Regulation of cAMP-Dependent Protein Kinase: Enzyme Activation without Dissociation†

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ABSTRACT: It has become axiomatic that, in contrast to other protein kinases, cAMP-dependent protein kinase (cAPK) is activated only when its catalytic (C) and regulatory (R^{II}_2) subunits dissociate. To directly evaluate this postulation, the ability of cAMP to dissociate the holoenzyme form of cAPK was examined by measuring the rotational mobility of the carboxyfluorescein-labeled C subunit (^{CF}C) complexed to the dimeric R^{II}_2 regulatory subunit under equilibrium conditions. The rotational mobility was determined from an analysis of the time-resolved emission anisotropy of the ^{CF}C subunit. The time-resolved anisotropy decays were best fitted by a sum of two exponentials for both the free ^{CF}C subunit and the $R^{II}_2{}^{CF}C_2$ complex (holoenzyme). In the absence of cAMP, the two rotational correlation times (ϕ_F and ϕ_S) were 1.7 ± 0.3 and 18.3 ± 0.7 ns for the free ^{CF}C subunit and 2.3 ± 0.1 and 93 ± 2 ns for the $R^{II}_2{}^{CF}C_2$ complex, respectively. The faster rotational correlation times can be attributed to the localized rotations of the label and the slower rotational correlation times to the global rotations of the entire molecule. The addition of cAMP had no significant effect on either the fast or the slow rotational correlation time of the $R^{II}_2{}^{CF}C_2$ complex (ϕ_F = 2.0 ± 0.2 ns and ϕ_S = 93 ± 9 ns). Control experiments established that the $R^{II}_2{}^{CF}C_2$ complex was fully activated by cAMP at the same concentrations (0.2−0.4 μM) used for the anisotropy measurements. Together, the results demonstrate (1) that cAMP can induce the catalytic activity of cAPK without subunit dissociation and (2) that cAMP binding to holoenzyme is insufficient to explain its *in vivo* dissociation.

Cyclic AMP-dependent protein kinase (cAPK)[1] is present in all eukaryotic cells where it mediates the phosphorylation of multiple protein substrates that are important regulators of cellular growth, metabolism, and homeostasis (Krebs, 1986; Taylor *et al.*, 1990; Walsh *et al.*, 1992). Although there are several isozymes of cAPK, all of them are structurally similar, with each being composed of a dimeric regulatory subunit (R_2) and two catalytic subunits (C). It is currently believed that cAPK activity is controlled by a unique dissociation/reassociation reaction in which the holoenzyme (R_2C_2) is catalytically inactive, but when cAMP binds to R_2, the C subunits dissociate and are then capable of phosphorylating substrates (Walsh & Cooper, 1979). While this mode of activation has no obvious advantage over conventional ligand-binding allosteric mechanisms, it could provide a means by which free C subunits (M_r ~40 000) are able to migrate between cellular compartments that are otherwise inaccessible to the larger holoenzyme (M_r ~190 000). The possibility of translocation into the nucleus is frequently cited to account for the fact that agents which elevate cAMP levels enhance transcription of several genes (Gonzalez & Montminy, 1989; Mellon *et al.*, 1989). Given this and the multiple roles of cAPK in cellular regulation, it is imperative to ascertain whether subunit dissociation is necessary for cAPK activation.

Evidence supporting the view that cAMP causes the dissociation of cAPK holoenzyme rests largely on a number of *in vitro* observations which demonstrate that, in the presence of cAMP and under nonequilibrium conditions, R_2 and C subunits come apart (Tao *et al.*, 1970; Reimann *et al.*, 1971; Rubin *et al.*, 1972; Corbin *et al.*, 1972; Brostrom *et al.*, 1971; Beavo *et al.*, 1974; Dills *et al.*, 1976). This conclusion has been challenged by the recent observation that cAMP does not reduce dipolar fluorescence resonance energy transfer (FRET) between heterochromatically labeled C and R_2 subunits to a level that is consistent with their physical dissociation (Johnson *et al.*, 1993). However, the inherent limitations in the FRET method [i.e., lack of knowledge of (i) the orientation of the donor transition dipole moment relative to the acceptor absorption transition dipole moment, (ii) the refractive index of the media between donor and acceptor, and (iii) the possibility that the acceptor alters the conformation of the protein in such a way that the emission properties of the donor are affected independently of FRET processes] dictate the development of an alternative and less problematic approach.

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; cAPK, cAMP-dependent protein kinase; C, catalytic subunit of the cAPK; FRET, fluorescence resonance (Förster) energy transfer; R^{II}_2, type II regulatory dimer of the cAPK; CF, 5,6-carboxyfluorescein succinimidyl ester; ^{CF}C, CF-labeled catalytic subunit; EDTA, ethylenediaminetetraacetic acid.

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We report here the use of the time-resolved emission anisotropy of the carboxyfluorescein-labeled C subunit (^{CF}C) to monitor the effect of cAMP on the association−dissociation reaction of cAPK under equilibrium conditions, in which no external dissociating force is applied to the enzyme. With time-resolved emission anisotropy it is possible to directly monitor (without the assumptions required of the FRET measurements) the rotational mobility of fluorescently labeled proteins and, therefore, the protein size, because small proteins rotate more rapidly than large proteins. Thus, if cAMP induces the dissociation of the holoenzyme, then the rotational mobility (measured as the rotational correlational time) of ^{CF}C should significantly increase because of the smaller size of the free C subunit relative to the holoenzyme. Specifically, we monitored the effect of cAMP on the rotational correlation times of ^{CF}C subunit both free in solution and complexed to type II regulatory dimer ($R^{II}_2{}^{CF}C_2$). We also measured the ability of cAMP to activate the $R^{II}_2{}^{CF}C_2$ complex at holoenzyme concentrations similar to those used for the anisotropy experiments to confirm that cAMP will activate the labeled holoenzyme at concentrations used for the anisotropy experiments and typically found intracellularly (Hofmann *et al.*, 1977).

EXPERIMENTAL PROCEDURES

Materials. 5,6-Carboxyfluorescein succinimidyl ester (CF) was obtained from Molecular Probes, Eugene, OR. All other reagents were reagent grade or better.

cAMP-Dependent Protein Kinase Purification. Bovine heart cAMP-dependent protein kinase subunits were purified to homogeneity by the procedure of Beavo *et al.* (1974) with the modifications described in Fletcher *et al.* (1986).

Modification of Catalytic Subunits. Purified C subunit was labeled with CF using a 17-fold molar excess of CF. The labeling was carried out in 0.2 mM $NaHCO_3$ (pH 8.2), 6 mM $MgCl_2$, and 4 mM ATP at room temperature. The reaction was stopped after 20 min by passing the reaction mixture through a Sephadex G-25 minicolumn equilibrated in 20 mM KH_2PO_4, pH 6.7, and 5 mM β-mercaptoethanol at 4 °C.

Holoenzyme Formation. Unless stated otherwise, ^{CF}C and the R^{II}_2 were recombined in the presence of 4 mM ATP and 6 mM $MgCl_2$ and incubated for 20 min at 30 °C.

Assays. When assaying low levels (<4 nM) of phosphotransferase activity, we used the method of Whitehouse and Walsh (1983) with kemptide as the substrate. When high concentrations of cAPK were assayed for phosphotransferase activity, this method was modified such that the reactions were initiated by taking the samples out of an ice bath and adding [γ-^{32}P]ATP and kemptide (final concentrations: 100 μM kemptide, 2 mM ATP, 20 mM $MgCl_2$). The reaction was then allowed to proceed for 1 min in a 30 °C water bath, after which it was terminated by addition of acetic acid (final concentration: 33%). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a protein standard. Fluorophore to protein ratios were estimated on the basis of a molar extinction coefficient of 66 000 (491 nm) for CF (Haugland, 1983).

Chromatography/Electrophoresis. To assess the proportion of the CF that was covalently bound to the C subunit, SDS−polyacrylamide gel electrophoresis of the ^{CF}C subunit was performed by the method of Laemmli (1970). Sephacryl S-200 gel filtration chromatography was carried out following Connelly *et al.* (1986) to separate free ^{CF}C from the $R^{II}_2{}^{CF}C_2$ holoenzyme.

Nanosecond Time-Resolved Fluorescence Anisotropy Decay. The time dependence of fluorescence anisotropy was determined by the single-photon-counting technique (Yguerabide, 1972; Hanson *et al.*, 1981) with an EEY scientific nanosecond spectrofluorometer (La Jolla, CA) equipped with a high-pressure hydrogen arc lamp. The vertically and horizontally polarized fluorescence decays were collected by exciting samples with vertically polarized light while orienting the emission polarizer (Polaroid HNP'B dichroic film) in a vertical [$I_{VV}(t)$] or horizontal [$I_{VH}(t)$] direction. Excitation and emission bands were selected with an Oriel 500-nm short-pass interference filter (catalog no. 59876) and a Corning 3-68 cut-on filter, respectively. Typically, 1×10^4 peak counts were collected (in 5−10 min) for the $I_{VV}(t)$ and $I_{VH}(t)$ decays. The total counts in the $I_{VV}(t)$ and $I_{VH}(t)$ curves were scaled with respect to each other with the steady-state ratio of integrated photon counts/1×10^6 lamp flashes that were detected when the samples were excited with vertically polarized light and the emission polarizer was oriented in the vertical (I_{VV}) or horizontal (I_{VH}) directions. (This was done to correct for fluctuations in the flash-lamp frequency and to account for differences in collection times.) To minimize convolution artifacts, lamp-flash profiles, $l_{VV}(t)$ and $l_{VH}(t)$, were recorded by removing the emission cut-on filter and replacing the sample with a suspension of latex beads. The photomultiplier's spectral time shift was corrected for by a computer during the data analysis. The correction factor (G), which corrects for differences in the sensitivity of the instrument to vertically or horizontally polarized light, was determined by measuring the integrated photon counts/1×10^6 lamp flashes that were detected when the samples were excited with horizontally polarized light and the emission polarizer was oriented in the vertical (I_{HV}) or horizontal (I_{HH}) directions.

Analysis of Fluorescence Anisotropy Decay Data. The time-resolved fluorescence anisotropy r(t) is defined as

$$r(t) = [i_{VV}(t) - i_{VH}(t)]/[i_{VV}(t) + 2i_{VH}(t)] \quad (1)$$

and

$$I_{VV}(t) = i_{VV}(t) \otimes l_{VV}(t) \quad (2)$$

$$I_{VH}(t) = i_{VH}(t) \otimes l_{VH}(t) \quad (3)$$

where $I_{VV}(t)$ and $I_{VH}(t)$ are the orthogonally polarized fluorescence decays measured in above experiments; $l_{VH}(t)$ and $l_{VH}(t)$ are the measured lamp-flash profiles when the emission polarizers are oriented in the vertical or parallel positions. The symbol $\otimes$ denotes the convolution product. Deconvolution from the finite-width lamp flash was done with the Globals Unlimited computer program (Beechem *et al.*, 1991). Goodness of fit was evaluated from the value of χ^2 and visual inspection of the difference between experimental and theoretical curves.

The fluorescence anisotropy data were best fitted by a sum of two exponentials

$$r(t) = r_o[f_F \exp(-t/\phi_F) + f_S \exp(-t/\phi_S)] \quad (4)$$

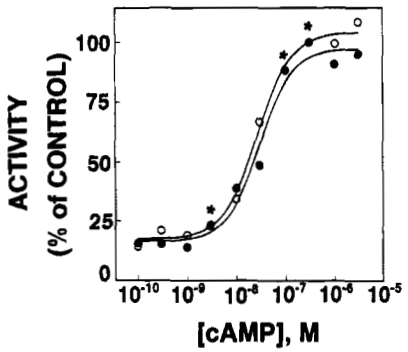

FIGURE 1: Concentration dependence of cAMP-induced catalytic activity of the native and CF-labeled cAPK holoenzymes. Protein kinase holoenzyme was prepared from unmodified subunits ($R^{II}_2C_2$)(○) as well as the fluorescently labeled C subunits ($R^{II}_2{}^{CF}C_2$) (●) by incubation of the subunits for 20 min at 30 °C in the presence of 6 mM $MgCl_2$ and 4 mM ATP. Equal amounts of each holoenzyme (10 nM) were incubated with varying concentrations of cAMP for an additional 10 min at 22 °C prior to quantification of catalytic activity by the kemptide assay procedure. Data (average of three determinations) are expressed as the percentage of the activity of R_2C_2 or $R^{II}_2{}^{CF}C_2$ activated by 0.3 μM cAMP, and the asterisks represent overlapping datum points.

where

$$f_F + f_S = 1 \quad (5)$$

and r_o is the limiting anisotropy, f_F and f_S are the preexponential weighting factors, and ϕ_F and ϕ_S are the fast and slow rotational correlation times, respectively.

RESULTS AND DISCUSSION

Characterization of the ^{CF}C Subunits. SDS−polyacrylamide gel electrophoresis of the ^{CF}C subunit indicated that greater than 95% of the fluorophore was covalently attached to the C subunit (data not shown). The stoichiometry of labeling for the various preparations ranged between 0.5 and 0.8 mol of CF/mol of C subunit. The concentration dependence of cAMP-induced phosphotransferase activity (Figure 1) and the k_{cat} (17 s^{-1}; data not shown) of the ^{CF}C subunit were indistinguishable from that of the native C subunit.

Separation of ^{CF}C Subunits from $R^{II}_2{}^{CF}C_2$ Complexes. $R^{II}_2{}^{CF}C_2$ complexes were prepared in the presence of MgATP with ^{CF}C and R^{II}_2 in a 1.7:1 molar ratio. The $R^{II}_2{}^{CF}C_2$ holoenzyme was separated from free ^{CF}C by Sephacryl-200 gel filtration (Figure 2). In the absence of cAMP, two peaks of ^{CF}C fluorescence were observed in the Sephacryl elution profile. The first peak was the larger $R^{II}_2{}^{CF}C_2$ complex and the second the smaller free ^{CF}C subunit. In the presence of cAMP, the intrinsic binding energy of the $R_2{}^{CF}C_2$ complex was reduced, and chromatography pulled apart the weakly associated subunits. Consequently, only a single fluorescent elution peak was observed that corresponds to free ^{CF}C subunits (Figure 2). In addition to separating the holoenzyme from the free ^{CF}C subunit, this demonstrates that cAMP can cause the dissociation of R_2 from the ^{CF}C subunits under nonequilibrium conditions that are routinely used to demonstrate that cAMP induces holoenzyme dissociation.

Catalytic Activity at High $R^{II}_2{}^{CF}C_2$ Concentrations. To evaluate whether the $R^{II}_2{}^{CF}C_2$ complexes were fully activated by cAMP at the same concentrations (0.2−0.4 μM) used for the anisotropy measurements, the effect of cAMP and R^{II}_2

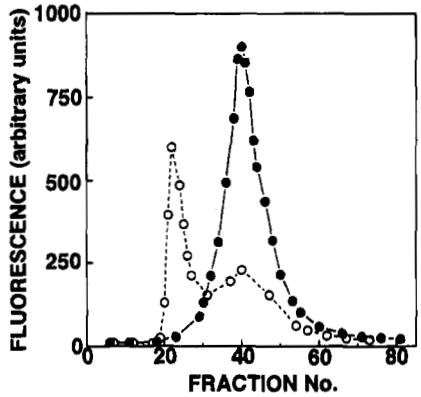

FIGURE 2: Sephacryl-200 elution profile of $R^{II}_2{}^{CF}C_2$ complexes in the absence and presence of cAMP. Shown are the elution profiles of ^{CF}C emission at 525 nm (with excitation at 470 nm) in the absence (○) and presence (●) of cAMP (100 μM) from a 1 × 19 cm Sephacryl-200 column equilibrated in 20 mM KH_2PO_4, 0.2 mM EDTA, and 5 mM β-mercaptoethanol, pH 6.7 at 4 °C. The amounts of the ^{CF}C and the R^{II}_2 subunits loaded onto the column were 0.25 and 0.15 nmol, respectively.

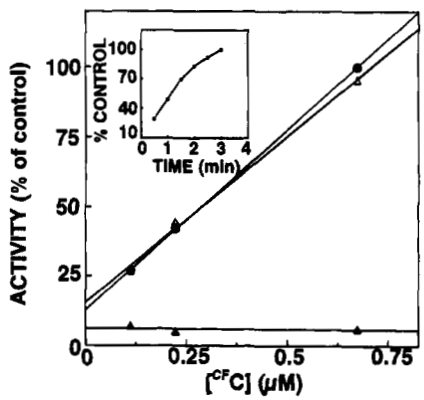

FIGURE 3: Effect of cAMP and R^{II} dimer on the concentration-dependent phosphotransferase activity of the ^{CF}C subunit . Shown is the concentration dependence of ^{CF}C subunit phosphotransferase activity (average of three determinations) in the absence of R^{II}_2 (●), in the presence of R^{II}_2 (▲), and in the presence of both R^{II}_2 and cAMP (50 μM) (△). The R^{II}_2:^{CF}C subunit ratio is 1.3:1. The inset shows the time course of product formation as a percent of catalytic activity relative to the 3-min datum point at 0.8 μM ^{CF}C (one determination).

on the catalytic activity of the ^{CF}C subunit was examined. Because the standard phosphotransferase assay conditions use a 100-fold lower cAPK concentration than the anisotropy measurements, which are done at levels comparable to those found intracellularly (0.2−2 μM; Hofmann *et al.*, 1977; Francis & Corbin, 1994), it was necessary to modify the published assay procedure (Whitehouse *et al.*, 1983) such that high concentrations of cAPK could be assayed. As shown in the inset in Figure 3, under these conditions product formation is linear during the first 1.5 min. Phosphotransferase activity during the first minute of the reaction (Figure 3) is also linearly dependent on the concentration of ^{CF}C both in the absence of R^{II}_2 and in the presence of R^{II}_2 plus cAMP (50 μM). This is the same concentration range of ^{CF}C subunit used in the rotational mobility experiments. As expected, in the absence of cAMP, R^{II}_2 inhibits the catalytic activity of ^{CF}C, presumably reflecting reassociation, while the addition of cAMP to the holoenzyme enhances catalysis to the same level observed for an equivalent molar amount of free ^{CF}C.

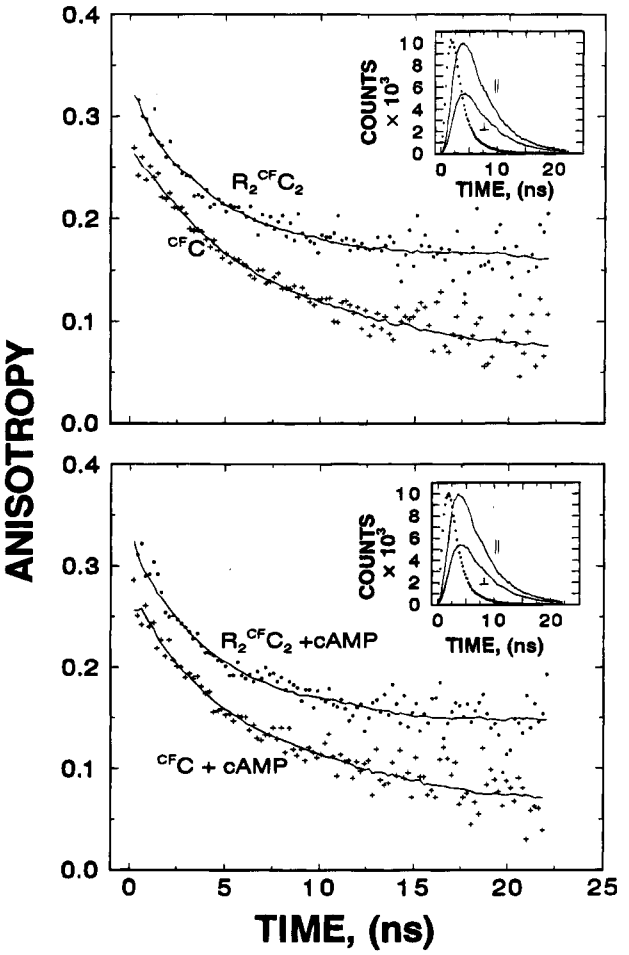

FIGURE 4: Time-resolved fluorescence anisotropy of $R^{II}_2{}^{CF}C_2$ complexes and ^{CF}C subunits in the absence (upper panel) and presence (lower panel) of cAMP (50 μM). Shown are the time courses of the observed anisotropy decay (dots and plus symbols) and theoretical decay with the best-fit parameters (solid lines). The time-resolved anisotropy decays were fitted to eq 4 with the Globals Unlimited program (Beecham *et al.*, 1991). The best-fit rotational correlation times (ϕ_F and ϕ_S) were 1.7 ± 0.3 and 18.3 ± 0.7 ns for the free ^{CF}C subunits and 2.3 ± 0.1 and 93 ± 2 ns for the $R^{II}_2{}^{CF}C_2$ complexes in the absence of cAMP, respectively. [The ratios of preexponential weighting factors (f_F/f_S) were 0.39 and 0.47, respectively.] In the presence of cAMP, the best-fit correlation times were 1.4 ± 0.2 and 19..0 ± 1.0 ns for the free ^{CF}C subunits and 2.0 ± 0.2 and 93 ± 9 ns for the $R^{II}_2{}^{CF}C_2$ complexes, respectively. [The ratios of preexponential weighting factors (f_F/f_S) were 0.40 and 0.43, respectively.] The insets show the time course of the excitation lamp pulses (dotted lines) and time decay of emissions (solid lines) of the $R_2{}^{CF}C_2$ complexes in the absence (upper panel) and presence (lower panel) of cAMP with the emission polarizer oriented either vertically (||) or horizontally (⊥) relative to the vertically polarized excitation beam. The fluorescence lifetime (4.1 ± 0.1 ns) of the various ^{CF}C subunit samples was unaffected by cAMP or state of association. Each time point represents 0.211 ns.

Effect of cAMP on the Rotational Mobility of cAPK. Figure 4 illustrates the effect of cAMP on the rotational mobility of the $R^{II}_2{}^{CF}C_2$ complex and ^{CF}C subunit that were isolated by gel filtration (see Figure 2). Shown are the observed anisotropy decay (dots) and theoretical decay kinetics using the best-fit parameters (solid lines). The observed time-resolved fluorescence anisotropy data were well fitted to an equation of a sum of two exponentials (eq 4) containing a fast and slow rotational correlation time, ϕ_F and ϕ_S, respectively. The fast rotational correlation time can be attributed to the fast internal motions of the protein, i.e., the localized rotations of the CF label, while the slow rotational correlation time represents the whole-body rotations of the holoenzyme ($R^{II}_2{}^{CF}C_2$). In the absence of cAMP, the two rotational correlation times were ϕ_F = 1.7 ± 0.3 ns and ϕ_S = 18 ± 1 ns for the free ^{CF}C subunit and ϕ_F = 2.3 ± 0.1 ns and ϕ_S = 93 ± 2 ns for the $R^{II}_2{}^{CF}C_2$ complex. These rotational correlation times are in reasonable agreement with the theoretical rotational correlation times for the holoenzyme (108−235 ns) and the free C subunit (13−20 ns) estimated by using the Stokes−Einstein equation and the various published values of the Stokes radii (Erlichman *et al.*, 1973; Zoller *et al.*, 1979; Cobb *et al.*, 1987; Olah *et al.*, 1993; Herberg *et al.*, 1994).

The addition of 50 μM cAMP had no significant effect on either the fast or the slow rotational correlation time of the $R^{II}_2{}^{CF}C_2$ complex (ϕ_F = 2.0 ± 0.2 ns and ϕ_S = 93 ± 9 ns). If cAMP had, in fact, induced dissociation of the $R^{II}_2{}^{CF}C_2$ complex, then the observed slow rotational correlation time of the holoenzyme (in the presence of cAMP) should have decreased from 93 to 19 ns, corresponding to the values observed with free ^{CF}C. These results unequivocally demonstrate that saturating concentrations of cAMP do not induce dissociation of the holoenzyme form of cAPK under equilibrium conditions. This is in contrast to situations like the Sephacryl chromatography experiments described above (Figure 2) where, in the presence of cAMP, the gel-filtration process pulls apart the weakly associated R^{II}_2 and ^{CF}C subunits.

There are two issues that must be considered in the interpretation of these findings. First, clearly, there are more than two rotational modes for fluorescent probes attached to asymmetric proteins; however, available techniques rarely resolve more than two correlation times [for review see Steiner (1991)]. Second, because the excited-state population of carboxyfluorescein molecules decays to zero during just the initial depolarization process, the fluorescent lifetime of carboxyfluorescein is, in reality, too short to accurately assess the rotational correlation time of the large $R^{II}_2{}^{CF}C_2$ complex. As a consequence, a broad range of ϕ_S values (93−208 ns) with low χ^2's can emerge from the nonlinear least-squares fitting routine utilized. Consequently, to compare the slower correlation times of the various holoenzyme samples, the fitting procedures involved an iterative process whereby the slow rotational correlation time was adjusted (fixed) down until the lowest ϕ_S value was obtained with the smallest χ^2. Although the accuracy of the resultant ϕ_S's can legitimately be questioned, inspection of the anisotropy decay (Figure 4) clearly reveals that cAMP does not significantly reduce the rotational mobility of the holoenzyme and cannot, therefore, have induced dissociation.

An additional issue is whether it is plausible for the holoenzyme to be catalytically active. cAPK substrates and inhibitor proteins/peptides share a consensus sequence that binds to the C subunit substrate recognition site. Consequently, the consensus sequences of substrates and inhibitors cannot simultaneously interact at the substrate recognition site. For the holoenzyme to be catalytically active, cAMP must induce a conformational change in R^{II} that moves its consensus sequence away from the C subunit substrate recognition site, allowing substrate binding. Mutational analyses of the C subunit have defined several regions in addition to the substrate/inhibitor consensus site that are important for the interaction of R with C subunits (Gibbs *et*

al., 1992; Cox & Taylor, 1994). More significantly, mutation of the consensus sequence of R^{II} ($Arg^{92} \rightarrow$ Ala and $Arg^{93} \rightarrow$ Ala) yields a mutated, stable holoenzyme that retains full catalytic activity both in the presence and in the absence of cAMP (Wang *et al.*, 1991). These results show (1) that R binding to C subunits depends on more than the C subunit consensus sequence and (2) that holoenzyme can be catalytically active.

Implications. While the present results reveal that cAMP-induced catalytic activation of cAPK can occur without holoenzyme dissociation, they also demonstrate that the cAMP-bound R_2 and C subunits will separate with a mild dissociating force. The question is—does this separation occur *in vivo*, and if so, what is the force that pulls apart the cAMP-activated holoenzyme?

Several lines of evidence, including morphological and biochemical studies (Krebs, 1986; Taylor *et al.*, 1990), form a compelling argument that cAPK can dissociate its subunits *in vivo*. One of the most direct examinations of this is the recent study using a cAPK composed of rhodamine-labeled type I regulatory dimer and fluorescein-labeled C subunits that, when associated, exhibited a large amount of FRET both *in vitro* and after being microinjected into viable cells (Adams *et al.*, 1991). Upon elevation of cellular cAMP levels, FRET decreased, indicating dissociation of the C subunits which then translocated into the nucleus. Importantly, within an hour, the labeled C subunits slowly returned to the cytoplasm and FRET was reestablished, apparently due to declining cAMP levels that allowed reassociation of subunits to form holoenzyme. In the context of results from numerous other studies, this observation is consistent with the *in vivo* dissociation of cAPK in the presence of cAMP.

In living cells there exist multiple factors such as cytoskeletal elements, membrane proteins, and cytosolic or nuclear proteins that could conceivably provide the external force needed to dissociate cAMP-activated holoenzyme. The only requirement would be that they are able to act upon cAPK. In this regard the cAPK anchor proteins (AKAPs) should be considered. These membrane proteins bind to the R^{II} subunits and are thought to target specific substrates for phosphorylation (Scott *et al.*, 1990). If the cAPK holoenzyme truly dissociates to become active, the need of AKAPs can be questioned. However, if, as the present results indicate, enzyme dissociation is unnecessary for catalytic activation, the physiologic significance of AKAPs becomes more tenable.

An important issue, raised by the present results, is whether there are different functional roles for cAMP-activated holoenzyme relative to the free C subunit. Enzymes that are controlled allosterically by the binding of small, rapidly diffusing ligands can more quickly cycle between inactive and active states than enzymes that are regulated by the slower mechanisms involving the combination of ligand binding followed by subunit dissociation and reassociation. For example, the cAMP-activated holoenzyme may play important roles in regulating the metabolic or ionic requirements of cells where rapid on—off responses are needed, while the role cAPK plays in transcriptional events could involve the slower cAMP-induced dissociation process. Overall, the present observations indicate that the regulation of cAMP-dependent protein kinase catalytic activity is more complex than is generally thought.

REFERENCES

Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S., & Tsien, R. (1991) *Nature 349*, 694—697.

Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38*, 299—308.

Beecham, J. M., Gratton, E., Ameloot, M., Knutson, J. R., & Brand, L. (1991) in *Topics in Fluorescence Spectroscopy: Principles* (Lakowics, J. R., Ed.) Vol. II, pp 241—305, Plenum Press, New York.

Bradford, M. (1976) *Anal. Biochem. 72*, 248—254.

Brostrom, C. O., Corbin, J. D., King, C. A., & Krebs, E. G. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2444—2447.

Cobb, C. E., Beth, A. H., & Corbin, J. D. (1987) *J. Biol. Chem. 262*, 16566—16574.

Connelly, P. A., Hastings, T. G., & Reimann, E. M. (1986) *J. Biol. Chem. 261*, 2325—2330.

Corbin, J. D., Brostrom, C. O., Alexander, R. L., & Krebs, E. G. (1972) *J. Biol. Chem. 247*, 3736—3743.

Cox, S., & Taylor, S. S. (1994) *J. Biol. Chem. 269*, 22614—22622.

Dills, W. L., Jr., Beavo, J. A., Bechtel, P. J., Myers, K. R., Sakai, L. J., & Krebs, E. G. (1976) *Biochemistry 15*, 3724—3730.

Erlichman, J., Rubin, C. S., & Rosen, O. M. (1973) *J. Biol. Chem. 248*, 7607—7609.

Fletcher, W. H., van Patten, S. M., Cheng, H. C., & Walsh, D. A. (1986) *J. Biol. Chem. 261*, 5504—5513.

Francis, S. H., & Corbin, J. D. (1994) *Annu. Rev. Physiol. 56*, 237—272.

Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S., Zoller, M. J. (1992) *J. Biol. Chem. 267*, 4806—4814.

Gonzalez, G. A., & Montminy, M. R. (1989) *Cell 59*, 675—680.

Hanson, D. C., Yguerabide, J., & Schumaker, V. N. (1981) *Biochemistry 20*, 6842—6852.

Haugland, R. P. (1983) in *Excited States of Biopolymers* (Steiner, R. F., Ed.) p 29, Plenum Press, New York.

Herberg, F. W., Dostmann, W. R. G., Zorn, M., Davis, S. J., & Taylor, S. S. (1994) *Biochemistry 33*, 7485—7494.

Hofmann, F., Bechtel, P. J., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 1441—1447.

Johnson, D. A., Leathers, V. L., Martinez, A.-M., Walsh, D. A., & Fletcher, W. H. (1993) *Biochemistry 32*, 6402—6410.

Krebs, E. G. (1986) in *The Enzymes: Control by Phosphorylation* (Boyer, P. D., & Krebs, E. G., Eds.) pp 3—20, Academic Press, Inc., New York.

Laemmli, U. K. (1970) *Nature 227*, 680—695.

Mellon, P. L., Clegg, C. H., Correll, L. A., & Mcknight, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4887—4891.

Olah, G. A., Mitchell, R. D., Sosnick, T. R., Walsh, D. A., & Trewhella, J. (1993) *Biochemistry 32*, 3649—3657.

Reimann, E. M., Walsh, D. A., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1986—1957.

Rubin, C. S., Erlichman, J., & Rosen, O. M. (1972) *J. Biol. Chem. 247*, 36—44.

Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D. Vitalis, E. A., & Mangili, J. A. (1990) *J. Biol. Chem. 265*, 21561—21566.

Steiner, R. F. (1991) in *Topics in Fluorescence Spectroscopy: Principles* (Lakowics, J. R., Ed.) Vol. II, pp 1—52, Plenum Press, New York.

Tao, M., Salas, M. L., & Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 408—418.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990) *Annu. Rev. Biochem. 59*, 971—1005.

Walsh, D. A., & Cooper, R. H. (1979) *Biochemical Actions of Hormones*, Vol. VI, pp 1—75, Academic Press, Inc., New York.

Walsh, D. A., Glass, D. B., & Mitchell, R. D. (1992) *Curr. Opin. Cell Biol. 4*, 241—251.

Wang, Y., Scott, J. D., McKnight, G. S., & Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2446—2450.

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693—3701.

Yguerabide, J. (1972) *Methods Enzymol. 26*, 498—578.

Zoller, M. J., Kerlavage, A. R., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 2408—2412.

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