absence of a signal, the enzymes are inactive. The exceptions are the oncogenic protein kinases, many of which are defective in their regulation and are constitutively active. cAMP-dependent protein kinase (cAPK)[1] is unusual in its activation mechanism. It was one of the first protein kinases to be purified (Walsh et al., 1968) and remains as one of the simplest. Its simplicity is due in large part to its mechanism of activation, which so far appears to be unique within the family. Like most protein kinases, cAPK is sequestered in an inactive form in the absence of the appropriate signal. The best studied inhibitors are the regulatory (R) subunits, with the release of the catalytic (C) subunit being triggered by the binding of cAMP (Gill & Garren, 1969). The inactive holoenzyme consists of two R- and two C-subunits, and this holoenzyme dissociates as follows in response to cAMP binding to the R-subunits:

$$R_2C_2 + 4cAMP \rightarrow R_2(cAMP)_4 + 2C$$

Once dissociated, the free C-subunit, in addition to phosphorylating proteins in the cytoplasm, can migrate to the nucleus, where it presumably plays a role in the regulation of gene expression (Meinkoth et al., 1990).

All cAPK holoenzymes are designated as Type I or II on the basis of the R-subunit (Beebe & Corbin, 1986; Hofmann et al., 1975; Rosen & Erlichman, 1975). The $R^I$- and $R^{II}$-subunits can be distinguished on the basis of elution from anion exchange resins (Corbin et al., 1975), amino acid sequence (Takio et al., 1984; Titani et al., 1984), and antigenicity (Kapoor et al., 1979; Mumby et al., 1985). Two genes have been identified in each category of R-subunit (Clegg et al., 1988; Jahnsen et al., 1986; Lee et al., 1983; Scott et al., 1987).

In addition to the R-subunits, there exists another family of inhibitors, although their physiological role remains unclear. These are the heat-stable protein kinase inhibitors (PKI's) (Van Patten et al., 1991; Walsh et al., 1971,1990). These relatively small proteins bind with a high affinity to the C-subunit, typically in the subnanomolar range. Whereas the inhibition of the C-subunit by the R-subunit is reversed by binding of cAMP, no physiological mechanism for releasing the C-subunit from PKI is known so far.

The mechanism for inhibition of the C-subunit is similar for both classes of inhibitors. As shown in Figure 1, both inhibitors contain a substrate-like sequence that occupies the peptide binding site of the C-subunit, thus rendering it inactive [for a review, see (Taylor et al., (1990)]. Both PKI and the R-subunits are, therefore, competitive inhibitors of protein substrates (Beebe & Corbin, 1986; Granot et al., 1980;

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[1] Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cAPK, cAMP-dependent protein kinase; R, regulatory subunit of cAPK; C, catalytic subunit of cAPK; PKI, heat-stable protein kinase inhibitor; ADP, Adenosine diphosphate; AMP-PCP, adenosine 5′-[$\beta,\gamma$-methylene]-triphosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; ATP, adenosine 5′-triphosphate; ATP-$\gamma$-S, adenosine 5′-thio[$\gamma$]triphosphate; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; FITC, fluorescein 5-isothiocyanate, HEPES , 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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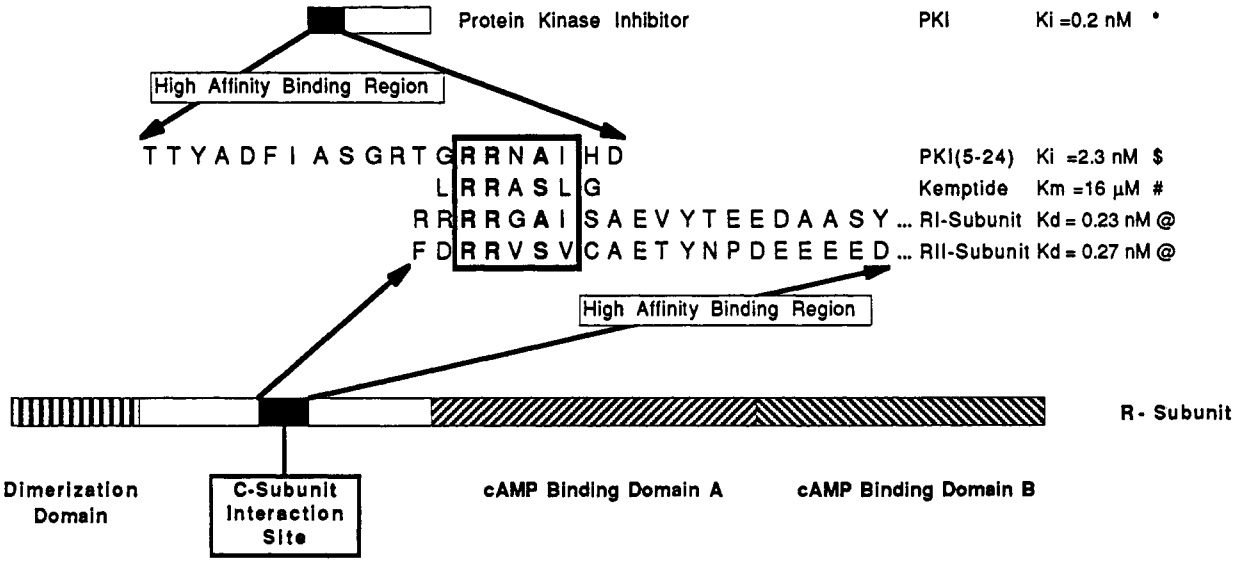


FIGURE 1: Sequence comparison of the physiological inhibitors and substrates of the C-subunit. The common consensus recognition sequence for substrates and inhibitors of this kinase, R-R-X-S/T/A-Y, is boxed. The apparent $K_m$'s and $K_d$'s were determined as follows: PKI·C-subunit (Whitehouse & Walsh, 1983), IP20·C-subunit (Cheng et al., 1986), heptapeptide substrate (Kemp et al., 1976), and R-subunit·C-subunit (Hofmann, 1980).

Whitehouse & Walsh, 1983). The $R^{II}$-subunits contain a Ser at their autoinhibitory site and are readily autophosphorylated upon holoenzyme formation (Rosen & Erlichman, 1975). In contrast, the $R^{I}$-subunits contain a pseudophosphorylation site where the Ser is replaced by Ala or Gly. Instead of being autophosphorylated, the type I holoenzymes bind MgATP with a high affinity (Hofmann et al., 1975). The PKI's, like the $R^{I}$-subunits, also contain a pseudophosphorylation site and bind ATP with a high affinity (Walsh et al., 1990). Tight binding of PKI and $R^{I}$-subunit to the C-subunit requires the synergistic binding of ATP (Corbin et al., 1975; Neitzel et al., 1991; Van Patten et al., 1986; Whitehouse & Walsh, 1983).

While the importance of MgATP for inhibition by the $R^{I}$-subunits and PKI has been recognized for some time, subunit interaction and $K_i$'s have always been evaluated on the basis of activity assays. Under these conditions the direct interaction of subunits in the absence of MgATP cannot be determined, because complex formation is measured indirectly by following loss of catalytic activity. In order to directly quantitate the role of MgATP in complex formation with the $R^{I}$-subunits and PKI, a method based on analytical gel filtration was developed that is independent of an activity assay. This method not only allowed us to quantitate the role of MgATP in complex formation but also provided a basis for evaluating competition between the various classes of inhibitors. Although both complexes bind MgATP with a high affinity, the ATP off rate for the type I holoenzyme is very slow compared to the PKI·C-subunit complex and probably accounts for the $R^{I}$-subunit being able to successfully compete for the C-subunit in the absence of cAMP.

## EXPERIMENTAL PROCEDURES

*Reagents.* The peptide substrate, LRRASLG, was obtained from the UCSD Peptide and Oligonucleotide Facility and purified by reverse-phase HPLC. ATP, ADP, AMP-PCP, and AMP-PNP were purchased from Sigma; ATP-γ-S, from Boehringer Mannheim; and yeast hexokinase (88 units/mg) and glucose, from Calbiochem. [γ-$^{32}$P]ATP (3000 Ci/μmol) was purchased from Amersham.

*Purification of Proteins.* Following overexpression in *Escherichia coli* (Slice & Taylor, 1989), the C-subunit was first purified by phosphocellulose chromatography (P11 Whatman) and then resolved into discrete isoelectric variants on a Mono S HR10/10 column using FPLC (Pharmacia/LKB) (Herberg et al., 1993). Isozymes I and II were used for these experiments. The purity of the protein was checked by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and by analytical gel filtration (see below). The specific activity of the C-subunit was 25 μmol/min/mg as measured by the coupled spectrophotometric method of Cook et al. (Cook et al., 1982) using the heptapeptide, LRRASLG, as a substrate. The purified C-subunit was stored at 4 °C in 20 mM potassium phosphate, 110 mM KCl, and 5 mM 2-mercaptoethanol, pH 7.0.

The recombinant $R^{I}$- and $R^{II}$-subunits were overexpressed in *E. coli* and purified as described previously (Saraswat et al., 1986). The $R^{I}$-subunits were purified first by ion-exchange chromatography on DEAE-cellulose (Buechler & Taylor, 1991). The $R^{II}$-subunits were purified first on cAMP–agarose by affinity chromatography (Scott et al., 1987, 1990). After the initial chromatography step, both R-subunits were purified further using a Mono Q 5/5 ion-exchange column. The R-subunits, typically from 4 L, were dialyzed against 50 mM Tris (pH 7.4), 5 mM 2-mercaptoethanol, and 2 mM EDTA and subsequently loaded onto a Mono Q column equilibrated in the same buffer. The R-subunits were eluted in the same buffer using salt gradients from 0 to 300 mM KCl for the $R^{I}$-subunit and from 0 to 500 mM KCl for the $R^{II}$-subunit.

The $R^I$- and $R^{II}$-subunits were frozen in 20 mM potassium phosphate, 2 mM EDTA, 30% glycerol, and 5 mM 2-mercaptoethanol, pH 6.5, and stored at −20 °C.

To obtain cAMP-free R-subunit, the R-subunits were treated with urea as described by Buechler et al. (1993). The skeletal muscle heat-stable protein kinase inhibitor (PKI) was expressed in *E. coli* and purified according to Thomas et al. (1991).

*Holoenzyme Formation and Purification.* Two methods were used to obtain holoenzyme. To obtain holoenzyme in the presence of MgATP, the C- and $R^I$-subunits in a molar ratio of 1.2:1 were dialyzed for 24 h at 4 °C against 20 mM potassium phosphate, 100 mM KCl, 5 mM 2-mercaptoethanol, 5% glycerol, pH 6.5, 100 μM ATP, and 1 mM $MgCl_2$. Holoenzyme free of MgATP was obtained by mixing FITC-labeled C-subunit and cAMP-free wild-type $R^I$-subunit in a molar ratio of 1.2:1 followed by incubation for 30 min at 22 °C. An alternative procedure was to dialyze the holoenzyme·ATP complex against buffer containing 1 mM EDTA at 4 °C for at least 48 h. The resulting holoenzyme (500 μL; approximately 1 mg/mL) was filtered through a 0.22-μm nylon filter (Rainin Instrument Co.) and loaded onto a Superose 12 HR10/30 column (Pharmacia/LKB) equilibrated with 20 mM MOPS, 50 mM KCl, and 2 mM 2-mercaptoethanol (buffer A), pH 7.0. Chromatography was performed at 22 °C with a flow rate of 0.8 mL/min, and the absorbance of the elutate was monitored at 280 nm. The holoenzyme was collected according to the retention time and stored as a stock solution at 4 °C. To obtain higher holoenzyme concentrations, the cAMP-free $R^I$-subunit and the unlabeled C-subunit were first concentrated separately to 4 mg/mL with a Centricon 30 filter (Amicon). The concentrated cAMP-free $R^I$-subunit was then combined with a mixture of unlabeled and FITC-labeled C-subunit. The molar ratio of unlabeled to labeled C-subunit was 50:1.

*Fluorescence Labeling.* The C-subunit (0.5–1 mg/mL) was labeled with fluorescein 5-isothiocyanate (FITC, Molecular Probes) using a 35-fold molar excess of FITC. The labeling was carried out in 100 mM HEPES (pH 8.0), 5 mM $MgCl_2$, and 5 mM ATP at 22 °C. The reaction was stopped after 30 min by passing the reaction mixture through a prepacked Sephadex G-25 column (NAP 10 column, Pharmacia/LKB) equilibrated with buffer A and stored at 4 °C. This FITC-labeled C-subunit retained more than 95% of its initial phosphotransferase activity and was able to form holoenzyme with the $R^I$-subunit in a manner that was indistinguishable from that of the unlabeled C-subunit. PKI was labeled under the same conditions as described above in the absence of MgATP. The labeling reaction was carried out at 37 °C but can also be done at 60 °C with the same results. The labeled PKI showed a slight increase in Stokes' radius (data not shown) but no difference in its ability to inhibit the C-subunit in the spectrophotometric activity assay. Both labeled and unlabeled PKI were stored at −20 °C.

*Analytical Gel Filtration.* Analytical gel filtration was carried out using a Superose 12 HR 10/30 column or a Superdex 75 HR10/10 column, both with a flow rate of 0.8 mL/min at 22 °C in buffer A. Buffers were freshly prepared, filtered, and degassed daily. Unless otherwise specified, buffer A was used. Most experiments were carried out in duplicate and on at least two separate occasions. For $K_d$ determinations, holoenzymes or PKI complexes at various concentrations were preincubated for 30 min and then injected using a 25-μL sample loop. Calibrations were performed with protein standards purchased from Sigma or Pharmacia/LKB. The void volume ($V_o$) was determined with blue dextran; and the internal volume ($V_i$) with tryptophan. The protein was detected in a double-detector system connecting a UV M monitor (Pharmacia; flow cell, 8 μL; 280 nm) with a fluorescence detector (Hitachi fluorescence spectrophotometer) using a 15-μL flow cell. The absorption wavelength was 493 nm; the emission wavelength was 520 nm. The derived chromatograms were plotted on a two-channel chart recorder, and additional data were collected with FPLC manager software on a 386 IBM clone PC. Data processing was performed with the same software.

*Calculations.* Analytical gel filtration readily distinguished the holoenzyme from the free C-subunit and the PKI·C complex from free PKI. The percentage of holoenzyme at various protein concentrations was calculated on the basis of the peak areas corresponding to both holoenzyme and C-subunit. The holoenzyme peak area was corrected since the formation of holoenzyme quenched the fluorescence signal. To obtain the correction factor, the fluorescence of various amounts of FITC-labeled C-subunit was determined at 22 °C, using an absorption of 493 nm and an emission of 520 nm. To each solution was added an equimolar amount of cAMP-free $R^I$-subunit. After 30 min the quench of fluorescence due to holoenzyme formation was measured. A quench factor of 0.77 was determined for the holoenzyme in the presence of MgATP, and a quench factor of 0.79 was determined for MgATP-free holoenzyme. Complex formation between PKI and the C-subunit led to an increase in the relative fluorescence rather than a quench. The correction factor was 1.1 in the presence of MgATP.

*ATP Analogs.* The same method described above was used to determine the effect of various analogs of ATP, including ADP. In order to remove any ATP contamination from the ADP stock solution, 5 mM ADP was incubated with 0.01 mg/mL yeast hexokinase and 15 mM glucose in buffer A for 15 h prior to use. The gel filtration column was equilibrated in buffer A containing 50 μM of each analog (10 μM for ADP) and 1 mM $MgCl_2$.

*ATP Off Rates.* Binding studies with [$\gamma$-$^{32}$P]ATP were performed using the method described by Døskeland and Øgreid (1988) for measuring the binding of cAMP. The stock solution of [$\gamma$-$^{32}$P]ATP was diluted to the desired concentration in buffer A containing 10 mM $MgCl_2$, pH 7.0, and C-subunit. After incubation for 5 min on ice, the physiological inhibitor, either cAMP-free R-subunit or PKI, was added in a 1.5-fold molar excess, and incubation was continued for 30 min on ice. Before addition of a 1000-fold molar excess of cold ATP as a chase, an aliquot (25 μL) was removed. Aliquots (25 μL) were then removed at various time points after initiation of the chase. Bound ATP was separated from free ATP by Millipore filtration after ammonium sulfate precipitation using prewetted HA filters (0.45 μm, Millipore, Bedford, MA). Since control experiments (data not shown) showed significant binding of MgATP to BSA (Døskeland & Øgreid, 1988), no BSA was added.

*Competition Experiments.* Competition experiments between PKI (FITC-labeled) and cAMP-free $R^I$- or $R^{II}$-subunit for the C-subunit were performed at room temperature using a Superose 12 gel filtration column equilibrated with buffer A. Equimolar amounts of two proteins, determined on the basis of titration and monitored by following the loss of activity (Cook et al., 1982), were preincubated at room temperature for 20 min. An equimolar amount of the third protein was then added, and incubation was continued for 15 min at room temperature. At that point the incubation mixture was loaded onto the gel filtration column, and the absorption at 280 nm

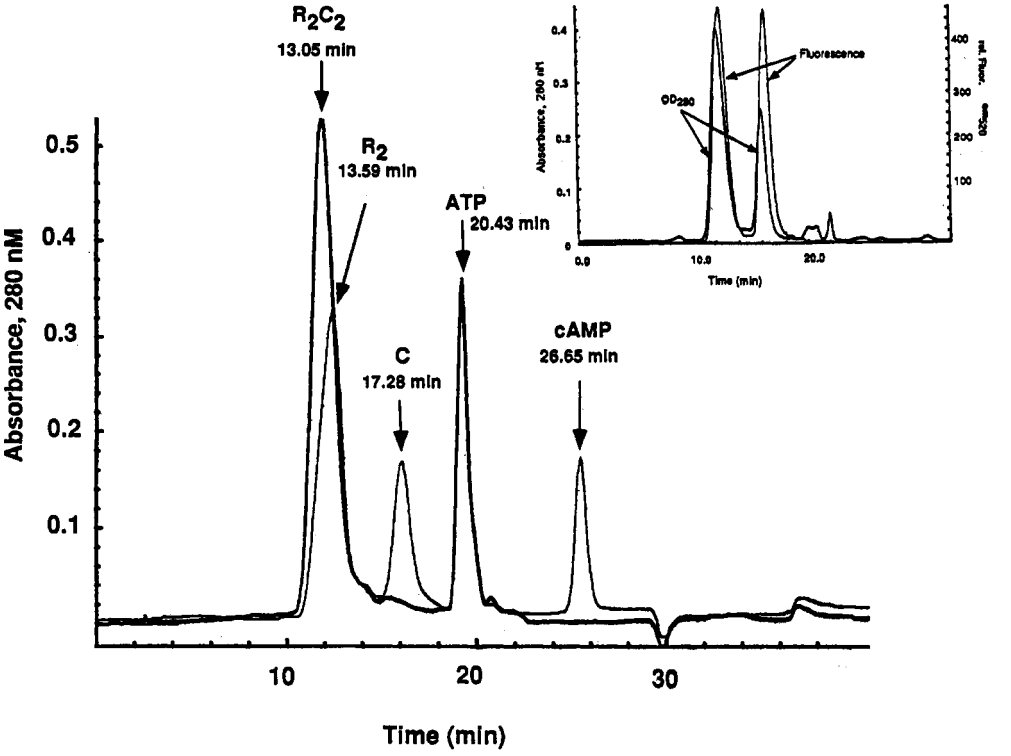


FIGURE 2: Elution of the type I holoenzyme from Superose 12 in the presence and absence of cAMP. Preformed type I holoenzyme was run on a Superose 12 column as described in the Experimental Procedures in the absence (thick line) and presence of cAMP (thin line). The various proteins and their retention times are indicated. The inset shows the elution of 230 nM FITC-labeled type I holoenzyme injected in a 25-$\mu$L loop on a Superose 12 column. The thick line shows the absorbance at 280 nm, while the thin line shows the elution detected by an on-line fluorimeter (excitation wavelength, 493 nm; emission wavelength, 520 nm). The correlation of peak heights with the concentrations of free C-subunit and holoenzyme is discussed in the text and in this experiment yielded 64.5% holoenzyme.

and the emission of FITC at 520 nm were monitored. The three proteins (C-subunit, R-subunit, and PKI) and the two complexes (C·PKI and $R_2C_2$) were distinguished on the basis of their retention times as well as by the fluorescence signal of the FITC-labeled PKI. For the $R^I$-subunit and PKI competition experiments, 1 $\mu$M of each protein was used; for the $R^{II}$-subunit experiments 0.5 $\mu$M of each was used.

## RESULTS

*Determination of Apparent $K_d$'s for Subunit Dissociation.* In order to determine the apparent $K_d$'s for subunit dissociation both in the presence and in the absence of MgATP, an analytical gel filtration method was developed. For these experiments a stock of MgATP-free holoenzyme (6–10 $\mu$M) was prepared by combining cAMP-stripped $R^I$-subunit and FITC-labeled C-subunit and subsequently diluting the mixture prior to gel filtration. The proteins could be monitored by either fluorescence or absorbance, although at low concentrations only fluorescence could be detected. The column was then run both in the presence and in the absence of MgATP.

As seen in Figure 2, the free subunits can be readily distinguished from the holoenzyme by using analytical gel filtration. The proteins associated with each peak were identified by assaying for activity and by SDS–gel electrophoresis. The relatively small shift in retention time of the free R-subunit in comparison to that of the holoenzyme demonstrated significant asymmetry of the R-subunit dimer. The $R_2$-dimer with a molecular mass of 85 600 Da had a Stokes radius of 43.2 Å, while the Stokes radius of the holoenzyme is only 46 Å, consistent with previous measurements (Zoller et al., 1979). A typical run where holoenzyme is dissociated approximately 35% is shown in the inset of Figure 2.

*Type I Holoenzyme.* The dissociation of the type I holoenzyme complex as a function of protein concentration is shown in Figure 3A. In the presence of MgATP, holoenzyme did not dissociate significantly even at subnanomolar concentrations (<0.05 nM). In the absence of MgATP, however, the apparent $K_d$ for the dissociation of the holoenzyme complex was 125 nM.

*Type II Holoenzyme.* When the type II holoenzyme was formed in the absence of MgATP, dissociation was only 87% at a concentration of 1 nM. When the type II holoenzyme was eluted in buffer containing MgATP, greater dissociation was observed, but it was still only 59% at 1 nM holoenzyme (Figure 3A).

*PKI·Subunit Complexes.* The stability of the C·PKI complex was determined using conditions identical to those described above for the holoenzymes. Instead of a Superose 12 column, a Superdex 75 gel filtration column was used. A 50 $\mu$M complex of recombinant C-subunit and FITC-labeled PKI was prepared, and the dissociation of this complex as a function of protein concentration is shown in Figure 3B. In the presence of 100 $\mu$M ATP and 1 mM $MgCl_2$, even at subnanomolar concentrations (0.5 nM) (data not shown), at least 80% of the complex was still intact. In the absence of MgATP, however, the apparent $K_d$ for dissociation was 2.3 $\mu$M.

*ATP Binding.* Analytical gel filtration was also used to determine the apparent $K_d$ for the binding of MgATP for holoenzyme formation with the type I R-subunit and for the formation of the recombinant C-subunit·PKI complex. To accomplish this, the Superose 12 column was equilibrated with buffer A containing varying concentrations of ATP and 1 mM $MgCl_2$. Holoenzyme was formed by combining R-subunit and C-subunit in the presence of MgATP followed

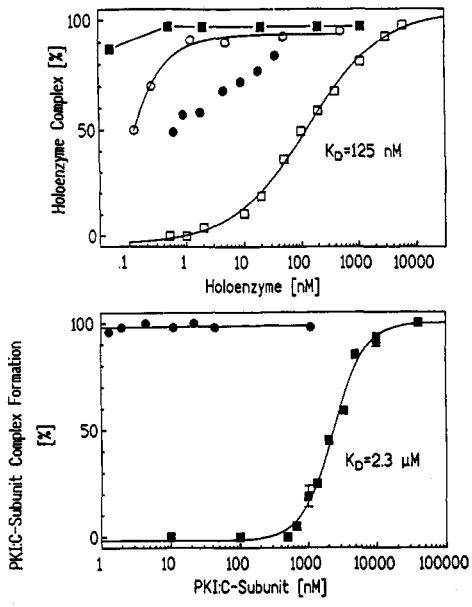


FIGURE 3: Apparent dissociation constants for various inhibitor complexes in the presence and absence of MgATP. Each data point was obtained from two independent gel filtration chromatograms. (A, top) Apparent dissociation constants (app$K_d$) for holoenzyme formation in the presence and absence of MgATP. The dissociation of both types of holoenzyme, I (■, □) and II (●, ○), is shown in the presence (filled symbols) and in the absence of MgATP (open symbols). (B, bottom) The apparent dissociation constant (app$K_d$) for the PKI·C-subunit complex in the presence and absence of MgATP. Dissociation curves were calculated in the presence (●) and absence of 100 μM ATP and 1 mM $MgCl_2$ (■) using a 25-μL loop. Error bars were used to evaluate duplicate experiments, although in most cases the error bars were hidden within the data point. When a large sample loop (500 μL) was used for injection, 20% complex dissociation was observed at 0.2 nM PKI·C-subunit concentration in the absence of MgATP.

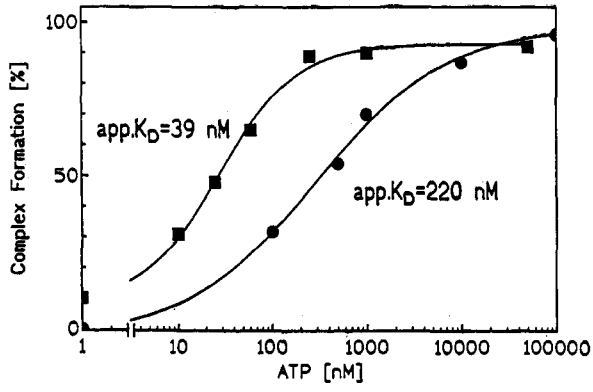


FIGURE 4: Apparent binding constants (app$K_d$) for MgATP binding to the inhibitor complexes. The dependency of complex formation on ATP concentration was determined for PKI (●) and the type I R-subunit (■) using gel filtration as described in Experimental Procedures.

by subsequent dialysis against EDTA-containing buffer to remove the ATP. After removal of the EDTA, 2.5 nM holoenzyme was incubated for 30 min at the indicated ATP concentration and then injected into the Superose 12 column. The amount of holoenzyme formed was determined by integrating the peak areas. The ATP concentration necessary to form 50% holoenzyme was 39 nM (Figure 4).

The MgATP dependency for complex formation with PKI was also determined using a Superdex 75 column. Protein (100 nM) from a 6.5 μM stock solution prepared in the absence of MgATP was incubated for 30 min at different ATP concentrations and then injected. The apparent $K_d$ for MgATP was 220 nM (Figure 4).

Table I: Effect of ATP Analogs on the Formation of $R^I$ Holoenzyme[a]

| analog | holoenzyme formation at 1 nM (%) |
|---|---|
| ATP | 95 |
| ADP | 30 |
| ATP-γ-S | 40 |
| AMP-PNP | 80 |
| AMP-PCP | 35 |

[a] All experiments were performed as gel filtration experiments under the conditions described in Experimental Procedures using 1 mM $MgCl_2$ and 50 μM (10 μM for ADP) of each analog.

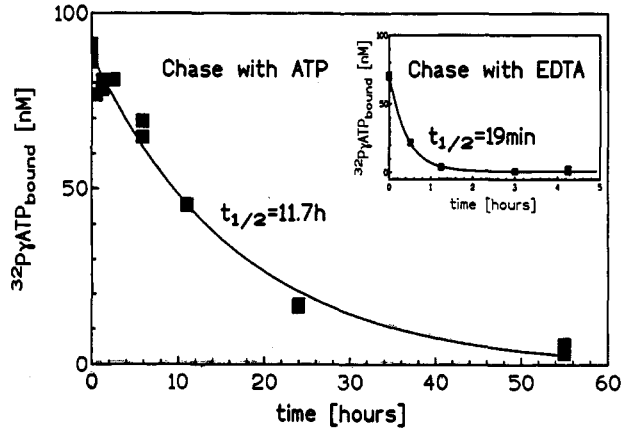


FIGURE 5: MgATP off rates for the type I holoenzyme. Off rates of MgATP from the type I holoenzyme were determined as described in Experimental Procedures. [γ-$^{32}$P]ATP (1 μM) was chased with 10 mM cold ATP or 10 mM EDTA (inset) at 1 mM $MgCl_2$. The holoenzyme concentration was 50 nM.

While MgATP has a dramatic effect on stabilization of the type I holoenzyme and the PKI complex, analogs of ATP were less efficient, as shown in Table I. Replacement of the bridging oxygen with nitrogen (AMP-PNP) or carbon (AMP-PCP) significantly reduced the capacity of the analog to stabilize the complex. ADP also was relatively inefficient in stabilizing the complex. When $Mn^{2+}$ was substituted for $Mg^{2+}$, however, the C-subunit·inhibitor complexes with the ATP analogs and ADP were significantly more stable (data not shown).

*ATP Off Rates.* The off rates for MgATP from the holoenzyme and from the PKI·C-subunit complex were determined as described in the Experimental Procedures. Once [γ-$^{32}$P]ATP was equilibrated with the holoenzyme complex, the bound ATP was then chased by adding at least a 1000-fold excess of cold ATP. Between 30 nM and 3 μM holoenzyme a linear dependency between protein concentration and [γ-$^{32}$P]ATP binding was observed. Physiological concentrations of cAPK are in the range of 0.2–0.3 μM (Beavo et al., 1975). Figure 5 shows the off rates for MgATP from holoenzyme, formed by combining the urea-stripped cAMP-free $R^I$-subunit with C-subunit and chasing the mixture with 1 mM ATP. Similar results were obtained when 10 mM ATP was used for the chase. The apparent half-maximal off rate for 100 μM $R^I$-stripped holoenzyme was determined to be 11.7 h.

A similar chase experiment was carried out for the C·PKI complex. [γ-$^{32}$P]ATP was bound to the complex and then chased as described above for holoenzyme. A linear relationship was observed for ATP binding and protein concentration in the range of 30 nM to 3 μM. The half-maximal off rate for MgATP from the PKI·C-subunit complex was 17 min (Figure 6).

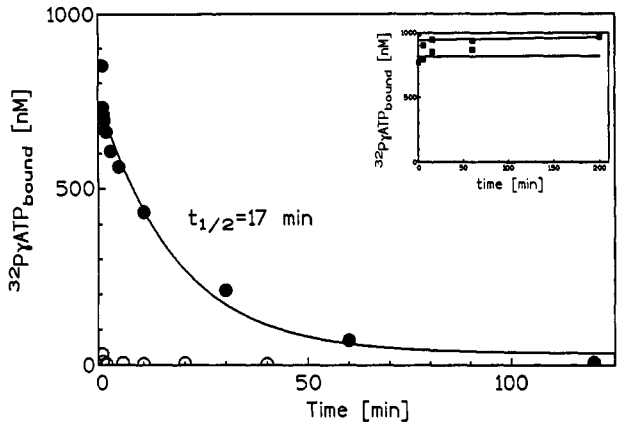


FIGURE 6: MgATP off rates for the PKI·C-subunit complex. An off rate of 17 min was determined for the PKI·C-subunit complex at a protein concentration of 700 nM. The filled circles (●) show a chase with 10 mM ATP; and the open circles (○), a chase with 10 mM EDTA. The inset shows the ATPase activity for both the holoenzyme (■) and the PKI·C-subunit complex (●). For the determination of ATPase activity, holoenzyme (350 nM) and the PKI·C-subunit complex (700 nM) were incubated with 1 μM [γ-$^{32}$P]ATP without a chase.

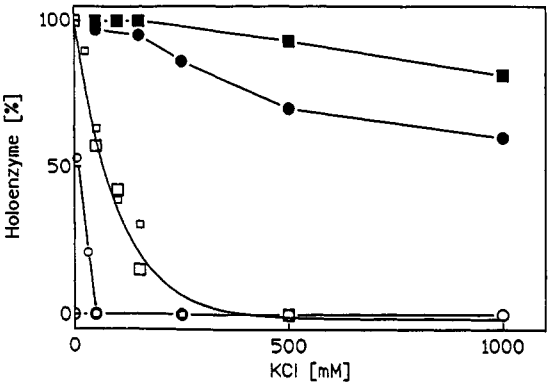


FIGURE 7: Effect of salt on the stability of complex formation. The type I holoenzyme (2 nM; ■, □) and the PKI·C-subunit complex (10 nM; ●, ○) were run on a Superose 12 column equilibrated in varying concentrations of KCl in the presence of 100 μM ATP and 1 mM $MgCl_2$ (filled symbols) or in the absence of MgATP in 1 mM EDTA (open symbols). An apparent value for half-maximal dissociation for the holoenzyme in the absence of MgATP was calculated to be 68 mM KCl. Also indicated is the dissociation of 100 nM holoenzyme (▫) and 200 nM PKI·C-subunit (○) in the absence of MgATP.

The free C-subunit shows significant ATPase activity (2.3 μmol/min/mg at room temperature). However, when the C-subunit is complexed with either PKI or the $R^I$-subunit, the ATPase activity, even at room temperature, is no longer detectable (inset, Figure 6). Therefore, under these conditions loss of ATP binding cannot be confused with hydrolysis of [γ-$^{32}$P]ATP.

Since MgATP binds so tightly to the holoenzyme and to the C·PKI complex, we determined whether EDTA could efficiently strip the nucleotide from the complex. For these experiments, the preformed holoenzyme was added to buffer containing 1 μM [γ-$^{32}$P]ATP and 1 mM $MgCl_2$, and the chase reaction was initiated with 10 mM EDTA. In the case of the holoenzyme a half-maximal off rate of 19 min was observed. For the C·PKI complex, the off rate, in the presence of EDTA, was too fast to measure.

*Effect of Salt on Dissociation of Holoenzyme and C·PKI.* The type I holoenzyme is relatively resistant to salt-induced dissociation. Analytical gel filtration was used to evaluate how important ATP was for this stability. For these experiments, the concentrations of the complexes used were in the range that would be found physiologically. Two holoenzyme concentrations were used, 5 and 100 nM; the C·PKI concentration was 200 nM. As seen in Figure 7, in the presence of 100 μM ATP and 1 mM $MgCl_2$ neither the type I holoenzyme nor the PKI·C-subunit complex dissociates significantly up to a salt concentration of 1 M KCl. Even at 3 M KCl half of the holoenzyme still exists as a complex (data not shown). In the absence of MgATP, however, complete dissociation of the holoenzyme is achieved in 250 mM KCl. The apparent salt concentration for half-maximal dissociation is approximately 68 mM. No complex formation occurred in the presence of 1 mM EDTA at salt concentrations above 25 nM for the PKI·C-subunit complex.

*Competition Experiments.* Since we ultimately would like to understand the physiological role of PKI, the two inhibitors, PKI and the R-subunit, were evaluated for their capacity to compete for the C-subunit in the presence of MgATP. As seen in Figure 8A, when the preformed type I holoenzyme was incubated with equimolar concentrations of FITC-labeled PKI, PKI could not compete at all for the C-subunit, similar to what has been observed previously (Ashby & Walsh, 1973). When C-subunit was added to an equimolar solution of both PKI and $R^I$, however, it preferentially associated with $R^I$. As seen in Figure 8B, under these conditions, approximately 70% of the C-subunit was found in the holoenzyme complex. Although PKI could not compete for the C-subunit when it was part of the holoenzyme complex, when the C·PKI·MgATP complex was preformed, the cAMP-free R-subunit could compete reasonably well for the C-subunit. The results shown in Figure 8C are similar to those obtained when the C-subunit was given a choice to associate with either cAMP-free $R^I$-subunit or PKI directly (Figure 8B). In this case 74% of the C-subunit was associated with $R^I$-subunit in a holoenzyme complex (Figure 8C). When the $R^I$-subunit is saturated with cAMP, however, it cannot compete for the C-subunit when C is associated with PKI (data not shown). In the absence of MgATP, PKI cannot compete with the holoenzyme under any of the conditions described above, and the results are indistinguishable from what is shown in Figure 8A.

The $R^{II}$-subunit is also capable of competing the C-subunit away from the C·PKI complex in a way similar to that of the $R^I$-subunit. Once the type II holoenzyme is formed, PKI is also not able to compete for the C-subunit in the presence of MgATP (data not shown).

## DISCUSSION

The C-subunit of cAPK can interact with several different types of inhibitor proteins. The best characterized are the R-subunits that bind with a high affinity in the absence of cAMP (Hofmann, 1980). The other inhibitors are the heat-stable protein kinase inhibitors (PKI's) (Walsh et al., 1990). MgATP has a direct effect on these inhibitor complexes. In fact, interaction with ATP is probably one of the most important distinguishing features.

The simplest inhibitors are the $R^{II}$-subunits since they do not require ATP for stable holoenzyme formation. Furthermore, since they contain an autophosphorylation site at their inhibitor domain, ATP binding results in autophosphorylation. The phosphorylated $R^{II}$-subunit has a somewhat reduced affinity for the C-subunit as reported previously (Rangel-Aldao & Rosen, 1976). In the intact cell the type II holoenzyme is presumably phosphorylated given the high levels of ATP in the cell (Erlichman et al., 1974). Following activation by cAMP, the $R^{II}$-subunit can be readily dephos-

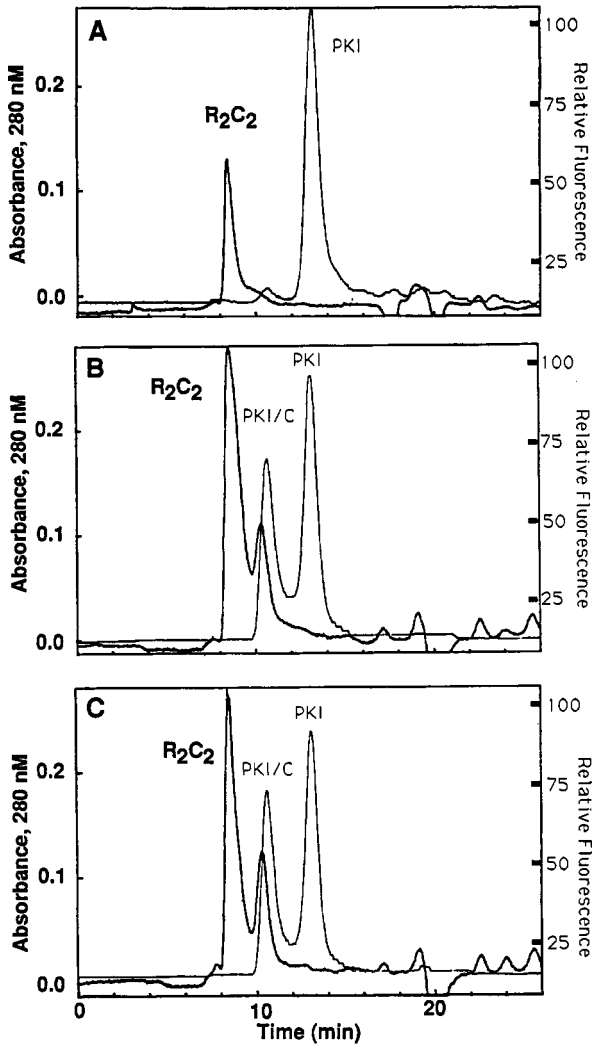


FIGURE 8: Competition between the C-subunit and physiological inhibitors. In panel A, FITC–PKI was added to preformed type I holoenzyme (1 $\mu$M). The mixture was then run over a Superose 10/30 column. The thick line shows absorbance at 280 nM; the thin line shows fluorescence emission at 520 nM due to the FITC–PKI. In panel B, the C·FITC–PKI complex (1 $\mu$M) was preformed and the $R^I$-subunit (1 $\mu$M) was then added. In panel C, C-subunit (1 $\mu$M) was added to a mixture of R-subunit (1 $\mu$M) and PKI (1 $\mu$M). After 15 min at room temperature the mixture was run on a Superose 12 column as described in panel A. All runs were performed in the presence of MgATP.

phorylated by protein phosphatases such as calcineurin (Blumenthal et al., 1986), and this would facilitate reassociation. Hence for the type II holoenzymes, complex formation does not depend on MgATP, and protein phosphatases can actually play a role in facilitating holoenzyme reformation.

In contrast to $R^{II}$, both the $R^I$-subunit and PKI require the synergistic binding of ATP to form a stable complex (Corbin et al., 1975; Neitzel et al., 1991; Van Patten et al., 1986; Whitehouse & Walsh, 1983). For the type I holoenzyme, ATP is not only important for stabilizing the holoenzyme but also essential for the formation of the type I holoenzyme under physiological conditions when cAMP is bound to the R-subunit (Neitzel et al., 1991). This dependency on ATP makes complex formation more difficult to dissect because one must consider not only the intrinsic $K_d$'s of the proteins for one another but also the binding of ATP and divalent cations that lock the C-subunit in a complex with its physiological inhibitors. Using analytical gel filtration, we have attempted to quantitate the effect of ATP on holoenzyme stabilization. In the case of the type I holoenzyme, the apparent $K_d$ for holoenzyme dissociation is reduced more than 3 orders of magnitude in the presence of MgATP. The stabilization is even greater for the C·PKI complex. Furthermore, as demonstrated previously for both complexes (Corbin et al., 1975; Van Patten et al., 1986; Whitehouse & Walsh, 1983), ADP and analogs of ATP will only partially substitute for ATP.

MgATP thus serves two roles for this protein kinase. For the free C-subunit, ATP binds as a simple substrate with a $K_m$ of 10 $\mu$M (Zoller et al., 1979). The $K_d$(ATP) for the free C-subunit is also 10 $\mu$M (Whitehouse et al., 1983). In the presence of the $R^I$-subunit or PKI, however, ATP serves as an allosteric effector because it stabilizes the inactive inhibited conformation. Perhaps a better term is "homosteric" inhibitor since ATP is not binding in this complex at a site that is distinct from the active site. It is binding at the same site in presumably a slightly different conformation (Hoppe et al., 1978; Zheng et al., 1993).

As demonstrated here, MgATP also stabilizes the type I holoenzyme against salt-induced dissociation. Furthermore, by quantitating this effect using purified proteins, we demonstrated unambiguously that, in the absence of MgATP, the type I holoenzyme dissociates readily under physiological salt conditions. In the absence of MgATP the C·PKI complex dissociates even more readily at physiological salt concentrations. This stringent requirement for MgATP leads to predictions that there may be an alternate mechanism for activating the type I holoenzyme or that there may be a specific, as yet unrecognized, mechanism for dissociating the C·PKI complex.

The MgATP dependency of the type I holoenzyme was first demonstrated using partially purified extracts (Corbin et al., 1978). These experiments also showed that in crude extracts reassociation of R- and C-subunits occurs rapidly in contrast to reassociation of purified subunits *in vitro*. Phosphodiesterases presumably account for this rapid holoenzyme reformation in crude extracts when cAMP is removed, and the addition of phosphodiesterases *in vitro* does enhance subunit reassociation (Rangel-Aldao & Rosen, 1977). Whether a physiological mechanism exists for rapidly removing tightly bound MgATP from the holoenzyme is not clear. However, if such a mechanism does exist, then both of these complexes could be activated under physiological conditions independent of cAMP levels.

Although both PKI and the $R^I$-subunit require ATP to form a stable complex with C, the two inhibitors differ in how they bind ATP. In both complexes, the ATPase activity seen with free C-subunit is abolished. ATP, however, exchanges at a relatively fast rate from the C·PKI complex, whereas at 350 nM holoenzyme no release of ATP could be measured over 2 h. Even at a 35 nM concentration of holoenzyme, somewhat below what is seen under physiological conditions (0.2–0.3 $\mu$M) (Beavo et al., 1975), a slow off rate could be measured.

We believe that the more rapid off rate of MgATP from the C·PKI complex probably explains why the cAMP-free R-subunit can compete effectively for the C-subunit when it is bound to PKI. This could be significant physiologically and certainly indicates that newly synthesized R-subunit could compete very well for the C-subunit in the cell when C was bound to PKI. The R-subunit might compete equally well under conditions where phosphodiesterases were active. PKI could thus serve as a shuttle. It rapidly reduces the kinase activity even when cAMP levels are high; however, according to this model, PKI would eventually return the C-subunit to a holoenzyme complex.

The *in vivo* microinjection of fluorescently tagged proteins also supports such a model (Fantozzi et al., 1992,1993; Meinkoth et al., 1990). On the basis of their results, neither the free $R^{I}$- and $R^{II}$-subunits nor the holoenzymes can enter the nucleus, whereas both PKI and the free C-subunit can enter the nucleus readily. A complex of PKI and the C-subunit also cannot enter the nucleus, but free PKI rapidly depletes the nucleus of C-subunit and thus can serve as a shuttle between the nucleus and the cytoplasm (Fantozzi et al., 1993). Whether PKI then returns the C-subunit to a holoenzyme complex *in vivo* remains to be established. Our results suggest that such a mechanism is at least feasible and that the formation of a C·PKI complex need not be a dead-end pathway.

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