Reconstitution of Types I and II Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

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ABSTRACT: Fluorescence intensity and anisotropy measurements using the fluorescent adenosine cyclic 3′,5′-phosphate (cAMP) analogue 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate (ε-cAMP) are sensitive to the dissociation of ε-cAMP which occurs when either the type I or the type II regulatory subunit (R^I or R^{II}) of cAMP-dependent protein kinase associates with the catalytic subunit. Studies using ε-cAMP show that MgATP has opposite effects on the reconstitution of both types of protein kinase: MgATP strongly stabilizes the type I holoenzyme while it slightly destabilizes the type II holoenzyme. The synthetic substrate Kemptide has a small inhibitory effect on the reconstitution of both holoenzymes when tested at 10 μM concentration. The protein kinase inhibitor has a larger effect which is especially pronounced in the reassociation of the type I enzyme. The diminished relative ability of the type I regulatory subunit to compete with the protein kinase inhibitor suggests that the combined effects of the two opposing equilibria (ε-cAMP and catalytic subunit binding) are different for the two types of regulatory subunits. Displacement experiments show that cAMP and ε-cAMP bind about equally well to the type I subunit. Slow conformational changes accompanying the binding of ε-cAMP by both regulatory subunits are greatly accelerated with the holoenzymes, suggesting that dissociation of the holoenzymes occurs via ternary complexes. The time courses of ε-cAMP binding also show the heterogeneity of binding characteristics of R^{II}. The 37 000-dalton fragment of type II subunit retains the ε-cAMP binding properties of the native subunit. However, only a fraction of the fragment preparation (~32% estimated from sedimentation measurements) binds the catalytic subunit well, suggesting heterogeneity of cleavage.

Living cells are capable of carrying out a variety of chemical reactions all of which need to be regulated in relation to one another. One such universal regulator is adenosine cyclic 3′,5′-phosphate (cAMP)[1] discovered by Sutherland and Rall [cf. reviews by Robinson et al. (1971) and Sutherland (1972)].

It is now generally accepted that the action of many hormones, particularly the catecholamines, is mediated through the production of cAMP as a second messenger [cf. review by Greengard (1978) and Krebs & Beavo (1979)]. Ultimately, this message provides a general mechanism for a variety of protein phosphorylations carried out by the cAMP-dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) [cf. reviews by Krebs (1972), Carlson et al. (1979), Hoppe & Wagner (1979), and Glass & Krebs (1980)]. Indeed, cAMP-dependent protein kinases are ubiquitously distributed in most tissues and play a central role in the regulation of energy metabolism and several other physiological processes (Krebs, 1972; Rubin & Rosen, 1975; Rosen et al., 1977; Cohen, 1978; Krebs & Beavo, 1979).

The holoenzyme of protein kinase is a tetramer composed of two regulatory subunits, which can each bind 2 equiv of cAMP, and two catalytic subunits. There are two distinct classes of regulatory subunits designated R^I and R^{II} which differ both in their physical properties (Erlichmann et al., 1973; Hofmann et al., 1975; Corbin et al., 1978) and in their tissue distribution (Corbin et al., 1975, 1977). In both types of holoenzyme, the binding of cAMP to the regulatory subunit causes the enzyme to dissociate into two catalytically active subunits and a dimeric, regulatory subunit–cAMP complex according to

$$R_2C_{2,\text{inactive}} + 4\text{cAMP} \rightleftarrows 2C_{\text{active}} + R_2\cdot(\text{cAMP})_4 \quad (1)$$

The structural and functional differences between the two types of kinase are due to differences in their regulatory subunits. The catalytic subunits are said to be identical (Hofmann et al., 1975). Type I protein kinase has a high-affinity binding site for MgATP which is absent in the type II holoenzyme (Beavo et al., 1975; Hofmann et al., 1975). However, the type II holoenzyme is capable of autophosphorylation of its regulatory subunit, whereas type I is not (Erlichmann et al., 1973; Rosen & Erlichmann, 1975). Despite these differences, the regulatory subunits have many structural similarities including binding to the catalytic subunit with equal affinity (Builder et al., 1979; Hofmann, 1980) and susceptibility to limited proteolysis yielding cAMP binding fragments of similar size (Srivastava & Stellwagen, 1978; Weber & Hilz, 1978; Corbin et al., 1978; Potter & Taylor, 1979a,b; Taylor et al., 1981). Although the sites susceptible to proteolysis are different in R^I and R^{II}, they are homologous (Potter & Taylor, 1980). R^I and R^{II} in the absence of cAMP inhibit the activity of the catalytic subunits by re-forming the holoenzyme as indicated by the reverse of eq 1. In addition, protein kinase activity can almost be entirely inhibited by a heat-stable protein inhibitor (Walsh et al., 1971; Demaille et al., 1977; Weber & Rosen,

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ε-cAMP, 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate; R^I and R^{II}, regulatory subunit of types I and II cAMP-dependent protein kinase, respectively; $37K^{II}$, carboxy-terminal fragment of the regulatory subunit of type II cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; PKI, cAMP-dependent protein kinase inhibitor; $\bar{A}$, anisotropy; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $\bar{n}$, number of moles of ligand bound per mole of protein; AMP-PNP, 5′-adenylyl imidodiphosphate; cAMP, adenosine cyclic 3′,5′-phosphate.

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1977; Szmigielski et al., 1977; Costa, 1977; Beale et al., 1977). This inhibitor binds to free catalytic subunit with a K_d in the nanomolar range and acts irrespectively of the concentration of cAMP (Demaille et al., 1977, 1979; Ferraz et al., 1979).

This report extends the studies of the two types of regulatory subunit by using 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate (ϵ-cAMP), the fluorescent analogue of cAMP first synthesized by Secrist et al. (1972a). ϵ-cAMP can substitute for cAMP in the activation of cAMP-dependent protein kinase (Secrist et al., 1972b). It has been applied as a sensitive spectroscopic probe of the cAMP binding sites on R^I and R^{II} (LaPorte et al., 1980; Builder et al., 1980a, 1981; Smith et al., 1981). The experiments described here utilize the fluorescence of ϵ-cAMP to further characterize the interaction of R^I and R^{II} and the proteolytic fragment of R^{II} with the catalytic subunit (C). The results show how molecules that bind to C—notably MgATP, the synthetic substrate, and the protein kinase inhibitor (PKI)—affect the R–C interaction.

Materials and Methods

Materials. [γ-^{32}P]ATP (>2000 Ci/mmol) was purchased from Amersham; cAMP, cGMP, and bovine serum albumin were from Sigma. ϵ-cAMP was obtained from P-L Biochemicals. The synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, or Kemptide, having the sequence of the phosphorylation site of porcine pyruvate kinase (Zetterqvist et al., 1976; Kemp et al., 1976) was purchased from Boehringer Mannheim. Sephacryl S-200 superfine and CM-Sephadex C-50 were obtained from Pharmacia, Piscataway, NJ, and DE-52 and P-11 cellulose were from Whatman. Spheroidal hydroxylapatite was purchased from BDH Chemicals, Ltd. The anion-exchange resin AG1-X8 (200–400 mesh, acetate form) and electrophoresis-grade urea were procured from Bio-Rad. Aquacide I-A was from Calbiochem. All other reagents were of the highest grade commercially available.

Protein Preparations and Assays. The catalytic subunit of type II cAMP-dependent protein kinase was prepared from bovine heart as previously described (Peters et al., 1977) with one additional purification step. The C subunit from the Sephacryl S-200 superfine column was chromatographed on hydroxylapatite as described by Sugden et al. (1976) with the following minor modifications. The column was equilibrated with 50 mM potassium phosphate, 50 mM sodium chloride, and 1 mM dithiothreitol, pH 6.8 at 4 °C. After application of the sample, the column was washed with the same buffer and eluted with a linear gradient of 50–400 mM potassium phosphate buffer, pH 6.8, containing 1 mM dithiothreitol. Fractions were pooled on the basis of absorption at 280 nm, concentrated by ultrafiltration with an immersible ultrafiltration unit (Millipore Immersible CX NMWL 10000 daltons), dialyzed against 1 volume of glycerol, and stored at −20 °C.

R^{II} was prepared from bovine heart essentially as described by Dills et al. (1979). Regulatory subunit free of cAMP was prepared according to Builder et al. (1980b) with the exception that longer times were used to complete the procedure and the buffer consisted of 5 mM Mops, 0.5 mM EDTA, and 15 mM β-mercaptoethanol, pH 6.9. When cAMP-free R^{II} was chromatographed on Sephacryl S-200 superfine, two peaks were routinely eluted. In both cases, $NaDodSO_4$ gel electrophoresis showed one major band at 56 kdalton. The first peak contained predominantly a high molecular weight aggregate of R^{II} on the basis of sedimentation velocity ultracentrifugation. When R^{II} was chromatographed on Sephacryl S-200 prior to urea treatment, two peaks were also observed. However, no difference was observed between them on the basis of molecular weight on $NaDodSO_4$ gels, sedimentation coefficient, or phosphate content. cAMP-free R^I from bovine skeletal muscle was a generous gift from Drs. Craig Hixon and Mary Hurwitz. R^I, like R^{II}, separated into two peaks on Sephacryl S-200 chromatography. Material in the second peak was routinely used with either R^I or R^{II}.

The cAMP binding fragment of R^{II}, designated $37K^{II}$, was obtained from endogenous proteolysis of R^{II}; it was purified by chromatography on Sephacryl S-200, and its molecular weight was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Type I and type II holoenzymes were prepared when needed by mixing equimolar quantities of regulatory and catalytic subunits at concentrations of 1–4 mg/mL.

The heat-stable protein inhibitor of cAMP-dependent protein kinase was prepared according to Demaille et al. (1977).

The purity of all proteins was greater than 95% on the basis of $NaDodSO_4$–polyacrylamide gel electrophoresis.

Protein concentration was routinely determined by UV absorption using values of $A_{280nm}^{0.1\%,1cm} = 1.49$ for C (Peters et al., 1977), 0.80 for R^I (Builder et al., 1980a), 0.60 for R^{II} (Corbin et al., 1978), and 0.92 for $37K^{II}$. In some cases involving R^I, the stoichiometry of ϵ-cAMP of 2 was used as a measure of protein concentration since it gave a well-defined end point in titrations with ϵ-cAMP (see Figure 1 below). The concentration of $37K^{II}$ was determined by amino acid analysis. The following molecular weights were used to calculate molarity from protein concentration: C = 39000, R^I = 49000, R^{II} = 56000, and $37K^{II}$ = 37000.[2]

Proteins were concentrated in dialysis bags at 4 °C by covering the membranes with Aquacide I-A or unswollen CM-Sephadex C-50. When Aquacide I-A was used, it was found to be a significant contaminant in the protein solution, as seen by the presence of a species of molecular weight 10000 in ultracentrifugation runs not found in those solutions concentrated by CM-Sephadex C-50. Aquacide I-A could not be removed by extensive dialysis.

Protein-bound phosphate was determined by the method of Itaya & Ui (1966) following ashing of the protein as described by Ames (1966).

Unless otherwise noted, the following buffers were used for the indicated proteins: R^I and R^{II}, 5 mM Mops, 0.5 mM EDTA, 0.1 M KCl, and 15 mM β-mercaptoethanol, pH 7.0; $R^I{}_2C_2$ and $R^{II}{}_2C_2$, 5–50 mM Mops, 0.5 mM EDTA, 0.1–0.15 M KCl, and 15 mM β-mercaptoethanol, pH 7.0.

The phosphotransferase activity of the catalytic subunit was measured by the filter paper method of Glass et al. (1978). Assay solutions contained 11.3 nM C, 4.2 mM magnesium acetate, 250 M [γ-^{32}P]ATP (100 cpm/pmol), 125 μM Kemptide, 0.17 mg/mL bovine serum albumin, 28 mM Mops, and 13 mM β-mercaptoethanol, pH 7.

Fluorescence Measurements. Measurements of fluorescence anisotropy[3] and fluorescence intensity ($I_\parallel + 2I_\perp$) were obtained by using the SLM 4000 fluorescence polarization spectrophotometer. The excitation wavelength was 310 nm for ϵ-cAMP. The fluorescence was observed through a Schott-KV filter with a 380-nm cutoff. Corrections were made for

[2] Determined by $NaDodSO_4$ gel electrophoresis.

[3] $\bar{A} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. $I_\parallel$ vibrates in the direction of propagation of the exciting light, and $I_\perp$ vibrates normally to the plane corresponding to the directions of excitation and observation. In a mixture of fluorescent species, the average anisotropy equals the sum of the individual anisotropies weighed by the individual fractional contributions to the total fluorescence intensity (Weber, 1952): $\bar{A} = \sum f_i A_i$.

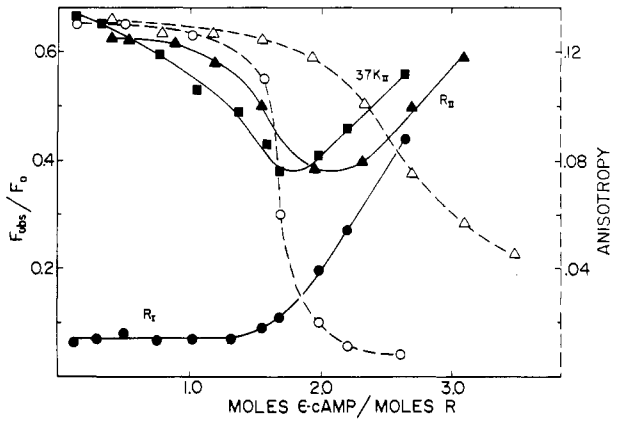

FIGURE 1: Titration of R^I, R^{II}, and $37K^{II}$ with ϵ-cAMP. Fluorescence intensity (F_{obsd}) (solid lines) and anisotropy measurements (dashed lines) were made in 5 mM Mops, 0.1 M KCl, 0.5 mM EDTA, and 15 mM β-mercaptoethanol, pH 7 at 25 °C, ~7 min after each addition of ϵ-cAMP. F_0 is the fluorescence of corresponding concentrations of ϵ-cAMP. Excitation, 310 nm; emission, Schott KV 380 filter. 2.5 μM R^I (●, ○); 1.0 μM R^{II} (▲, △); 2.5 μM $37K^{II}$ (■).

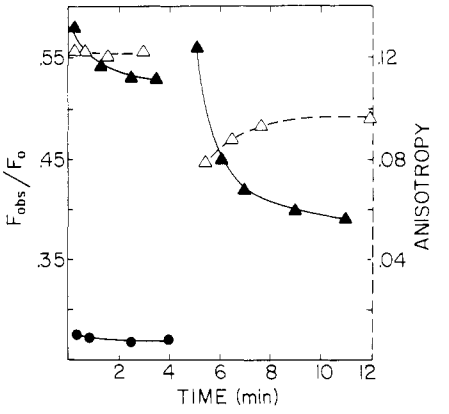

FIGURE 2: Time course of ϵ-cAMP binding to R^I and R^{II}. At zero time, 2 μM ϵ-cAMP was added to a 0.85 μM solution of R^I (●), and 1 μM ϵ-cAMP was added to 1.0 μM R^{II} (▲, △). After a 5-min incubation, a second mole of ϵ-cAMP was added to R^{II}. Solid lines, fluorescence intensities; dashed lines, anisotropies. For other conditions, refer to the legend of Figure 1.

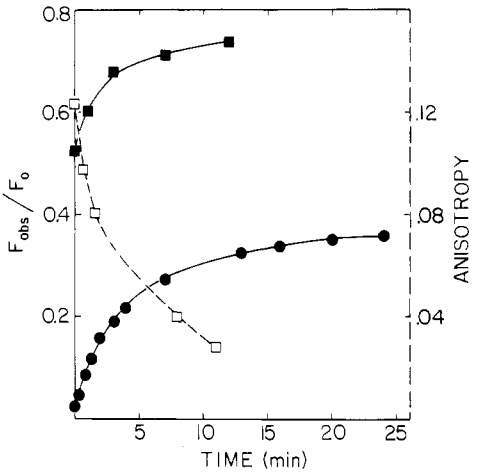

FIGURE 3: Time course of displacement of ϵ-cAMP from R^I and R^{II} by cAMP. R^I and R^{II} (1 μM) were incubated with 0.86 μM ϵ-cAMP for 13 and 6 min, respectively. At zero time, cAMP was added to give 3.1 μM. R^I (●); R^{II} (■, □). For other conditions, see the legend of Figure 1.

background fluorescence in the case of R^1.

Ultracentrifugation. Sedimentation velocity experiments were performed at 4 °C with a Beckman-Spinco Model E analytical ultracentrifuge equipped with an interference optical system and a photoelectric scanner. The absorption scanner was linked to a PDP-12 computer (Digital Equipment Corp.) for data collection and analysis. Rayleigh patterns were recorded on Kodak II-G photographic plates, and the plate readings and computations were performed by using computer programs developed by Teller (1973).

The viscosity and density of buffers were calculated by addition of the values for the individual buffer components (Holcenberg et al., 1972) determined experimentally and from *International Critical Tables* (1933). The relative viscosity at 20 °C of 0.1 M Mops was determined to be 1.0410 with a capillary flow viscometer (Schachman, 1957). The relative density at the same temperature was 1.01226. Partial specific volumes of 0.7434 for C, 0.7248 for R^{II}, 0.7305 for $37K^{II}$, and 0.7325 mL/g for $R^{II}_2C_2$ were calculated from the amino acid composition of the proteins (Corbin et al., 1978; Takio et al., 1980; Shoji et al., 1981) according to Cohn & Edsall (1943).

Sedimentation velocity studies were moving boundary experiments. The sedimentation coefficients were computed from the inflection point of the boundary and from equivalent boundary calculations (Teller, 1973).

Results

ϵ-cAMP Binding to the Regulatory Subunits. LaPorte et al. (1980) and Builder et al. (1980a) showed that distinctive changes in the fluorescence of ϵ-cAMP occur on its binding to both types of regulatory subunit (R^I and R^{II}). Those studies were extended here. Figure 1 illustrates the characteristic changes in the relative fluorescence yield and anisotropy obtained under our experimental conditions. The titration of R^I indicates that considerable quenching (>90%) of ϵ-cAMP fluorescence occurs, with an end point being reached near 1.7–1.8 mol of ϵ-cAMP. The decrease in fluorescence during titration of R^{II} takes place in distinct stages, with an additional decline in yield accompanying the binding of the second mole of ϵ-cAMP. These effects are consistent with those reported by the previous investigators. Saturation is accompanied by a large decrease in anisotropy, giving a sensitive indication of the appearance of unbound ϵ-cAMP. The titration of the 37 000-dalton fragment of R^{II}, $37K^{II}$, is nearly superimposable with that of R^{II} when differences in protein concentration due to possible errors in the extinction coefficients are considered.

The binding of ϵ-cAMP by both regulatory subunits requires 5–10 min to reach equilibrium. The effects of this slow binding are especially conspicuous with R^{II}, where the second binding site is occupied at even a lower rate than the first (Figure 2). The addition of cAMP slowly displaces ϵ-cAMP at about the same rate for R^I and R^{II} (Figure 3), though some ϵ-cAMP remains bound at equilibrium. Since the binding of ϵ-cAMP by R^I is approximated by a constant quantum yield, the exchange can be evaluated according to the following equation:

$$R^I(\epsilon\text{-cAMP}) + \text{cAMP} \overset{K}{\rightleftharpoons} R^I(\text{cAMP}) + \epsilon\text{-cAMP}$$

in which the distribution coefficient (K) equals the ratio of the dissociation constants ($K_{\epsilon\text{-cAMP}}/K_{\text{cAMP}}$). Figure 3 shows that ~40% of the ϵ-cAMP is free at equilibrium. By assuming that sites not occupied by ϵ-cAMP are saturated with cAMP, one can estimate a $K \approx 0.6$, indicating that cAMP and ϵ-cAMP bind about equally well to R^1.[4]

Reassociation of the Regulatory Subunits with the Catalytic Subunit. Since the activation of cAMP-dependent protein kinase is reversible, the association of R with C can be followed

[4] ϵ-cAMP = 0.4 × 0.86 μM; R^I(cAMP) = 2 × 1 μM − 0.6 × 0.86 μM; cAMP = 3.1 μM − R^I(cAMP); R^I(ϵ-cAMP) = 0.6 × 0.86 μM.

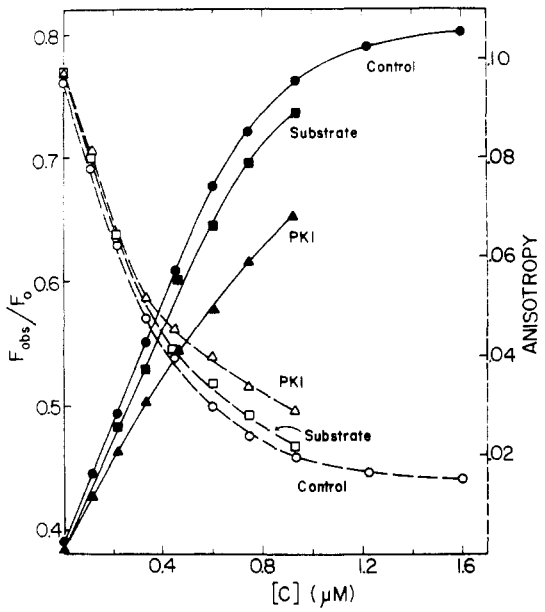

FIGURE 4: Effect of PKI and Kemptide on the reassociation of R^{II} and C. R^{II} (1.0 μM) was incubated with 1.7 μM ϵ-cAMP for 8 min before addition of C. A 54 μM solution of C was preincubated with 100 μM Kemptide and a 29 μM solution of C with 49 μM PKI for ~30 min before the titrations were begun. The cuvette solution of R^{II} and ϵ-cAMP also contained 10 μM Kemptide for the substrate experiment and no PKI for the PKI experiment. For other conditions, see the legend of Figure 1. Solid lines, fluorescence intensities; dashed lines, anisotropies. Control (no PKI or Kemptide) (●, ○); Kemptide (■, □); PKI (▲, △).

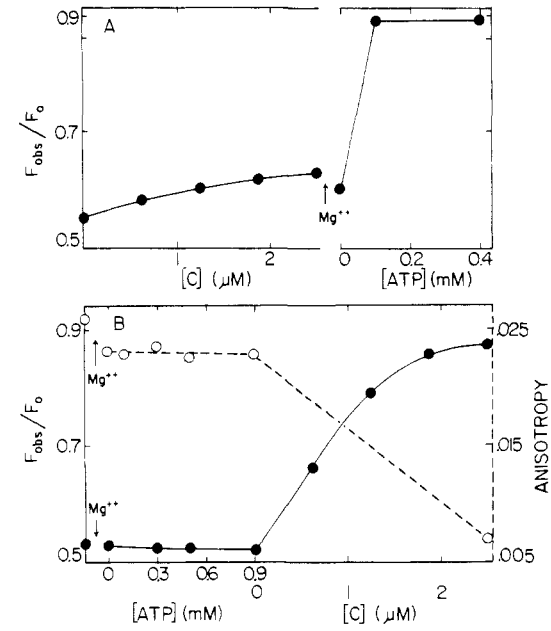

FIGURE 5: Effect of Mg^{2+} and ATP on the reassociation of R^I and C. (A) R^I (1.7 μM) plus 5 μM ϵ-cAMP was titrated with C. Magnesium acetate (4.6 mM) was added after the final addition of C as indicated by the arrow. The mixture was then titrated with ATP. (B) Magnesium acetate (4.6 mM) was added to a solution of 1.7 μM R^I plus 5 μM ϵ-cAMP as indicated by the arrows. The mixture was titrated with ATP followed by C. See the legend of Figure 1 for other conditions.

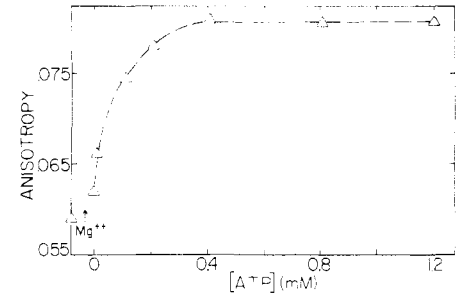

FIGURE 6: Effect of Mg^{2+} and ATP on the reassociation of R^{II} and C. Magnesium acetate (4.6 mM) was added to a mixture of 2.5 μM R^{II}, 5 μM ϵ-cAMP, and 1.25 μM C as indicated by the arrow. The mixture was then titrated with ATP. Other conditions are given in the legend of Figure 1.

by the appearance of free cAMP (Brostrom et al., 1971); the use of ϵ-cAMP instead of cAMP offers the advantage of a sensitive detection technique. Titrations of $R^I(\epsilon\text{-cAMP})_2$ and $R^{II}(\epsilon\text{-cAMP})_2$ with the catalytic subunit demonstrate considerable differences in behavior. R^{II} shows a linear increase in fluorescence with C alone, reaching an end point at ca. 0.9 mol of C per mol of R as shown in the control experiment of Figure 4. No such response is observed with R^I unless MgATP is added (Figure 5). It was previously reported that MgATP facilitates the recombination of R^I and C (Bechtel & Beavo, 1974; Beavo et al., 1975; Hofmann et al., 1975). Figure 5A shows that upon addition of 4.6 mM Mg^{2+} followed by ATP, there is an intermediate release of ϵ-cAMP as indicated by a rise in fluorescence to a maximum value. The release is not due to a simple displacement of ϵ-cAMP by ATP as shown in the left portion of Figure 5B: the increase in fluorescence occurs only after addition of C. Under these conditions, R^I behaves similarly to R^{II}, displaying a linear response to C with an end point around 1 mol of C per mol of R. The displacement of ϵ-cAMP from both R^I and R^{II} occurs within seconds in contrast to the slow equilibria observed with the regulatory subunits alone.

MgATP has opposite effects on the binding of C by R^I and R^{II}. In contrast to R^I, it causes dissociation of the $R^{II}{}_2C_2$ complex as indicated by a ca. 30% increase in anisotropy with a half-maximum response obtained at 50 μM ATP (Figure 6). The effect of MgATP is most likely the result of phosphorylation of R^{II} since AMP-PNP, a nonhydrolyzable analogue of ATP, had no effect on the dissociation of the complex at concentrations up to 1 mM.

Effect of the Protein Kinase Inhibitor and Substrate on the Binding of R^I and R^{II} to C. Both the protein kinase inhibitor (PKI) (Walsh et al., 1971; Demaille et al., 1977) and R^{II} (Severin et al., 1975; Witt & Roskoski, 1975; Todhunter & Purich, 1977; Flockhart et al., 1980) act as strong competitive inhibitors of C with respect to protein substrates. R^I probably behaves similarly. The competition for common binding sites implies that the presence of either PKI or protein substrates may block the formation of the holoenzyme. The effects of PKI and the synthetic peptide substrate, Kemptide, on the formation of types I and II holoenzyme are illustrated in Figures 4 and 7. The inhibitory effect of 10 μM Kemptide is barely detectable in both cases. On the other hand, PKI, which has an affinity for C of the same order of magnitude (Ferraz et al., 1979) as that of R^I and R^{II} (Builder et al., 1979; Hofmann, 1980), has a larger inhibitory effect which is especially pronounced with R^I. PKI and ϵ-cAMP should act in concert in the dissociation of the holoenzyme according to the following reaction:

$$R_2C_2 + 4\epsilon\text{-cAMP} + 2\text{PKI} \overset{K}{\rightleftharpoons} R_2(\epsilon\text{-cAMP})_4 + 2\text{PKI}\cdot\text{C}$$

In the case of simple equilibria, ignoring possible binding of ϵ-cAMP by R_2C_2, the distribution coefficient (K) is related to the individual dissociation constants by

$$K = \frac{K_{R_2C_2}}{K_{PKI}K_{\epsilon\text{-cAMP}}}$$

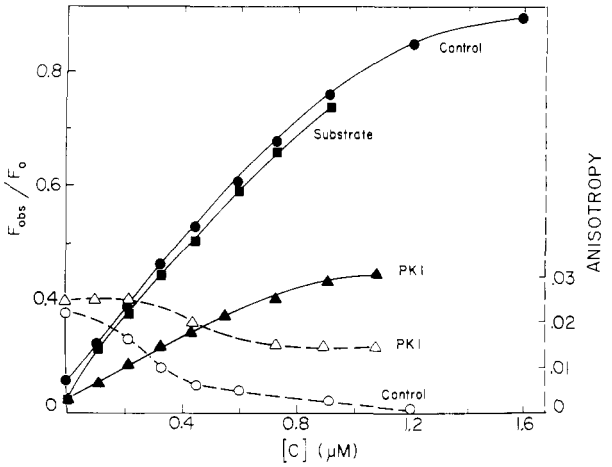

FIGURE 7: Effect of PKI and Kemptide on the binding of R^I to C. R^I (1.0 μM) was incubated with 1.7 μM ϵ-cAMP, 1 mM magnesium acetate, and 0.1 mM ATP for 8 min before the titration was begun. Other conditions are given in the legend of Figure 4. Control (○, ●); Kemptide (■, □), and PKI (▲, △).

where $K_{R_2C_2}$, K_{PKI}, and $K_{\epsilon\text{-cAMP}}$ are the dissociation constants for the holoenzyme, C–PKI, and the $R_2(\epsilon\text{-cAMP})_4$ complex, respectively. Since the catalytic subunits are the same in both types I and II protein kinases, the differing sensitivities of these two types of enzymes to PKI probably reflect differences in $K_{R_2C_2}$ and/or $K_{\epsilon\text{-cAMP}}$. Under conditions approximating ours (150 mM NaCl and 1 mM Mg^{2+} for R^I and no Mg^{2+} for R^{II}), Hofmann (1980) found that $K_{R_2C_2}$ is ~63% larger for R^I than for R^{II} and that $K_{\epsilon\text{-cAMP}}$ is approximately 16% larger for R^{II} than for R^I. These variations act to give a factor of 1.4 in favor of the dissociation of the type I holoenzyme as compared to that of type II.

Observations on the 37K Fragment of R^{II}. When the 37 000-dalton fragment of R^{II} ($37K^{II}$) is titrated with C, one observes a high anisotropy value, indicating that considerable amounts of ϵ-cAMP remain bound even at concentrations of C that would cause a nearly complete dissociation of the $R^{II}_2(\epsilon\text{-cAMP})_4$ complex. The increase in fluorescence intensity is only 10% of that found with R^{II}. Titrations obtained at a 3-fold lower concentration of $37K^{II}$ gave similar results. The incomplete dissociation of ϵ-cAMP probably does not stem from a failure to saturate available binding sites with C.

Sedimentation velocity experiments were performed on a mixture of $37K^{II}$ and C to estimate the extent of complex formation. The plots obtained with the absorption scanner at 280 nm show that the mixture of $37K^{II}$ and C sediments well ahead of either protein alone (data not shown). The sedimentation coefficients are listed in Table I. The value for $37K^{II}$ is lower than expected for a molecule of that size, indicating probable asymmetry.

The percentage of $37K^{II}$ and C in the complex was 31.9%. This was calculated by using the relation $\sum c_i s_i = c_t s_{obsd}$, where c_i and s_i are the concentration and sedimentation coefficients, respectively, of each sedimenting species, c_t is the total concentration of all sedimenting material, and s_{obsd} is the sedimentation coefficient observed for the mixture of all sedimenting components. The sedimentation coefficients of $37K^{II}$ and C in the mixture were assigned the value determined for each component alone under the assumption that the sedimentation coefficient did not change significantly with concentration. The sedimentation coefficient for the $37K^{II}$–C complex, 5.27 S, was derived from the relation $s = KM_r^{2/3}$, where K is a constant and M_r the molecular weight. K was taken as the average of the K's calculated for $37K^{II}$ and C.

Table I: Sedimentation Coefficients of $37K^{II}$, C, and the $37K^{II}$–C Complex[a]

protein	[cAMP] (μM)	$\bar{s}_{20,w}$ (S)
$37K^{II}$		3.01 ± 0.10
$37K^{II}$	135	2.51 ± 0.05
C		3.67 ± 0.08
C	135	3.66 ± 0.09
$37K^{II}$ + C		3.98 ± 0.05
$37K^{II}$ + C	135	3.23 ± 0.03

[a] Sedimentation velocity experiments were performed at 60 000 rpm on 0.25 mg/mL $37K^{II}$, 0.25 mg/mL C, and a mixture of 0.25 mg/mL each of $37K^{II}$ and C in 5 mM Mops, 0.5 mM EDTA, 0.1 M KCl, and 15 mM β-mercaptoethanol, pH 7.0 at 4 °C. Sedimentation coefficients are weight-average values.

cAMP caused dissociation of the $37K^{II}$–C complex as indicated by a reduction in the rate of sedimentation of a mixture of $37K^{II}$ and C in the presence of a 20-fold excess of cAMP over $37K^{II}$. The sedimentation coefficients are listed in Table I. The value for the $37K^{II}$–C complex in the presence of cAMP is the same within 5% as that which would be expected for a mixture of $37K^{II}$ and C under conditions where the two species would not interact, indicating that cAMP causes complete dissociation of the $37K^{II}$–C complex.

The sedimentation coefficients of $37K^{II}$ in the absence and presence of cAMP are strikingly different. The smaller value obtained in the presence of cAMP indicates that binding of the nucleotide to $37K^{II}$ causes a conformational change that results in a more extended structure. A similar though smaller change in the sedimentation coefficient of R^{II} with and without bound cAMP (from 3.9 to 4.2 S) was also noted. The binding of cAMP causes conformational changes in both $37K^{II}$ and R^{II}. R^{II} is apparently restricted from undergoing as large a conformational change as $37K^{II}$ by its amino-terminal region, which also maintains the dimeric structure.

The $37K^{II}$ fragment inhibits catalytic activity approximately 10 times less effectively than R^{II}. For molar ratios of $37K^{II}$/C of 1, 2, 4, and 8, inhibitions of 10, 35, 71, and 93%, respectively, were observed. In contrast, R^{II}/C molar ratios of 1.0 and 1.1 yielded 90 and 94% inhibition, respectively.

Discussion

ϵ-cAMP binding experiments with R^I, R^{II}, and $37K^{II}$ show that approximately 2 mol of ϵ-cAMP is bound per mol of regulatory subunit. This stoichiometry for R^I agrees with that reported by Corbin et al. (1978), who used a modified Millipore filtration technique, with that of Builder et al. (1980a), who utilized ϵ-cAMP as well as equilibrium dialysis, and with that of Bohnert et al. (1982), who followed the displacement of 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate][bis-(ANS)] from R^I and R^{II} by cAMP. The quenching of the ϵ-cAMP fluorescence was different for R^I and R^{II}, with R^{II} displaying a biphasic quenching pattern coinciding with the binding of 1 and 2 equiv of ϵ-cAMP. Similar effects have been previously described by LaPorte et al. (1980).

These results demonstrate clearly that the two intrachain cAMP binding sites of R^{II} are different. While no evidence was obtained here for binding heterogeneity in R^I, it has been reported that the two sites differ in exchange experiments using labeled cyclic nucleotide (Rannels & Corbin, 1980; Corbin et al., 1981).

The physiological significance for the existence of two different cAMP binding sites is unknown. cGMP-dependent protein kinase, which appears to be homologous to R^I (Hashimoto et al., 1982), binds only one cGMP per R subunit. However, in this case, there is no dissociation of the RC

complex [cf. review by Glass & Krebs (1980) and Gill et al. (1981)]. The existence of two cAMP binding sites per monomer permits cooperativity to occur and may allow one site to be under control by ATP in the case of R^I or by phosphorylation–dephosphorylation in the case of R^{II}. In this way, the two sites could display different physiological functions under different cellular conditions.

Both R^I and R^{II} display time-dependent binding of ϵ-cAMP. The affinity of R^I for ϵ-cAMP appears to be very similar to that for cAMP, in contrast to the activation process which, according to Secrist et al. (1972b), requires 10-fold higher concentrations of ϵ-cAMP compared to cAMP. This could reflect the assay conditions; alternatively, ϵ-cAMP could be less effective than cAMP in dissociating the holoenzyme. Recent reports (Smith et al., 1981) estimate a K_d of 0.6 nM for ϵ-cAMP, a value that is surprisingly lower than that reported for cAMP [ca. 2.5 nM of Hofmann (1980)]. It appears that the ϵ-cAMP sites on R^I are identical with those of cAMP since almost all other cAMP derivatives bind preferentially to either site 1 or site 2, depending on whether the modification is at C-8 or N-6 on the adenine ring (Corbin et al., 1981); the same authors find no difference between cAMP and ϵ-cAMP binding to the two sites and report almost equal competition between the two nucleotides in studies utilizing [^{3}H]cAMP.

While the binding of ϵ-cAMP to R^I and R^{II}, as well as its displacement by cAMP, was slow (minutes), the binding of the catalytic subunit (C) to the R·ϵ-cAMP complex was relatively fast (seconds). Since C displaces ϵ-cAMP from R^I, as shown by the drop in anisotropy, the relative rates of these reactions indicate that C binds to R before ϵ-cAMP is released according to eq 2. The existence of a ternary complex of

$$R{\cdot}\epsilon\text{-cAMP} + C \rightleftarrows R{\cdot}\epsilon\text{-cAMP}{\cdot}C \rightleftarrows RC + \epsilon\text{-cAMP} \quad (2)$$

R·C·cAMP must be involved in the activation of the holoenzyme by cAMP as well as in the formation of the holoenzyme from individual subunits since the reaction is reversible and must proceed through the same intermediate. Evidence for such an intermediate has been obtained by ESR measurements using spin-labeled cAMP derivatives (Hoppe et al., 1978; Kaiser et al., 1981) and on the basis of the kinetics of association (Tsuzuki & Kiger, 1978; Builder et al., 1980b, 1981). The ternary complex should be in rapid equilibrium with the holoenzyme and individual subunits since activation by cAMP is rapid (Builder et al., 1980b).

Because of ternary complex formation, the rates of the activation (dissociation) and interaction (association) will be highly dependent on the concentration of the reacting species. Further study will be needed to determine if the intermediate is active or partially active and if this complex can explain the rapidly oscillating processes involving cAMP. The slow binding of ϵ-cAMP to R^{II} and the ultracentrifugation data indicate that a change in conformation of the protein takes place upon binding of cyclic nucleotides. It is now accepted that proline residues can make a kinetically significant contribution to protein unfolding and refolding (Brandts et al., 1977). The potential complexity and slowness of protein folding due to cis–trans isomerization of proline bonds increase dramatically with the number of proline residues. R^{II} has 20 proline residues, a number of which are in a *pro–pro* configuration in the N-terminal half of the molecule (K. Titani, unpublished experiments). It has been estimated that a protein with 20 proline residues will have a predicted half-time for refolding of nearly 10 min (Creighton, 1978). It would be interesting to measure the rate of binding of cAMP with the 14–18K fragment that binds the nucleotide (Rannels & Corbin, 1979): if proline isomerization were involved in these slow equilibria, one would predict a higher rate of association of that fragment with ϵ-cAMP.

The possibility that holoenzyme formation is inhibited by substrate or PKI was investigated by using ϵ-cAMP bound to the regulatory subunits. The peptide substrate caused a slight inhibition of formation of both type I and type II holoenzymes. PKI, however, brought about a significant decrease in the reassociation of R^I and C; both PKI and R^I appeared to bind equally well to C. PKI also inhibited the reassociation of R^{II} and C, but to a smaller extent than for R^I. Therefore, R^{II} must compete more effectively than R^I with PKI for C.

The small inhibition of reassociation of both R^I and R^{II} with C in the presence of substrate is not surprising since the dissociation constant of the peptide is approximately 5 orders of magnitude higher than that for R^I and R^{II}; Builder et al. (1980b) found that the substrate did not affect the rate of reassociation of the holoenzyme. There are several possible explanations for the differing abilities of R^I and R^{II} to compete with PKI for C. The dissociation constant of R^I–C may be higher than that of R^{II}–C. Using the dissociation constants obtained by Hofmann (1980) on the basis of activity measurements, we estimate that the difference in sensitivity to PKI can be attributed at least in part to differences in the affinity of the two regulatory subunits for C.

A less likely explanation for the disparate behavior of R^I and R^{II} is that R and PKI bind noncompetitively to C. Ashby & Walsh (1973) have reported that PKI does not bind to type I holoenzyme. R^{II}, however, may be able to bind to C simultaneously with PKI. No information on the binding of PKI to type II holoenzyme is available.

Anisotropy measurements show that MgATP has opposite effects on the state of association of type I and II holoenzymes; it strongly favors the reassociation of type I kinase while promoting the dissociation of the type II enzyme. This provides further evidence for the suggestion that MgATP may contribute to the control of protein kinase activity in vivo independently of cAMP. Type I protein kinase is located primarily in skeletal muscle, which relies principally on glycogenolysis for energy production and contraction. After extended periods of contraction, the level of ATP is lowered, and the enzyme would tend to dissociate, triggering the cascade pathway leading to glycogen degradation and restoration of the basal level of ATP. Type II protein kinase is found predominantly in cardiac muscle which relies heavily on oxidative phosphorylation for energy production. Variations in the concentration of ATP would not be as drastic as in skeletal muscle and would tend to maintain the enzyme in its active state.

Limited proteolysis has provided information on the functional domains of the protein kinase, in particular its regulatory subunits. Both R^I and R^{II} can be split by various proteases, yielding cAMP binding fragments of 31–40 kdalton from the carboxy-terminal region of the molecule and smaller fragments of 7–14 kdalton from the NH_2 terminus that can still dimerize (Sugden et al., 1976; Corbin et al., 1978; Potter & Taylor, 1979a,b; Weber & Hilz, 1979; Srivastava & Stellwagen, 1978). In addition, a 14-kdalton fragment has been isolated, presumably from the carboxy terminus, that can bind 1 mol of cAMP (Rannels & Corbin, 1979). In all cases except two (Weber & Hilz, 1979; Srivastava & Stellwagen, 1978), these fragments were reported not to inhibit the activity of the C subunit. However, binding measurements were not carried out. In the present study, the 37-kdalton fragment of R^{II} was found to interact with the catalytic subunit on the basis of fluorescence measurements, ultracentrifugation, and inhibition of catalytic activity. Additional evidence for this interaction

is provided in the following paper in which bis(ANS) is used (Bohnert et al., 1982).

The 37-kdalton fragment binds more weakly to C than R^{II}. This could be explained in part by heterogeneous cleavage of R^{II} during generation of the fragment. The protease-sensitive region of R^{II} is near the autophosphorylation site, the so-called "hinge region" (Takio et al., 1980; Potter & Taylor, 1979b; Flockhart et al., 1980). Taylor et al. (1981) have shown that chymotrypsin treatment generates a fragment with the NH_2-terminal sequence NH_2-Asx-Arg-Arg-Val-Se$\overset{P}{}$r-Val-Cys-X while trypsin treatment produces major and minor fragments with sequences NH_2-Val-Se$\overset{P}{}$r-Val-Cys and NH_2-Arg-Val-Se$\overset{P}{}$r-Val-Cys, respectively. As reviewed by Glass & Krebs (1980), an Arg-Arg structure is important for substrate recognition, so these two fragments would be expected to have greatly altered affinities for the catalytic subunit.

An analogous hinge region has also been detected in R^{I}. In this instance, the homologous serinyl residue is only phosphorylated by the cGMP-dependent protein kinase (Geahlen & Krebs, 1980). Phosphorylation reduces the ability of this subunit to inhibit the catalytic subunit and lowers the binding of cAMP from 2 to 1 mol per mol of R^{I} (Geahlen et al., 1981). This suggests that the fragment of R^{I} would have similar properties to that of the R^{II} reported here.

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Binding of 5,5′-Bis[8-(phenylamino)-1-naphthalenesulfonate] by the Regulatory Subunits of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

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ABSTRACT: Binding to the regulatory subunits of types I and II adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase (R^I and R^{II}, respectively) produces large distinctive increases in fluorescence and optical activity of 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate] [bis(ANS)]. Both specific and nonspecific interactions are involved. Association of the regulatory subunits with either the catalytic subunit or cAMP results in dissociation of a major portion of the bound bis(ANS) as detected by changes in fluorescence and circular dichroism. The results are consistent with the accepted cAMP binding properties of R^I and R^{II}, showing cooperativity in the case of R^I and two heterologous binding sites for R^{II}. cGMP has the same overall effect on bis(ANS) binding as cAMP. However, very high concentrations are required for complete dissociation of bis(ANS) from R^{II}, consistent with the observation that cGMP is inefficient in bringing about the dissociation of the type II holoenzyme. Magnesium binding to sites having dissociation constants of ca. 12 mM increases the interaction of bis(ANS) with both of the isolated regulatory subunits. Experiments involving the 37 000-dalton fragment of R^{II} indicate that the limited proteolytic cleavage was heterogeneous, with only 24–39% of the resulting population interacting strongly with the catalytic subunit.

In the preceding paper (Bohnert et al., 1982), the fluorescent probe 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate (ϵ-cAMP)[1] was used to study the interaction of the regulatory subunits of protein kinase with the catalytic subunits (C) and to demonstrate the effects of molecules which bind to C (MgATP, substrate, and protein kinase inhibitor) on the R–C interaction.

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[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; ϵ-cAMP, 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate; bis(ANS), 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate]; ANS, 8-anilino-1-naphthalenesulfonate; $NaDodSO_4$, sodium dodecyl sulfate; R^I and R^{II}, regulatory subunit of types I and II cAMP-dependent protein kinase, respectively; $38K^I$ and $37K^{II}$, carboxy-terminal fragment of the regulatory subunit of types I and II cAMP-dependent protein kinase, respectively; C, catalytic subunit of the cAMP-dependent protein kinase; CD, circular dichroism; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $\bar{n}$, number of moles of ligand bound per mole of protein; BSA, bovine serum albumin.

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