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Biochemistry

Cyclic AMP-dependent protein kinase I: Cyclic nucleotide binding, structural changes, and release of the catalytic subunits

(activation/deactivation/fluorescence/circular dichroism)

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ABSTRACT **Type I cyclic AMP (cAMP)-dependent protein kinase is composed of a dimeric regulatory subunit (R_2) and two catalytic subunits (C subunits). The R_2 dimer binds four cAMP molecules to release the two C subunits. To characterize the cAMP binding sites and elucidate their role in the release of the C subunits, the R_2 dimer has been studied by equilibrium methods. The cAMP titration of R_2 was monitored by endogenous tryptophan fluorescence, and the results suggest one class of binding sites. The titration plot is monotonic for saturation of four sites per R_2. A similar titration monitored by near-UV circular dichroic changes exhibited profound changes in the region of the ^1L_b tyrosine and ^1L_a and ^1L_b tryptophan transitions; a plot of these data also showed a linear monotonic response. Thus, the fluorescence and circular dichroic changes show that cAMP binding to R_2 induces a conformational or structural change. The one apparent class of binding sites implies that all binding sites are characterized by similar K_d values or by K_d values much less than the receptor concentration. The reactivity of the cysteine sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) showed that saturation with cAMP indirectly protects one sulfhydryl group per R monomer. Analysis of cAMP activation of the holoenzyme, detected by phosphotransferase assays, showed that saturation of both cAMP binding sites per R monomer is necessary to effect the release of the C subunit. By using a fluorescent analog of cAMP, 1,N^6-etheno-cyclic AMP (ε cAMP), the (ε cAMP)$_4$·R_2 complex was titrated with C subunit, causing the release of ε cAMP. The titration showed that the release of ε cAMP was a positive cooperative process; its Hill plot had a slope of 2.6 and the K_{a_1} and K_{a_2} values obtained by extrapolation were 2.1×10^7 M^{-1} and 5.0×10^8 M^{-1}, respectively. The calculated ΔΔG for first and last site coupling was 1.9 kcal/mol (1 cal = 4.18 J) of holoenzyme.**

Cyclic AMP (cAMP)-dependent protein kinase has been shown to catalyze the phosphorylation of a large number of enzymes and proteins (1, 2), many of which have modified activities indicating their involvement in regulatory processes. This covalent regulation of enzymes in various metabolic pathways is in turn regulated by the state of activation of the cAMP-dependent protein kinase. It has been established that the mechanism of activation of the enzyme involves a complex interaction of four protein subunits and four cAMP molecules. The enzyme (R_2C_2) is composed of a dimeric regulatory subunit (R_2) and two catalytic subunits (C subunits). The R_2 dimer binds four molecules of cAMP which, in some manner, effects the release of the two C subunits (see Eq. 1).

$$R_2C_2 + 4\ \text{cAMP} \rightleftarrows (\text{cAMP})_4\cdot R_2C_2 \rightleftarrows (\text{cAMP})_4\cdot R_2 + 2C. \quad [1]$$

cAMP-dependent protein kinase exists in two forms, type I and type II. These types differ primarily in their R_2s. The type I R_2 has $M_r \approx 98{,}000$, and the type II R_2 has $M_r \approx 112{,}000$. The type I enzyme is the predominate form in skeletal muscle and is the subject of this study.

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Although the overall scheme for cAMP activation of cAMP-dependent protein kinase has long been known (3), the detailed steps have not been investigated. The function of the two recently discovered cAMP-binding sites on the R monomer (i.e., four sites per R_2) is unknown (4–7). It has been suggested that the binding of cAMP to R_2 and R_2C_2 exhibits positive homotropic cooperativity (8, 9), but a detailed characterization of the cyclic nucleotide binding and the release of the C subunit has not been presented. Because the C subunit efficiently catalyzes the exchange of [^{3}H]cAMP bound to R_2 with excess free cAMP in solution, it appears that, at physiological concentrations, the complex representative of both cAMP and C subunit binding to R_2 [(cAMP)$_4$·R_2C_2] should be, at best, metastable (10, 11). By using denatured-renatured R_2 and native C subunit, we have carried out a variety of titrametric experiments in which intrinsic and extrinsic fluorescence, intrinsic circular dichroism, cysteine sulfhydryl reactivity, and cAMP-activated phosphotransferase activity were used.

Our experiments have characterized the cyclic nucleotide binding sites, the structural changes in the R_2 subunits, and the roles of the cAMP binding sites in the release of the C subunits and of the C subunits in the release of 1,N^6-etheno-cyclic AMP (ε cAMP).

EXPERIMENTAL PROCEDURES

All chemicals used were reagent grade and were obtained from Sigma, Pierce, Kodak, or Vega. The [γ-^{32}P]ATP was enzymatically synthesized using [^{32}P]phosphate (12). The substrate peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly was synthesized with a Beckman Model 900D peptide synthesizer, and its composition was confirmed by amino acid analysis (see below). Buffer A contained 5 mM morpholinepropanesulfonic acid/0.5 mM dithiothreitol/0.5 mM EDTA/100 mM KCl, with the pH adjusted to 7.0.

The R_2 and C subunits of type I cAMP-dependent protein kinase were purified by published procedures from bovine skeletal muscle (13, 14), and their purities were determined by NaDodSO$_4$/polyacrylamide electrophoresis in a Tris buffer system.

These experiments required the use of cAMP-free R_2. The only practical way to remove bound cAMP involves denaturing the protein, separating the cAMP, and then renaturing the protein (7). Although such harsh treatment is not desirable, it was necessary. The cyclic nucleotide-free R_2 so obtained binds four

Abbreviations: cAMP, cyclic AMP; R_2 and C subunit, regulatory and catalytic subunits, respectively, of type I cAMP-dependent protein kinase; ε cAMP, 1,N^6-etheno-cyclic AMP; CD, circular dichroism; cGMP, cyclic GMP; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); K_a, apparent association constant.

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molecules of cAMP per R_2 and also completely inhibits C subunit kinase activity in stoichiometric amounts—i.e., the holoenzyme (R_2C_2) can be reconstituted from R_2 and 2 C subunits. The holoenzyme so formed can in turn be activated by cAMP in a predictable manner.

The protein concentrations of the homogeneous stock solutions and the composition of the substrate peptide were determined by amino acid analyses, after hydrolysis by using a Durrum D-500 amino acid analyzer. The proteins and peptides were first hydrolyzed *in vacuo* for 24 hr using 1 ml of distilled 5.7 M HCl and a crystal of phenol. The molar extinction coefficient of the R monomer at 280 nm was $\approx$43,500 M^{-1} cm^{-1}.

Fluorometric Titrations. Fluorescence titrations were monitored with a Turner model 430 or an Aminco SPF-500 scanning spectrofluorometer at 30°C. All additions were made in small volumes relative to the sample volume, and the data were corrected for volume changes. After each addition, the sample was incubated in the dark until equilibrium had been achieved. The concentrations of the cyclic nucleotide solutions were determined by spectrophotometric methods.

Circular Dichroism. The circular dichroic (CD) experiments were completed with a Roussel Jouan Dichrograph Mark III that had been calibrated with twice-recrystallized *d*-10-camphorsulfonic acid. All calculations were done by using a DEC-10 computer and previously published programs (15). The near-UV CD-monitored titration of R_2 was performed in buffer A using a 1.0-cm-path-length cuvette by recording a complete spectrum after each addition of cAMP.

Cysteine Reactions. The accessibility of the cysteines in R_2 was investigated by their reactivity with 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB). These reactions were carried out by using published procedures (16) and a Beckman model 25 spectrophotometer and anaerobic solutions.

Phosphotransferase Assays. The phosphotransferase assays were conducted as described (17), with several modifications. The concentration of the reconstituted holoenzyme was 1.0 nM, and the specific activity of the [γ-^{32}P]ATP was 20,000 dpm/pmol (1 dpm = 16.7 mBq). The [^{32}P]phosphate incorporated in the substrate peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was determined by scintillation counting using a Beckman LS-9000 instrument.

C Subunit Titration of (ε cAMP)$_4$·R_2. The titration of (ε cAMP)$_4$·R_2 by the C subunit was followed by the release of ε cAMP, as shown by increased fluorescence. This reaction is analogous to deactivation of the enzyme. Small aliquots of a concentrated C subunit solution in buffer A were added to a 0.15 μM solution of (ε cAMP)$_4$·R_2 in buffer A. After equilibrium was reached, the emission spectrum of ε cAMP was recorded and the relative fluorescence values were corrected for volume changes.

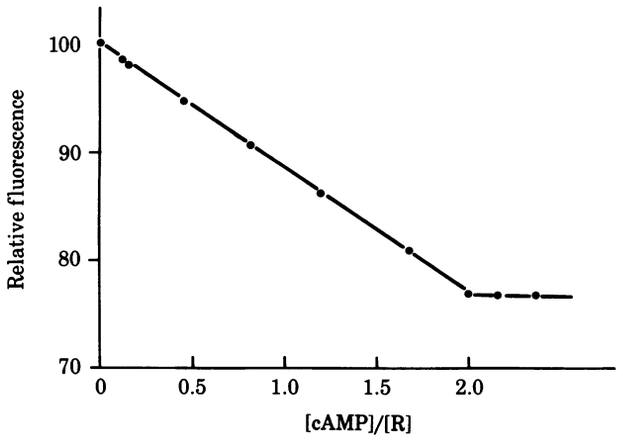

FIG. 1. Tryptophan fluorescence-monitored titration of 0.5 μM R_2 with cAMP. The experiment was carried out in buffer A, and the wavelengths of excitation and emission were λ_{ex} = 293 nm and λ_{em} = 332 nm, respectively.

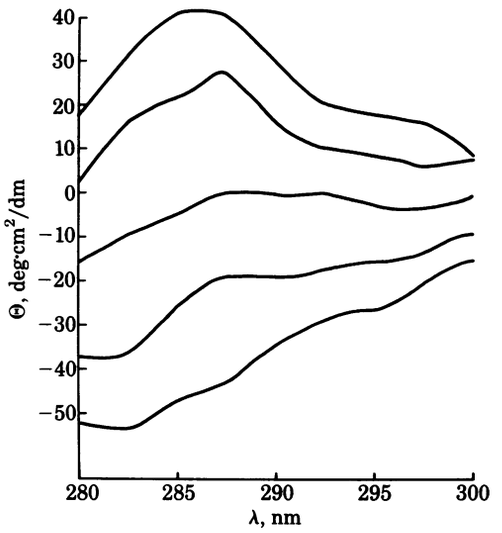

FIG. 2. Near-UV CD-monitored titration of 4.0 μM R_2 with cAMP. Ellipticity is expressed as mean residue. From bottom to top fractional saturations per R monomer are 0, 0.2, 0.5, 0.7, and 1.0, respectively.

RESULTS

Fluorescence Measurements. The fluorescence of the tryptophans in the polypeptide chains of type I R_2 is partially quenched when the binding sites of the R_2 are saturated with cAMP but that of the tryptophans of type II R_2 is essentially unaffected by cAMP binding (5, 15). A graph of the cAMP titration of type I R_2, as monitored by fluorescence changes, is shown in Fig. 1. The fluorescence quenching was linear for both cAMP binding sites on the R monomer (the four sites on the R_2 dimer).

CD Changes. To determine whether the binding of cAMP to R_2 induced a conformational change, the titration of R_2 with cAMP was monitored by CD changes at 280–300 nm. A representative collection of recorded spectra is shown in Fig. 2. Changes in the CD spectra of proteins at 280–300 nm have been attributed to tyrosine and tryptophan residues. For many pro-

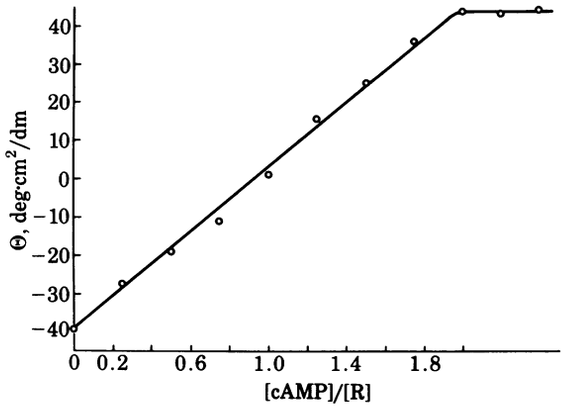

FIG. 3. CD-monitored titration of R_2 with cAMP, λ = 288 nm. For experimental conditions, see Fig. 2.

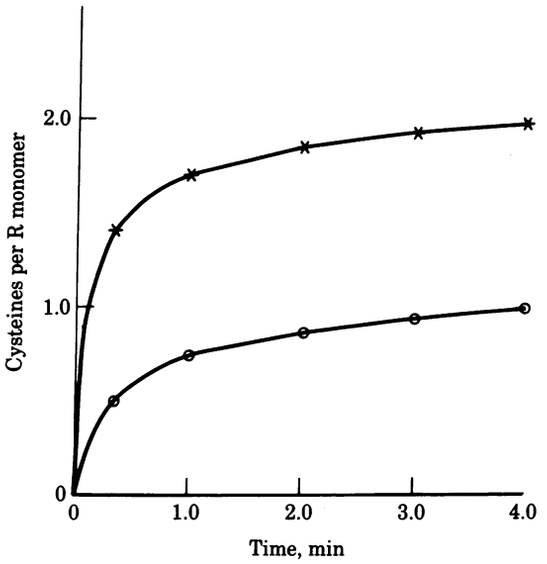

FIG. 4. Cysteine modification of 1.84 μM R monomer by DTNB in the absence (×) or presence (○) of cAMP.

teins, the 0-0 ^1L_b transition of tyrosine occurs at 281–289 nm and the 0-0 ^1L_b and ^1L_a transitions of tryptophan occur at 289–303 and 288–293 nm, respectively (19). The ellipticity increased in a linear manner with each addition of cAMP. This is shown by the titration plot (Fig. 3) of the ellipticity change at 288 nm, which should represent both tyrosine and tryptophan changes.

To support the described near-UV CD changes as being intrinsic, due to the protein, the CD spectrum of the R_2 was recorded in the presence of cyclic GMP (cGMP) and compared with those of cGMP and of R_2. The CD changes resulting from the binding of cGMP to R_2 (not shown) were similar to those recorded with cAMP.

Cysteine Reactivity. The reaction of the cysteine residues of R_2 with DTNB in the presence and absence of cAMP is shown in Fig. 4. In the absence of cAMP, two cysteine residues were modified per R monomer within 4 min but, in the presence of cAMP, only one reacted within this time. To study the ε cAMP binding properties of DTNB modified R_2, 2.5 nmol of R_2 was anaerobically modified by DTNB until 2.0 cysteines were modified per R monomer. After anaerobic dialysis of the protein, the modified R_2 was used to carry out a fluorescence titration of 1.0 μM ε cAMP. The observed relative fluorescence values were corrected for volume changes and internal absorption. A titration plot of the corrected data (not illustrated) showed that 2.0 ε cAMP molecules bind per R monomer.

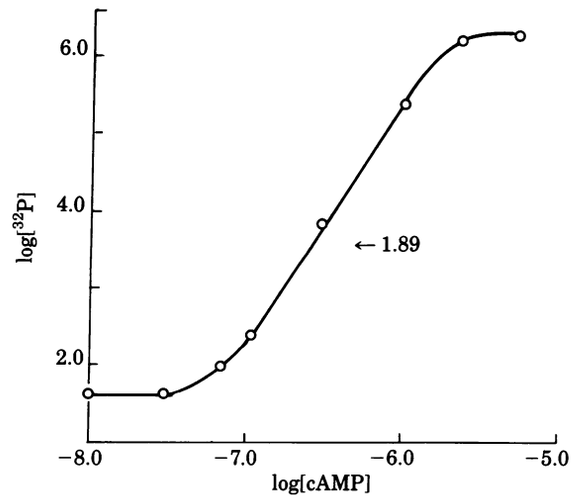

FIG. 5. Activation of reconstituted type I holoenzyme as a function of free cAMP in buffer A.

Release of the C Subunits. The release of the C subunits from type I cAMP-dependent protein kinase holoenzyme was determined by phosphotransferase assay as a function of free or unbound cAMP. The net or overall equilibria for the activation of cAMP-dependent protein kinase is given by Eq. 1. Many of the possible postulated steps, summarized by Eq. 1, are given in Eqs. 2–7:

$$\text{cAMP} + R_2C_2 \overset{K_{a_1}}{\rightleftarrows} \text{cAMP}\cdot R_2C_2 \quad [2]$$

$$\text{cAMP} + \text{cAMP}\cdot R_2C_2 \overset{K_{a_2}}{\rightleftarrows} (\text{cAMP})_2\cdot R_2C_2 \quad [3]$$

$$\text{cAMP} + (\text{cAMP})_2\cdot R_2C_2 \overset{K_{a_3}}{\rightleftarrows} (\text{cAMP})_3\cdot R_2C_2 \quad [4]$$

$$\text{cAMP} + (\text{cAMP})_3\cdot R_2C_2 \overset{K_{a_4}}{\rightleftarrows} (\text{cAMP})_4\cdot R_2C_2 \quad [5]$$

$$(\text{cAMP})_4\cdot R_2C_2 \overset{K_{D_1}}{\rightleftarrows} (\text{cAMP})_4\cdot R_2C + C \quad [6]$$

$$(\text{cAMP})_4\cdot R_2C \overset{K_{D_2}}{\rightleftarrows} (\text{cAMP})_4\cdot R_2 + C. \quad [7]$$

This set of equations was used as a detailed equilibrium model for the cAMP activation of the holoenzyme. The assumption implicit in this model is that two cAMP molecules must bind to each R monomer before a C subunit is released. The result of an algebraic equilibrium derivation, using Eqs. 2–7 is given by Eq. 8.

$$[C]^2 = \frac{K_{a_1}\cdot K_{a_2}\cdot K_{a_3}\cdot K_{a_4}\cdot K_{D_1}\cdot K_{D_2}\cdot[R_2C_2][\text{cAMP}]^4}{[(\text{cAMP})_4\cdot R_2]} \quad [8]$$

Eq. 8 shows that the square of the concentration of free C subunit is proportional to the cAMP concentration to the fourth power. Hence, [C] would be proportional to $[\text{cAMP}]^2$. If this model describes the activation of cAMP-dependent protein kinase under equilibrium conditions, a plot of log[C] vs. log[cAMP] should have a slope of ≈2.0.

Phosphotransferase assays were conducted at low concentrations of reconstituted type I holoenzyme using the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly. The results are shown as a log-log plot in Fig. 5. The slope of the line through the experimental data is 1.89, which is within experimental error of the predicted slope.

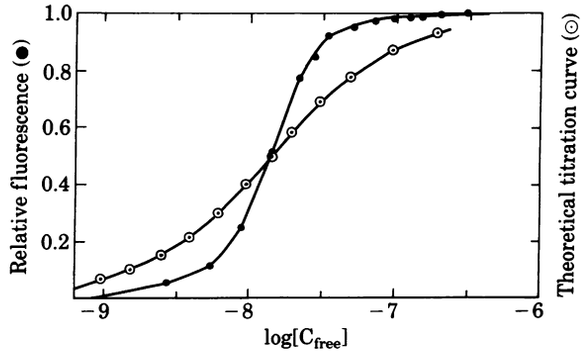

FIG. 6. Fluorescence-monitored titration of $(\varepsilon\ \text{cAMP})_4\cdot R_2$ by the C subunit. The experiment was carried out in buffer A containing 0.125 mM ATP and 12.5 mM $MgCl_2$. Wavelengths of excitation and emission were 320 nm and 410 nm, respectively. R_2 = 0.15 μM.

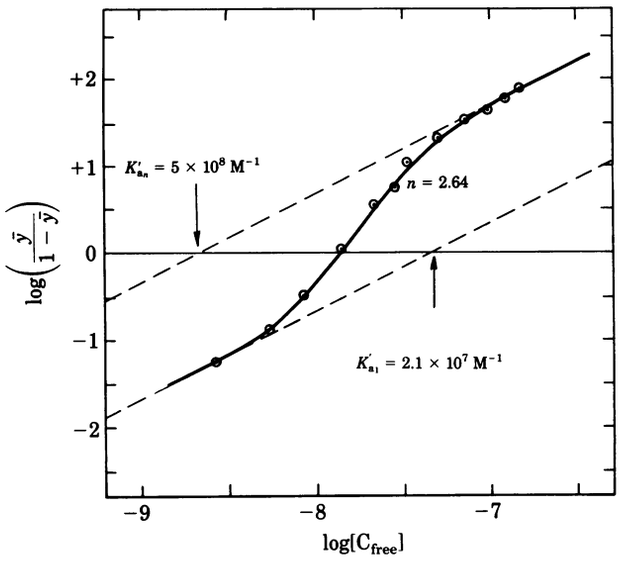

FIG. 7. Hill plot of C subunit titration of (ε cAMP)$_4$·R_2. For details, see Fig. 6.

Titration of (ε cAMP)$_4$·R_2 with the C Subunit. To study the interaction of the C subunits and the extrinsic fluorescent probe ε cAMP with R_2, the following experiment was performed; the (ε cAMP)$_4$·R_2 complex (0.15 μM) was titrated by incremental addition of a stock solution of C subunit in the presence of 100 μM MgATP, monitoring the release of ε cAMP after equilibrium was achieved. The fluorescence change is plotted in Fig. 6. It is obvious that the experimental titration takes place over a more narrow C subunit concentration range than the theoretical change. The $K_{0.5}$ occurred at 12.6 nM free C subunit. This represents the point at which half of the ε cAMP is free or unbound and half is still noncovalently bound to R_2. Analysis of the titration data by a Hill plot (Fig. 7) gave a Hill coefficient, n, of 2.64, and extrapolation of the linear portions of the plot to $\log \bar{y}/(1-\bar{y}) = 0$ showed that $K_{a_1}' = 2.1 \times 10^7\ M^{-1}$ and $K_{a_n}' = 5.0 \times 10^8\ M^{-1}$. The K_a' values are apparent association constants reflecting the release of ε cAMP from R_2 as the result of C subunit binding.

DISCUSSION

The binding of small molecules to many enzymes is known to regulate their activity (for review, see ref. 20). Such activity changes are often due to allosteric conformational changes and frequently result in changed environments of the side chains of the amino acids in the protein. For example, changes in the tryptophan emission spectra have been noted in certain enzymes whose conformations or structures have been changed in response to ligand binding (21; for review, see ref. 22). It was previously reported that the tryptophan fluorescence of type I R_2 is quenched by addition of cAMP, and this quenching was attributed to direct interaction between a tryptophan residue at a cAMP binding site and bound cAMP (5, 18). Recently, however, we presented preliminary data suggesting that the binding of cAMP to R_2 apparently induces a conformational or structural change in the protein (23) and that this change may cause the tryptophan fluorescence quenching. Further evidence for this is presented here.

The tryptophan fluorescence-monitored titration of R_2 showed the quenching was a linear function of added cAMP (see Fig. 1). This linear dependence suggests that there are not two or more classes of cAMP binding sites described by sequential K_d values. On the contrary, the data given in Fig. 1 may be described as reflecting a single class of binding sites, the binding of cAMP must be described as positive homotropic cooperativity, or the K_d values must be much smaller than the R_2 concentration.

If the K_d values for receptor-ligand interactions are much smaller than the receptor concentration, all the ligand added will bind to the receptors and free ligand will be present only at a very low concentration. Hence, the K_d values for the binding of cAMP to R_2 cannot easily be determined by the fluorescence or CD data presented here. Because the R_2 concentration was 500 nM in the tryptophan-monitored experiment and the K_d values for cAMP are $\approx$1–0.1 nM, all the cAMP added should bind to R_2. The fluorescence quenching would be expected to be linear with each addition of cAMP to the saturation point.

To gain direct evidence of the postulated conformational change on binding of cAMP, the titration of nucleotide-free R_2 was carried out by using near-UV CD to monitor changes in the asymmetric environment of the aromatic portions of the tyrosine or tryptophan residues. As shown in Fig. 2, changes occur in both the tyrosine and the tryptophan CD signals. The latter changes agree with the tryptophan fluorescence-quenching results. The R monomer contains $\approx$14 tyrosine residues, so the profound changes in the tyrosine CD signals suggest a change in the asymmetric environment of a significant number of these residues, most probably due to a conformational or structural change in the protein. A titration plot of the CD data (see Fig. 3) is similar to Fig. 1 in that it is linear and monotonic over its entire range. To ascertain whether the CD change shown in Fig. 2 was an extrinsic change (24) due to the bound cAMP itself, cGMP was used instead of cAMP and an analogous CD change was observed. The adsorption spectra of cGMP and cAMP are significantly different (25) and should give rise to different CD spectra even when bound to R_2 unless the absorption spectra of the bound cyclic nucleotides were coincidental. Hence, it appears the CD changes shown in Fig. 1 and 2 are intrinsic changes due to the tyrosine and tryptophan residues.

Structural changes in the R_2 of type II cAMP-dependent protein kinase that result from cAMP binding have previously been proposed on the basis of differential cysteine reactivity with DTNB (26). The time courses of type I R_2 cysteine sulfhydryl modification by DTNB in the absence and presence of cAMP (see Fig. 4) showed that not only the extent but also the rate of modification of the cysteines was changed by the presence of saturating levels of cAMP. The protection of one cysteine residue per R monomer can be explained in two ways. Either binding of cAMP may directly block access to one cysteine residue or it may induce a conformational change in R_2 that affords indirect protection of the one cysteine under consideration. To distinguish between direct and indirect protection of the one cysteine residue in the R monomer, nucleotide-free R monomer was modified with DTNB and separated from DTNB and 5-thio-2-nitrobenzoic acid by rapid dialysis. The resulting protein, in which two cysteines had been modified per R monomer, was used to titrate ε cAMP and showed 2.0 ε cAMP bound per R monomer. This result suggests that the cysteine protected from DTNB modification by cAMP is not located at a cAMP binding site and thus is afforded only indirect protection. Such indirect protection has traditionally been interpreted as evidence of a ligand-induced conformational change. However, other factors may be involved that could cause a change in the chemical reactivity of one or more of the cysteine sulfhydryl groups (22).

The effect of the binding of cAMP to the R_2 of type I cAMP-dependent protein kinase is the release of the C subunits, which is referred to as activation. Therefore, we studied the activation

process in detail to determine whether the conformational change seen in the binding of cAMP by pure R_2 related directly to the release of the C subunits from the holoenzyme. The activation of type I cAMP-dependent protein kinase as a function of cAMP concentration is shown in Fig. 5. The slope of the log-log plot is ≈2.0, which agrees with Eq. 8. The prediction of the slope of the experiment shown in Fig. 5 by Eq. 8 strongly suggests that the C subunits of the type I holoenzyme are released in response to saturation of the two cAMP binding sites on each R monomer. Equilibrium analysis of such subunit interactions shows only the smallest ratio of whole numbers describing the interaction of these subunits. This analysis cannot be used to ascertain whether the RC complexes in the holoenzyme act independently or whether all four cAMP sites must be saturated before the two C subunits are released.

The equilibrium binding of cyclic nucleotide and C subunit to R_2 was studied by monitoring the release of ε cAMP from $(\varepsilon\ \text{cAMP})_4 \cdot R_2$ as a function of free C subunit in the presence of MgATP (see Fig. 6). Comparison of the experimental curve and a theoretical curve obtained by assuming that all the binding constants for ε cAMP are equal and that ε cAMP and the C subunits cannot simultaneously bind to R_2 shows that the titration reaches an end point over a narrower concentration range of C subunit than expected, which suggests positive cooperativity. The Hill plot (see Fig. 7) of the data shown in Fig. 6 shows that $n = 2.64$ and that K_{a_1}' and K_{a_n}' are $2.1 \times 10^7\ M^{-1}$ and $5 \times 10^8\ M^{-1}$, respectively (27). This n value (2.64) is indicative of significant positive cooperativity—for example, the n value for hemoglobin, another four-binding-site protein, is 2.8 (28). The values of K_{a_1}' and K_{a_n}' indicate an overlap difference in the values for the first and last sites of ≈24-fold.

These K_as cannot be attributed to specific molecular associations such as ε cAMP binding or C subunit binding to R_2. Rearrangement of Eq. 8 to the form of a Hill plot suggests that K_{a_1}' and K_{a_n}' could be a complex product of constants. However, by using the determined K_a' values to calculate a $\Delta\Delta G$, one obtains a value of −1.9 kcal/mol (1 cal = 4.18 J). This value is a measure of the first- and last-site free energy of interaction.

cAMP-free R_2 apparently has one class of cAMP binding sites, and saturation of these binding sites causes a structural or conformational change in the protein. Because activation of the enzyme occurs concomitantly with a structural change in R_2, it is probably that a conformational change in R_2 is responsible for the release of the C subunits from the holoenzyme.

The C subunit titration of ε cAMP R_2 saturated in the presence of MgATP showed that the release of the cyclic nucleotide is a cooperative process. Hence, deactivation of the enzyme is a cooperative process, as is activation (8, 9).

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