Fluorescence Resonance Energy Transfer within a Heterochromatic cAMP-Dependent Protein Kinase Holoenzyme under Equilibrium Conditions: New Insights into the Conformational Changes That Result in cAMP-Dependent Activation†

David A. Johnson,‡ Valerie L. Leathers,§ Anna-M. Martinez,§ Donal A. Walsh,⊥ and William H. Fletcher*,§

Division of Biomedical Sciences and the Department of Neuroscience, University of California, Riverside, California 92521, Department of Anatomy, Loma Linda University, and Molecular Cytology Research, Veterans Administration Medical Center, Loma Linda, California 92357, and Department of Biological Chemistry, University of California, Davis, California 95616

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ABSTRACT: Previous studies of the ligand regulation of the cAMP-dependent protein kinase have demonstrated the cAMP-mediated dissociation of the holoenzyme by using nonequilibrium techniques; i.e., gel filtration, ion-exchange chromatography, and differential centrifugation. While physically mild, these could have caused weakly associated species to dissociate, thereby providing a potentially flawed interpretation of the mechanism of activation of the protein kinase. To assess this, the activation of the cAMP-dependent protein kinase has been monitored under equilibrium conditions using dipolar fluorescence energy transfer to measure changes in the proximity relations between the catalytic (C) and regulatory (R) subunits that compose the holoenzyme. Specifically, we prepared a heterochromatically labeled protein kinase type II holoenzyme, with the regulatory and catalytic subunits labeled with sulforhodamine and carboxyfluorescein, respectively, and monitored the exchange of electronic excitation energy between the C and R subunits by both donor lifetime and steady-state fluorescence. Biochemically, the heterochromatic holoenzyme was closely identical to the native protein with regard to cAMP-induced increase in catalytic activity, reassociation of C and R subunits, inhibition of catalytic activity by the specific protein kinase inhibitor (PKI), and observed dissociation examined by gel filtration upon cAMP addition. However, under equilibrium conditions, the energy-transfer measurements revealed that the addition of cAMP to this heterochromatic reporter complex promoted an estimated 10-Å increase in the distance between the derivatization sites on C and R but not a dissociation of these subunits. Addition of PKI plus cAMP promoted full dissociation of the two subunits. Addition of a high-affinity substrate ((Ser 21)PKI(14–22)-amide) had no significant effect on energy transfer and therefore the distance between derivatization sites on C and R. These results demonstrate (1) that, *in vitro*, cAMP does not decrease the binding affinity between the subunits of the holoenzyme as much as is generally assumed and (2) that PKI, but not a high-affinity substrate, can affect holoenzyme dissociation. To what extent these regulatory events occur in viable cells is currently being examined.

From *in vitro* studies, the cAMP-dependent protein kinase appears unique among regulatory proteins, because its activity is controlled by a dissociation/reassociation reaction, reflecting a conformational change of far greater magnitude than that of conventional allosteric control. In the absence of cAMP, the protein kinase is maintained as an inactive holoenzyme composed of two monomeric catalytic (C) subunits and a dimeric regulatory (R) subunit.[1] An elevation in cAMP and its subsequent binding to the two distinct sites on the R subunit induce conformational changes that promote dissociation of the holoenzyme and yield active free catalytic subunit. Notably, dissociation has been identified *in vitro* only after subjecting the holoenzyme to one of a variety of physical separation methods under nonequilibrium conditions, such as sucrose gradient ultracentrifugation (Reimann *et al.*, 1971; Tao *et al.*, 1970), ion-exchange chromatography (Brostrom *et al.*, 1971; Corbin *et al.*, 1972; Rubin *et al.*, 1972), gel filtration (Beavo *et al.*, 1974), or cAMP affinity chromatography (Dills *et al.*, 1976). Although these procedures would impose only a relatively mild force, such methods can nevertheless induce dissociation of weakly associated subunits. Physiologically, the reason for this complex dissociation mechanism has remained an enigma but could have extremely important ramifications. Specifically, dissociation appears to be counter to the recent data, indicating that at least the R_{II} subunits are responsible for localizing the protein kinase to specific subcellular environments (Scott *et al.*, 1990). Of note, the highly homologous cGMP-dependent protein kinase, in which the regulatory and catalytic domains are contiguous on the same polypeptide chain, is activated by a simple

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* Corresponding author: Molecular Cytology Research—151, Veterans Administration Medical Center, Loma Linda, CA 92357; voice (909) 825-7084 ext. 2268, FAX (909) 796-4508.

‡ University of California, Riverside.

§ Veterans Administration Medical Center.

⊥ University of California, Davis.

[1] Abbreviations: C, catalytic subunit of the cAMP-dependent protein kinase; R_{II}, type-II regulatory subunit of the cAMP-dependent protein kinase; CF, 5,6-carboxyfluorescein succinimidylester; TR, Texas Red sulfonyl chloride; ^{CF}C and ^{TR}C, CF-labeled and TR-labeled catalytic subunit, respectively; $^{TR}R_{II}$ and $^{CF}R_{II}$, TR-labeled and CF-labeled regulatory subunit, respectively; $^{TR}R_{II}{}_2{}^{CF}C$ the heterochromatic holoenzyme containing derivatized R_2C_2; EDTA, ethylenediaminetetraacetic acid; MES, 2-morpholinoethanesulfonic acid; PKI, inhibitor protein of the cAMP-dependent protein kinase; PKI(5–24), the peptide containing residues 5–24 of PKI; (Ser 21)PKI(14–22)-amide, high-affinity substrate with a structure of (Ser 21)PKI(14–22)-amide; cAMP, adenosine cyclic 3′,5′-monophosphate; OD, optical density.

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conformational change (Gill *et al.*, 1977; Lincoln *et al.*, 1978). Because of these observations, it is important to examine the ligand-induced dissociation of the cAMP-dependent protein kinase under equilibrium conditions. Using a spectrofluorometric approach with a novel heterochromatic protein kinase holoenzyme, we have directly monitored the effects of cAMP on holoenzyme dissociation, which is seen as a decrease in fluorescence resonance energy transfer under equilibrium conditions. These new data suggest that the mechanism of activation of the cAMP-dependent protein kinase by cAMP-induced dissociation requires reconsideration.

MATERIALS AND METHODS

Materials. Texas Red sulfonyl chloride (TR) and 5,6-carboxyfluorescein succinimidyl ester (CF) were obtained from Molecular Probes, OR. The high-affinity substrate (Ser 21)-PKI(14-22)-amide was synthesized following procedures described elsewhere (Glass *et al.*, 1989). All other compounds were reagent grade or better.

cAMP-Dependent Protein Kinase and Inhibitor Protein 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). The inhibitor protein of cAMP-dependent protein kinase was purified from rabbit skeletal muscle as detailed previously (Walsh *et al.*, 1990).

Modification of Catalytic and Regulatory Subunits. Catalytic and regulatory subunits were recombined to form holoenzyme, which was freshly prepared for each derivatization. Purified catalytic subunit in 2.5 mM Tris chloride, pH 7.4, 0.1 mM EDTA, 7.5 mM β-mercaptoethanol, 50 mM NaCl, and 50% (v/v) glycerol was added to purified regulatory subunit in 5 mM MES, pH 7.4, 0.2 mM EDTA, 15 mM β-mercaptoethanol, 150 mM NaCl and incubated 20 min at 30 °C in the presence of 4 mM ATP, 6 mM $MgCl_2$. The relative amounts of C and R subunits were chosen so that there would be less than 5% of the phosphotransferase activity in the absence *versus* presence of cAMP. Recombined holoenzyme (~2 mg/mL) was eluted from a Sephadex G-25 mini-column equilibrated in 0.1 M $NaHCO_3$, pH 8.4, for buffer exchange as previously detailed (Fletcher *et al.*, 1986). In the case of the 5,6-carboxyfluorescein (CF) derivatization, 2 μL of CF/mg of protein was added from a stock of 1.5 mg of CF/300 μL of dimethyl sulfoxide. The sample was incubated for 7 min at 24 °C and immediately buffer exchanged through a Sephadex G-25 minicolumn equilibrated in 20 mM KH_2PO_4, pH 6.7, 5 mM β-mercaptoethanol and the eluate incubated with cAMP (0.5 mM) for 20 min at 4 °C to dissociate the derivatized holoenzyme. Free subunits were resolved from holoenzyme with a Sephadex CM-50 minicolumn (0.2 mL of resin/0.3 mL of sample) equilibrated in 20 mM KH_2PO_4, pH 6.7, 5 mM β-mercaptoethanol. Derivatized R subunit does not bind to the cation exchange resin, whereas the C subunit is retained and can be eluted with 250 mM KH_2PO_4, pH 6.7, 5 mM β-mercaptoethanol.

The procedure used with Texas Red (Sulforhodamine, TR) was identical to that described for CF derivatization with the exception of the actual reaction conditions with the fluorophore. After buffer exchange into 0.1 M $NaHCO_3$, pH 8.4, holoenzyme was incubated with TR (5 μL/mg of protein from a stock of 1 mg/100 μL of dimethylformamide) for 1 h at 4 °C. The derivatization was terminated by processing as described above for CF. Following the Sephadex CM-50 chromatographic step, the $^{TR}R_{II}$ was buffer exchanged using a Sephadex G-25 minicolumn equilibrated in 20 mM KH_2PO_4, pH 6.7, 5 mM β-mercaptoethanol so as to remove excess cAMP. Derivatized subunits were stored at 4 °C and utilized for experimentation within 2 weeks of modification.

Formation of Heterochromatic Holoenzyme. Unless stated otherwise, CF-labeled catalytic subunit (^{CF}C) and TR-labeled regulatory subunit ($^{TR}R_{II}$) were recombined in the presence of 4 mM ATP and 6 mM $MgCl_2$ and incubated 20 min at 30 °C. The relative amounts of ^{CF}C and $^{TR}R_{II}$ were chosen such that the $^{TR}R_{II}$·^{CF}C holoenzyme had less than 5% of the catalytic activity in the presence of cAMP as compared to the absence of cAMP.

Where noted, ATP and Mg^{2+} were removed from the heterochromatic holoenzyme using a G-25 minicolumn equilibrated in 20 mM KH_2PO_4, pH 6.7, 5 mM β-mercaptoethanol using the centrifugation/chromatography conditions described previously (Fletcher *et al.*, 1986). To assess the efficiency of this step, the OD A_{260}/A_{280} ratio was obtained prior to and after the minicolumn. This procedure removed >95% of ATP, which means that residual ATP could be no more than 0.6 μM in the fluorescence lifetime instrument and about one-fifth that during steady-state observation.

Assays. Phosphotransferase activity was quantitified by the method of Whitehouse and Walsh (1983) using Kemptide as the substrate. Protein concentrations were determined by the method of Bradford (1976) with IgG as a protein standard or by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Fluorophore-to-protein ratios were estimated based on molar extinction coefficients of 66 000 (491 nm) and 85 000 M^{-1} cm^{-1} (596 nm) for CF and TR, respectively (Haugland, 1983).

Chromatography/Electrophoresis. SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Sephacryl S-300 gel filtration chromatography was carried out following Connelly *et al.* (1986).

Steady-State Spectroscopy. Steady-state fluorescence measurements were made with a Perkin-Elmer MPF-66 spectrofluorometer. Emission correction factors were generated with the aid of a calibrated 150-W reference lamp from Optronics Laboratories, Inc. (Orlando, FL).

Fluorescence Lifetime. Fluorescence lifetimes were determined by the time-correlated single-photon-counting technique with an EEY scientific nanosecond fluorometer (La Jolla, CA) equipped with a high-pressure hydrogen arc lamp. Fluorescence decay rates were initially resolved and assessed as a single exponential function using the method of moments fitting procedure. The analysis of the fluorescence decay was continued with the Globals Unlimited computer program (Laboratory for Fluorescence Dynamics, Urbana, IL) to fit the decays to a unimodal gaussian distribution function. Excitation and emission bands were selected with Oriel 500-nm short-pass (no. 59876) and 540-nm narrow-band (no. 54361) interference filters, respectively. (This filter combination permitted <3% of the $^{TR}R_{II}$ emission from bleeding into the ^{CF}C signal.) In addition to the chromatic filters, a Polaroid HNP'B dichroic film polarizer (Norwood, MA) was placed in the observation path and rotated at an angle of 55° from vertical to minimize anisotropic contributions to the observed decay. The instrumental arrangement and principles of data treatment have been discussed in detail elsewhere (Yguerabide, 1972).

Fluorescence Energy Transfer. The efficiency of dipolar resonance energy transfer between discrete donors and suitable acceptors is related to the distance (R) separating the two

molecules by eq 1 (Förster, 1959)

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \quad (1)$$

where R_0 is the Förster distance and represents the distance at which transfer efficiency is 50% and is evaluated according to

$$R_0 = 9.765 \times 10^3 (\kappa^2 J Q_D n^{-4})^{1/6} \quad (2)$$

The overlap integral, J, is defined by the expression

$$J = \frac{\sum I_D(\lambda)\, \epsilon_A(\lambda)\, \lambda^4 \Delta\lambda}{\sum I_D(\lambda) \Delta\lambda} \quad (3)$$

where $\epsilon_A(\lambda)$ is the molar extinction coefficient of the energy acceptor and $I_D(\lambda)$ is the relative donor emission spectrum, Q_D denotes the donor quantum yield in the absence of acceptor group, n represents the refractive index of the medium between donor and acceptor, λ is the wavelength in centimeters, and κ^2, the orientation factor, accounts for the relative orientation of the donor emission and acceptor absorption transition moments.

In the case where donor and acceptor are at separate sites on a macromolecule, efficiency of energy transfer (E) can be measured as the extent of the reduction of the donor steady-state emission or fluorescence lifetime:

$$E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (4)$$

I_{DA} and I_D are the fluorescence intensities of the donor (D) in the presence and absence of the acceptor (A), and τ_{DA} and τ_D are the fluorescence lifetimes of the donor in the presence and absence of acceptor, respectively.

For the case where there are multiple donors and acceptors at various distances from one another, the donor decay in the presence of acceptor can be written as

$$I_{DA}(t) = \sum_{k=1}^{m} A_k \int P_k(r) \sum_{i=1}^{n} \alpha_i e^{-(t/\tau_i)(1+(R_0/r)^6)}\, dr \quad (5)$$

where $P_k(r)$ is the probability distribution of distances for one population, $\int P_k(r)\, dr = 1$, m = the total number of populations, and A_k is the amplitude or relative concentration of the kth population (Rice *et al.*, 1991). The probability distribution of $P_K(r)$ is modeled by a Gaussian fit where $\bar{r}_k$ is the mean distance and σ_k is related to the full width at half-maximum height (half-width) by $2.355\sigma_k$ (Rice *et al.*, 1991):

$$P_k(r) = \frac{1}{\sigma_k\sqrt{2\pi}} \exp\left(-\frac{(r - \bar{r}_k)^2}{2{\sigma_k}^2}\right) \quad (6)$$

RESULTS

Biochemical Characterization of Derivatized Subunits. We have previously described the derivatization of the cAMP-dependent protein kinase catalytic subunit by fluorescein isothiocyanate (Fletcher *et al.*, 1986). That study showed that the optimum derivatization (i.e., without loss of catalytic activity) was best accomplished by protection of the catalytic site by the presence of ATP and regulatory subunit (i.e., as holoenzyme), even though this required the subsequent separation and purification of the free subunits. We have employed the same strategy here and have also isolated the derivatized regulatory subunit that is produced concurrently. Moreover, for these studies we have used fluorophores of potentially greater quantum yields. To obtain uniquely labeled catalytic and regulatory subunits, holoenzyme derivatization was undertaken with both Texas Red and 5,6-carboxyfluorescein. This could allow us to construct donor–acceptor pairs of either $^{CF}R_{II}$.^{TR}C or $^{TR}R_{II}$.^{CF}C, but this strategy also has the added advantage of allowing us to select the pair with the least degree of contamination with the other similarly derivatized subunit. Characteristics of the preparations are provided by Table I. The holoenzyme retained full catalytic activity and full cAMP-dependency following conjugation with either fluorophore (Table I), demonstrating that the protein was not adversely affected by the derivatization procedure.

Table I: Catalytic Activity throughout Texas Red and Carboxyfluorescein Derivatization[a] of the Protein Kinase A Holoenzyme

		phosphotransferase activity		
		total activity (μmol min^{-1})		
	species	−cAMP	+cAMP	% yield
CF				
holoenzyme	RC	39	1660	100
derivatized holoenzyme	$^{CF}R_{II}$.^{CF}C	67	1710	103
CM-50: flow-through	$^{CF}R_{II}$	122	288	
CM-50; 250 mM KH_2PO_4 eluate	^{CF}C	272	274	16.5
TR				
holoenzyme	RC	64	1794	100
derivatized holoenzyme	$^{TR}R_{II}$.^{TR}C	110	1645	92
CM-50; flow-through	$^{TR}R_{II}$	22	47	
CM-50; 250 mM KH_2PO_4 eluate	^{TR}C	212	219	12.2

[a] Conditions for fluorescence labeling and assay are described in Materials and Methods. Following derivatization, each fraction was assayed for phosphotransferase activity in the presence and absence of 62 μM cAMP.

The overall yields through the purification for both C (by activity) and R (by protein determination) were ~10–15%, similar to what we have obtained previously (Fletcher *et al.*, 1986). The C subunit, when eluted from CM-50, exhibited equal activity in the presence or absence of cAMP with either derivatization procedure, indicating the absence of regulatory subunit. The regulatory subunit from carboxyfluorescein derivatization had some catalytic subunit contamination but catalytic activity of the species conjugated with Texas Red was at the level of background. Because of these results, we elected to use the donor-acceptor pair of $^{TR}R_{II}$.^{CF}C for all future studies. The preparations of ^{CF}C and $^{TR}R_{II}$ were examined by SDS gel electrophoresis; each was homogeneous, not contaminated with the other subunit or free fluorophore, and of the expected size (Figure 1). For the derivatization procedure described here, the molar ratios of fluorophore to protein were approximately 0.5 and 1.4 for ^{CF}C and $^{TR}R_{II}$, respectively. Of importance for the energy-transfer measurements was the lack of residual ^{TR}C in the $^{TR}R_{II}$ fraction or $^{CF}R_{II}$ in the ^{CF}C preparation.

Characterization of the $^{TR}R_{II}$.^{CF}C holoenzyme and its constituent subunits is depicted in Figure 2. The ability of the derivatized subunits to associate into holoenzyme is demonstrated by the titration of ^{CF}C by $^{TR}R_{II}$: the profile obtained was exactly equivalent to unmodified C and R (panel A); each had a closely similar specific activity. The activation of heterochromatic holoenzyme by cAMP was very similar to that displayed by the native holoenzyme with just a modest shift in cAMP sensitivity (K_a values 130 and 90 nM, respectively; panel B). Both ^{CF}C and native C were equally titrated by the high-affinity protein kinase inhibitor PKI (panel C). Collectively, the results of Figure 2 demonstrate that the mutual affinities of C and R subunits are not appreciably

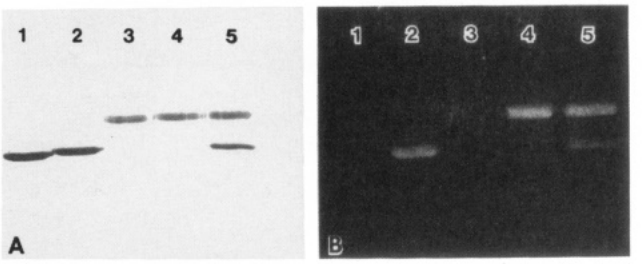

FIGURE 1: SDS-PAGE characterization of fluorescently labeled cAMP-dependent protein kinase subunits. Derivatized catalytic and regulatory subunits were prepared as described in Materials and Methods. Samples (5–10 μg) were electrophoresed on a 10% SDS-PAGE slab gel (1.0 mm) by the method of Laemmli (1970). A fluorograph of the gel was obtained after a 6-s exposure of Polaroid 667 film while the gel was on a UV light box (Panel B). A Coomassie Blue stained gel is shown in panel A. Lane 1: unmodified catalytic subunit. Lane 2: CF-labeled catalytic subunit (^{CF}C). Lane 3: unmodified regulatory subunit. Lane 4: TR-labeled regulatory subunit ($^{TR}R_{II}$). Lane 5: heterochromatic holoenzyme ($^{TR}R_{II}$.^{CF}C).

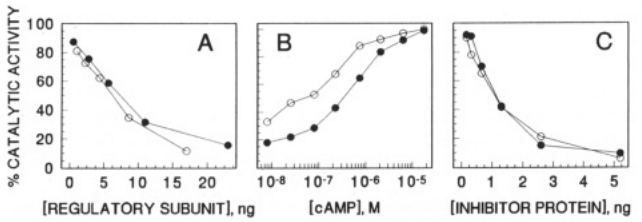

FIGURE 2: Enzymological characterization of $^{TR}R_{II}$.^{CF}C. Panel A: effect of derivatization on the ability of catalytic and regulatory subunits to form holoenzyme. Equal amounts of ^{CF}C (filled circle) or C (open circle) (6 ng) were incubated, respectively, with incremental amounts of $^{TR}R_{II}$ or R_{II}. After 10 min at 22 °C, loss of catalytic activity as a result of holoenzyme formation was quantified by the Kemptide assay. Panel B: effect of derivatization on the ability of the heterochromatic holoenzyme to dissociate upon addition of cAMP. Protein kinase holoenzyme was prepared from unmodified subunits (R_2C_2) (open circles) as well as the fluorescently labeled subunits ($^{TR}R_{II}$.^{CF}C) (filled circles) following incubation of the subunits for 15 minutes at 30 °C in the presence of 0.4 mM $MgCl_2$, 0.6 mM ATP. Equal amounts of each holoenzyme based on specific activity in the presence of 140 μM cAMP 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. Panel C: effect of derivatization on the ability of catalytic subunit to interact with protein kinase inhibitor protein (PKI). Equal amounts of ^{CF}C (filled circles) or C (open circles) (3 ng) were incubated with increasing concentrations of PKI for 10 minutes at 22 °C. Loss of catalytic activity as a result of PKI binding at the substrate site was assayed as described in Materials and Methods. Data are the average of duplicate determinations, relative to a control sample of native C or ^{CF}C.

altered by their conjugation with fluorophores. Likewise, the native and heterochromatic enzymes bind and phosphorylate equivalent amounts of substrate in response to cAMP (i.e., they have similar specific activities). This, along with the fact that the PKI inhibits the catalytic activities of the fluorescently labeled and native proteins to comparable levels, indicates that the substrate binding sites of these two species are not detectably different. As described previously (Fletcher *et al.*, 1986), fluorescenation does not significantly alter the dissociation of ^{3}H-cAMP from the regulatory subunit upon titration with ^{CF}C.

Steady-State Fluorescence Spectroscopy. The corrected excitation and emission spectra of ^{CF}C and $^{TR}R_{II}$.^{CF}C are shown in Figure 3. Because R_{II} was nonspecifically labeled with sulforhodamine, there was not a single absorption spectrum used in the calculation of the Förster distances. Instead, the excitation spectrum of $^{TR}R_{II}$ was taken to represent the spectral profile of the acceptor absorption spectra with the maximum

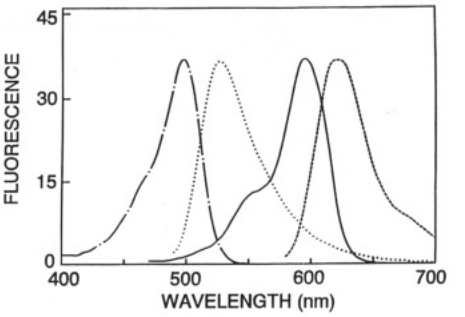

FIGURE 3: Spectral overlap of donor ^{CF}C and acceptor ($^{TR}R_{II}$). Shown are the corrected excitation (dot/dash line) and emission (dotted line) spectra of ^{CF}C and the excitation (solid line) and emission (dashed line) spectra of $^{TR}R_{II}$ in 50 mM MES, 5 mM β-mercaptoethanol, pH 6.8.

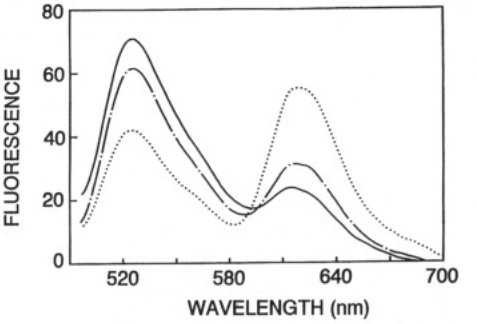

FIGURE 4: Effect of cAMP and PKI on the steady-state emission spectra of the heterochromatic holoenzyme. Carboxyfluorescein-labeled catalytic (^{CF}C)(donor) and Texas Red-labeled regulatory ($^{TR}R_{II}$) (acceptor) subunits were reassociated to form $^{TR}R_{II}$.^{CF}C as described in Materials and Methods. The sample was diluted with 50 mM MES, pH 6.8, 5 mM β-mercaptoethanol to a final concentration of 20 nM in 0.35 mL. Emission spectrum of the donor–acceptor pair (dotted line) is compared with the spectrum generated after the addition of cAMP (20 μM) (dot/dash line) and then again after the addition of PKI (300 nm) (solid line). Samples were excited at 470 nm.

extinction set equal to 85 000 M^{-1} cm^{-1}. The calculated overlap integral for $^{TR}R_{II}$–^{CF}C donor–acceptor pair was 2.41 $\times 10^{-13}$. Thus, assuming a donor (^{CF}C) quantum yield of 0.5 and κ^2 equal to $^2/_3$, the Förster critical distance (R_0) was 51.3 Å.

The steady-state fluorescence spectra of the heterochromatic holoenzyme alone, plus cAMP, and plus cAMP + PKI are shown in Figure 4 for excitation at 470 nm. In the absence of cAMP the donor emission (^{CF}C) (λ_{max} = 525 nm) is ~30% less than in the presence of cAMP, indicating donor quenching. Reciprocally, acceptor emission ($^{TR}R_{II}$) (λ_{max} = 620 nm) is ~70% greater in the absence of cAMP than in its presence, showing the existence of acceptor sensitization. Steady-state spectra were variable with respect to individual $^{TR}R_{II}$.^{CF}C holoenzyme preparations suggesting a dependence on the extent and position of fluorophore labeling. Examining only the change in the donor spectra ±cAMP, the average efficiency of energy transfer was 21% ± 11% in six determinations.

When PKI was added to the cuvette containing $^{TR}R_{II}$.^{CF}C and cAMP the donor emission increased an additional 15% while acceptor emission declined by a further 25%, indicating an additional reduction of energy transfer between donors and acceptors and a greater increase in the average distance separating the $^{TR}R_{II}$ and ^{CF}C subunits (Figure 4).

To further characterize the heterochromatic holoenzyme, the concentration dependence of cAMP-induced dissociation of this construct was monitored as a reduction in acceptor sensitization, and the results are illustrated in Figure 5.

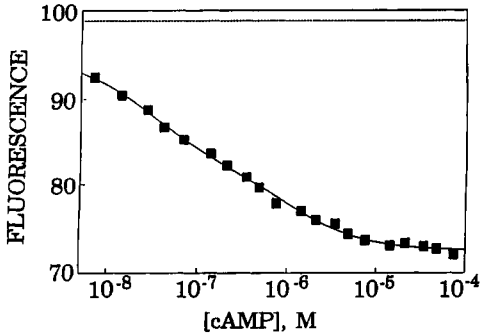

FIGURE 5: Concentration dependence of cAMP-induced reduction in acceptor sensitization. $^{TR}R_{II}$·^{CF}C was prepared as described in Materials and Methods. The sample was diluted to 20 nM with 50 mM MES, pH 6.8, 5 mM β-mercaptoethanol (final volume 0.35 mL) prior to steady-state fluorescence measurements. cAMP titration was initiated by the addition of increasing concentrations of the nucleotide. Decreased acceptor sensitization as a result of apparent holoenzyme dissociation was monitored at an excitation of 470 nm and emission of 615 nm.

Incremental amounts of cAMP were titrated into a solution of $^{TR}R_{II}$·^{CF}C, and the samples were excited at 470 nm and the emission monitored at 615 nm. The EC_{50} for cAMP to induce a change in energy transfer between the subunits of the heterochromatic holoenzyme ($^{TR}R_{II}$·^{CF}C) was 90 nM, a value very similar to that for the cAMP-dependent activation of this species (Figure 2B). This equal cAMP sensitivity is an indication that both the fluorescence measurement and the increased catalytic activity change are reflecting the same changing interaction between the derivatized regulatory and catalytic subunits. The decrease in the energy transfer from the donor ^{CF}C to the acceptor $^{TR}R_{II}$ upon cAMP addition reflects an increase in distance between the derivatization sites on the two subunits. However, even with saturating amounts of cAMP, significant $^{TR}R_{II}$ emission remains with 470 excitation (data not shown), which is far greater than what would be expected if there was no energy transfer. cAMP addition thus appears to have diminished the energy transfer between the donor ^{CF}C and the acceptor $^{TR}R_{II}$ but not to have fully abolished it, as would have been expected if the heterochromatic protein kinase holoenzyme had been totally dissociated by cAMP into its component R and C subunits. This was investigated further by lifetime rather than steady-state fluorescence measurements.

Donor Lifetime Measurements. We extended these studies of the interaction between the subunits in the heterochromatic holoenzyme by an analysis of donor lifetimes, because the lifetime reduction method to measure dipolar energy transfer is more quantitative and less susceptible to extraneous factors, e.g., pH changes produced by added reagents. Given the amine reactivity of the fluorescent labels utilized in this study and the numerous amines on each subunit potentially available for derivatization, more than one site on each subunit can be expected to be labeled and, therefore, more than one distance between donors and acceptors on adjacent subunits should be anticipated. The heterogeneity of interlabel distances will produce a distribution of fluorescence lifetimes, as energy transfer occurs with varying efficiencies between the different site of labeling. Because of this, the fluorescence decays were fit to a unimodal Gaussian distribution function. The exchange of electronic excitation energy between otherwise well-separated electronic systems will reduce the fluorescence lifetime of the donor, and in a system with multiple distances between donors and acceptors an increase in the width of the distribution of lifetimes is also to be expected.

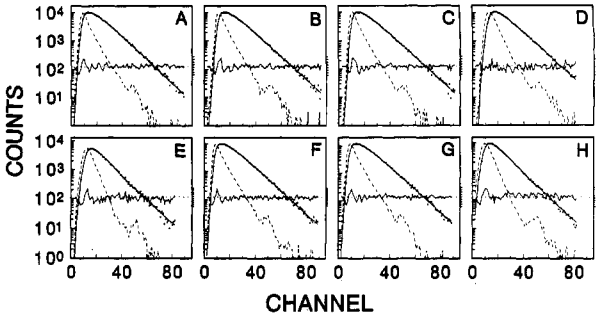

FIGURE 6: Fluorescence decay curves of free donor (^{CF}C) and donor–acceptor pair ($^{TR}R_{II}$·^{CF}C) in the presence and absence of cAMP and PKI. Panels A, B, C, and D: free ^{CF}C (200 nM) incubated with no other additions (A), with 140 μM cAMP (B), 300 nM PKI, and 140 μM cAMP (C), or 30 μM (Ser 21)PKI(14–22)-amide. Panels E, F, G, and H: $^{TR}R_{II}$·^{CF}C (200 nM) incubated with no other additions (E), with 140 μM cAMP (F), 300 nM PKI, and 140 μM cAMP (G), or 30 μM (Ser 21)PKI(14–22)-amide. The smooth lines through experimental data were generated with best-fit parameters given in Table II. The shape of the lamp pulses is shown as dashed lines. Weighted residuals are drawn across the center of each panel. Each channel is 0.422 ns. Details of the procedures are described in Materials and Methods. For E, F, G, holoenzyme was reconstituted in the presence of Mg^{2+} and ATP as described in Materials and Methods, leading to a concentration of 200 and 140 μM, respectively, under the conditions of the fluorescence experiments. For H, the Mg^{2+} and ATP was removed by gel filtration–centrifugation.

Plots of the fluorescence decays of the donor ^{CF}C are shown in Figure 6 with the calculated center and half-width of the distribution of lifetimes summarized in Table II. In the absence of acceptor ($^{TR}R_{II}$) the donor ^{CF}C fluorescence decay was well fit to a narrow unimodal Gaussian distribution with a center of the lifetimes at 4.3 ± 0.1 ns and a half-width <0.01 ns (six determinations). The addition of cAMP and PKI, either alone or together had no significant effect on the fluorescence decay rate indicating that neither reagent affects the fluorescence lifetime of the free catalytic subunit. As a component of the $^{TR}R_{II}$·^{CF}C holoenzyme complex, the center of the donor lifetime (^{CF}C) distribution was reduced to 3.7 ± 0.1 ns and the half-width increased to an average of 0.8 ns demonstrating energy transfer between the CF and TR fluorophores due to close proximity (i.e., association) of C and R subunits. The average efficiency of energy transfer by lifetime analysis was 0.14. This was somewhat less than the values determined by steady-state donor quenching (0.21) but the difference is due to the higher concentrations (5-fold) of samples used in the lifetime instrument, which is necessary to keep the data collection time reasonable. When lower concentrations of samples were used with the lifetime instrument and the data accumulation time appropriately lengthened, the energy-transfer efficiencies were about the same as those measured with the steady-state donor quenching method (data not shown). These concentration-dependent differences in energy-transfer efficiencies might reflect some concentration dependence of aggregation not detectable by traditional methods.

The addition of cAMP to the $^{TR}R_{II}$·^{CF}C holoenzyme complex resulted in a significant lengthening of the center of the lifetime distribution to 4.0 ± 0.1 ns and a narrowing of the distribution half-width to 0.4 ns. Importantly, however, even though the concentration of cAMP (140 μM) was >10-fold above the maximum required to either activate the protein kinase (Figure 2B) or alter the steady-state fluorescence (Figure 5), the center and half-width of the lifetime distribution did not return to the values of the free ^{CF}C (4.3 and <0.01 ns, respectively). If the only event modulating energy transfer was the dissociation of the holoenzyme to yield free ^{CF}C, then these

Table II: Unimodal Gaussian Analyses of the Effect of cAMP (140 μM), PKI (300 nM), and the High-Affinity Substrate (Ser 21)PKI(14–22)-amide (30 μM) on the Distribution of Fluorescence Decay of Carboxyfluorescein-Labeled C-Subunit (^{CF}C) from the cAMP-Dependent Protein Kinase[a]

		τ ± SD (ns)	half-width (ns)	n
A	^{CF}C	4.3 ± 0.1	<0.01	6
	^{CF}C + cAMP	4.2 ± 0.1	<0.01	6
	^{CF}C + PKI	4.3 ● 0.1	<0.01	6
	^{CF}C + cAMP & PKI	4.3 ± 0.1	<0.01	6
	$^{TR}R_{II}$.^{CF}C	3.7 ± 0.1	0.82	5
	$^{TR}R_{II}$.^{CF}C + cAMP	4.0 ± 0.1	0.40	5
	$^{TR}R_{II}$.^{CF}C + PKI	4.0 ● 0.1	0.50	3
	$^{TR}R_{II}$.^{CF}C + cAMP & PKI	4.3 ± 0.1	<0.01	5
B	$^{TR}R_{II}$.^{CF}C	3.7 ± 0.1	0.8	4
	$^{TR}R_{II}$.^{CF}C + (Ser 21)PKI(14–22)-amide	3.8 ± 0.2	1.0	3
	$^{TR}R_{II}$.^{CF}C + (Ser 21)PKI(14–22)-amide & cAMP	4.1 ± 0.1	0.5	3
	$^{TR}R_{II}$.^{CF}C + cAMP	4.1 ± 0.1	0.6	4

[a] For the experiments presented in A, holoenzyme was reconstituted in the presence of Mg^{2+} and ATP as described in Materials, leading to a concentration of 200 and 140 μM, respectively, under the conditions of the fluorescence measurements. For the experiment presented in B, the Mg^{2+} and ATP was removed by gel filtration–centrifugation.

data would indicate that the maximum degree of cAMP-induced dissociation was only ~50%. [This assumes that there is a linear relation between the amplitude of dissociation and relative energy transfer efficiency.]

To further characterize ligand modulation of the holoenzyme dissociation, the effects of PKI were examined. PKI specifically inhibits the catalytic subunit of cAMP-dependent protein kinase through competitive high-affinity binding to the substrate site (Ashby & Walsh, 1972 and 1973; Demaille *et al.*, 1977; Whitehouse & Walsh, 1983; Knighton *et al.*, 1991b). When the ^{CF}C subunit was free in solution, PKI had no effect on either the center or the half-width of the fluorescence lifetime distribution (4.3 ± 0.1 and <0.01 ns, respectively; Table II). Addition of PKI to the donor–acceptor pair ($^{TR}R_{II}$.^{CF}C) lengthened the center of the lifetime distribution to 4.0 ± 0.1 ns (Table II, section A) and narrowed the distribution half-width to 0.5 ns (three determinations), about the same extent as when cAMP was used alone. When both cAMP and PKI were added to solutions of $^{TR}R_{II}$.^{CF}C, the center and half-width of the donor lifetime distribution increased to values identical to those observed for free ^{CF}C (4.3 ± 0.1 and <0.01 ns, respectively; five determinations), indicating an apparent full dissociation of the holoenzyme.

Assuming a Förster critical distance of 51.3 Å and adding or subtracting one-half of the half-width of the lifetime distributions to calculate the range of energy-transfer efficiencies, the range of the average intersite distances for the $^{TR}R_{II}$.^{CF}C alone is 62–85 Å, while those for the heterochromatic holoenzyme plus cAMP or plus PKI are, respectively, 72–96 and 71–107 Å. Using the center of the lifetime distributions and a value of 51.3 Å for the Förster critical distance, the average distances between donor and acceptor increased ~10 Å when either cAMP or PKI was added to $^{TR}R_{II}$.^{CF}C. Because no energy transfer could be detected when both cAMP and PKI were added to the $^{TR}R_{II}$.^{CF}C complex, and because the resolution of the lifetime instrument is only 0.1 ns, the average distance between donors and acceptors when both cAMP and PKI are present would be >96 Å.

Given the results obtained with PKI, we have also examined the effect of a protein kinase substrate. The substrate chosen was (Ser21)PKI(14–22)-amide, which is the most potent substrate for this protein kinase so far detected with K_m (0.14 μM) and V_{max} values 30-fold lower and 2-fold higher, respectively, than for the well-characterized substrate Kemptide (Glass *et al.*, 1989). The concentration of (Ser21)PKI-(14–22)-amide used for these experiments (30 μm) is 200-fold greater than the K_m value, which, from a comparison of the K_m and K_d values of Kemptide (Whitehouse *et al.*, 1983), should be more than sufficient to saturate the kinase. To avoid formation of phosphorylated substrate which would no longer interact with ^{CF}C (Glass *et al.*, 1989), it was necessary to eliminate the ATP from the heterochromatic holoenzyme. This was accomplished by using a "minicolumn" centrifugation/gel filtration procedure (Methods), which decreased ATP levels to less than 1 μM. Identical results were obtained with either preparation of the heterochromatic holoenzyme. This was so whether the preparations were used alone, plus cAMP, plus PKI, or plus both (not shown). In the absence of MgATP, (Ser21)PKI(14–22)-amide addition did not mimic the results obtained with PKI (Table II, section B). It had no effect on the energy transfer of the holoenzyme alone and did not modulate the effects observed with cAMP. (Ser21)-PKI(14–22)-amide differs from the PKI in three significant ways. First, it lacks Phe-10, a key binding determinant for PKI but not PKI-based substrates (Mitchell, Glass and Walsh, unpublished). Second, the peptide, but not PKI, possesses a seryl substrate hydroxyl which has been proposed to be an important substrate recognition determinant as well as being the phosphoryl acceptor (Reed *et al.*, 1985). Finally, the peptide lacks the rest of the PKI molecule that contributes at least 15% of the binding energy (Glass *et al.*, 1989).

Gel Filtration Analysis of the $^{TR}R_{II}$.^{CF}C Holoenzyme. The observations with both steady-state fluorescence and donor lifetimes and the apparent absence of cAMP-mediated dissociation raised the question of whether the derivatization of the two subunits in $^{TR}R_{II}$.^{CF}C might have affected the protein such that it was no longer comparable to native holoenzyme (i.e., would not dissociate upon cAMP addition to yield separate subunits under nonequilibrium conditions). To test this, the heterochromatic holoenzyme was formed in the presence of a slight excess of ^{CF}C and chromatographed on a Sephacryl S-300 column (1 × 19 cm) in the absence or the presence of 100 μM cAMP. The column was monitored by fluorescence at 525 nm. As shown in Figure 7, $^{TR}R_{II}$.^{CF}C holoenzyme in the absence of cAMP gave two peaks eluting in the expected positions for holoenzyme and free catalytic subunit. Addition of cAMP fully dissociated the protein into free catalytic subunit. The peak fractions of holoenzyme (eluted – cAMP) and catalytic subunit (eluted + cAMP) were further evaluated by steady-state fluorescence. The emission spectra of the holoenzyme aliquot (left inset, Figure 7) indicated the presence of both $^{TR}R_{II}$ and ^{CF}C, and strong energy transfer between the two fluorophores. The inset also shows that the addition of cAMP (100 μM) diminished energy transfer as shown by

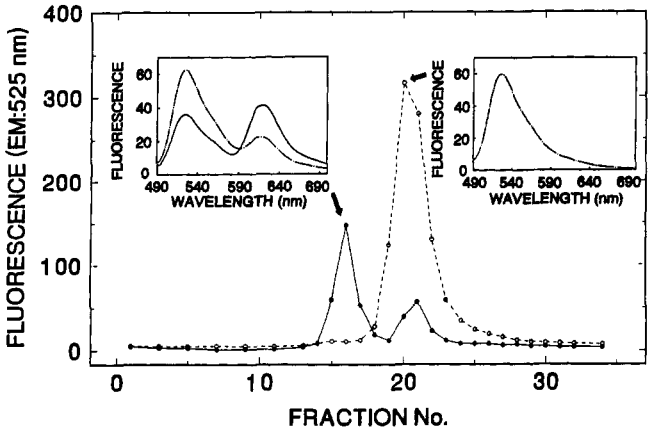

FIGURE 7: Sephacryl S-300 elution profile of $^{TR}R_{II}$.^{CF}C complex eluted in the absence and presence of cAMP. Heterochromatic holoenzyme was prepared with 0.27 nmol $^{TR}R_{II}$ and 0.34 nmol ^{CF}C as discussed in Materials and Methods and eluted in the absence (• – •) and presence (– – –) of cAMP (100 μM). The elution buffer was KH_2PO_4 (20 mM), β-mercaptoethanol (5 mM), EDTA (1 mM), pH 6.8; cAMP (100 μM) was added to this as required. Left inset: steady-state emission spectra of the first peak fraction of the sample (arrow) that was eluted in the absence of cAMP. Spectra were recorded before (solid line) and after (dash line) the addition of cAMP (100 μM). Right inset: steady-state emission spectrum of the peak fraction of the sample (arrow) that was eluted in the presence of cAMP.

the ~50% enhancement of emission from CF and a comparable reduction of emission from TR. These data further confirm the presence of holoenzyme in this fraction. The spectrum of the catalytic subunit peak (right inset, Figure 7) was clearly that of ^{CF}C alone. These sets of results show that $^{TR}R_{II}$.^{CF}C will completely dissociate when subjected to physical conditions that yield free subunits, as has been frequently demonstrated for native holoenzyme (see references in the Introduction).

DISCUSSION

In the studies described here a heterochromatic cAMP-dependent protein kinase has been prepared that by the several criteria tested is closely equivalent to underivatized native holoenzyme. The interaction between the two derivatized subunits appears to have been fully preserved, as evidenced by the equal titration of ^{CF}C activity by $^{TR}R_{II}$ and of C by R, (Figure 2A), and also by the very close similarity of cAMP-dependency for activation of each form of holoenzyme (Figure 2B and Figure 5). Both ^{CF}C and native C were equally titrated by PKI reflecting the integrity of the catalytic site. Many studies of the native holoenzyme have shown that when, after cAMP addition, it is then subjected to mild nonequilibrium conditions, the R and C subunits can be separated (Reimann *et al.*, 1971; Tao *et al.*, 1970; Brostrom *et al.*, 1971; Corbin *et al.*, 1972; Rubin *et al.*, 1972; Beavo *et al.*, 1974; Dills *et al.*, 1976; and many studies subsequently). This same property is exhibited by the $^{TR}R_{II}$.^{CF}C holoenzyme (Figure 7). Thus, by these criteria $^{TR}R_{II}$.^{CF}C mimics native enzyme, and its integrity does not appear to have been compromised by the derivatization procedures. The extent of derivatization is reasonable with a level of only 0.5–1.5 mol of fluorophore per subunit.

This study represents the first assessment of the changes in holoenzyme structure induced by cAMP under strictly equilibrium conditions. When assessed by steady-state fluorescence (Figure 5) or fluorescence lifetimes (Figure 6 and Table II), energy transfer between the CF-labeled catalytic subunits (donor) and TR-labeled regulatory subunits (acceptor) was readily apparent. The addition of cAMP resulted in two major consequences. First, there was a clear decrease in energy transfer between the two subunits reflected by a decline in both donor quenching (525 nm) and acceptor sensitization (615 nm), and these changes occurred with the same cAMP-dependency as did enzyme activation (Figures 2B and 5). Second, even with a maximum amount of cAMP a significant degree of energy transfer remained (Table II). Three possibilities could account for the presence of significant energy transfer even upon maximum cAMP addition:

(1) Under the conditions of the fluorescence experiments only a portion of the protein kinase dissociates into free $^{TR}R_{II}$ and ^{CF}C subunits.

(2) cAMP promotes the formation of a partially dissociated intermediate, which is the end point under equilibrium conditions. The further dissociation to form free subunits only occurs when the protein is subjected to one of the reported nonequilibrium conditions.

(3) Under equilibrium conditions, cAMP promotes a conformational change in the heterochromatic protein kinase, but not its dissociation into free subunits.

Alternative 1, despite being compatible with observed results under non-equilibrium conditions appears untenable. As noted, the steady-state fluorescence changes exhibited a cAMP dependence equal to that of enzyme catalytic activation and had clearly reached a maximum level of change at the highest cAMP concentration examined (Figure 5). Further, the fluorescence donor lifetime experiments (Table II) were undertaken with a cAMP concentration that was more than 10-fold higher than this maximum value. Very clearly in these lifetime determinations, despite this maximum amount of cAMP, the holoenzyme did not attain the characteristics of 100% free C subunit. If the changes in donor lifetime reflect only the dissociation of the protein kinase to free subunits, the degree of dissociation would be only ~50%. It is not consistent with a simple cAMP-mediated dissociation mechanism for the effects of cAMP to be maximal for both the change in energy transfer and protein kinase activation but for dissociation to have only attained ~50%.

Alternative 2 merits scrutiny. A ternary species of R_2-$CcAMP_2$ has been described by Connelly *et al.* (1986) in studies of the reassociation of isolated catalytic and regulatory subunits. If, under the equilibrium conditions present in the fluorescence experiments, cAMP addition were to lead solely to the formation of this complex, it would lead to a decrease in energy transfer in the presence of a maximal concentration of cAMP but not its elimination (as was observed, Table II). The formation of a trimeric species with cAMP addition would also provide a suitable explanation of the results obtained with PKI. PKI addition to a trimeric R_2CcAMP_2 plus C mixture would most likely lead to further dissociation (and in consequence further decrease in energy transfer) by binding to the free catalytic subunit and promoting a shift of the equilibrium in favor of further dissociation of the trimeric complex. This would then result, as was observed, in a total elimination of energy transfer in the presence of PKI plus cAMP but not with cAMP alone (Table II). The formation of this trimeric species, however, is incompatible with well-established data on the specific enzymatic activity of holoenzyme and free catalytic subunit. In particular as reported by Hofmann (1980) and Schewechheimer and Hofmann (1977) but also with extensive data from other investigators, the specific activity of (holoenzyme + cAMP) is identical to that of free catalytic subunit (+ or – cAMP). Further, if the

reassociation of R and C is examined, an identical specific activity is attained following reassociation upon addition of cAMP, as is exhibited by the original catalytic subunit prior to regulatory subunit addition. (All specific activities are quoted per mole of catalytic subunit.) Were, in contrast, the trimeric species R_2C to be the end product of holoenzyme activation by cAMP, the specific activity obtained would be only one-half, that of initial free catalytic subunit, since the activity of the trimeric species is still cAMP-dependent (Connelly *et al.*, 1986). This would appear to eliminate the possibility that this trimeric species is the end point of holoenzyme activation under the equilibrium conditions of the fluorescence experiments reported. Other partial intermediates, in addition to that described by Connelly *et al.* (1986) can also be envisioned; thus, this alternative cannot be totally excluded.

Alternative 3 appears to be the most likely explanation for the observations of this report. Supported by studies using a variety of approaches (Builder *et al.*, 1980; Chau *et al.*, 1980; Armstrong & Kaiser 1978; Tsuzuki & Kiger, 1978), the activation mechanism of both type I and type II cAMP-dependent protein kinase has been proposed to occur via the formation of an $R_2C_2cAMP_4$ intermediate species in accord with eq 7; with the possibility that an inactive $R_2C_2cAMP_2$

$$R_2C_2 + 4cAMP \rightleftharpoons R_2C_2cAMP_4 \rightleftharpoons R_2cAMP_4 + 2C \quad (7)$$

might precede the formation of the $R_2C_2cAMP_4$ complex (Cobb *et al.*, 1987). Both the lifetime and steady-state fluorescence data are fully compatible with the possibility that under equilibrium conditions cAMP produces the species $R_2C_2cAMP_4$, in which there has been a conformational rearrangement of subunits to increase the distance between the fluorophores, but not a full dissociation into the individual subunits. Therefore, under equilibrium conditions with cAMP addition alone the end point of the reaction would be $R_2C_2cAMP_4$, and this would account for why energy transfer was diminished by cAMP addition but not eliminated, even at very high cyclic nucleotide concentrations (Table II). The possibility of a stable holoenzyme conformation in which saturating concentrations of cAMP did not dissociate holoenzyme has been previously suggested from NMR spectroscopy studies (Granot *et al.*, 1980) but apparently not further investigated. This extended holoenzyme would be much more weakly complexed than unliganded holoenzyme. The fluorescence studies allow an estimate of the extent of conformational change induced by cAMP. Assuming that energy transfer occurs only between single donor–acceptor pairs and that the Förster distance (R_0) is 51.3 Å, then, from eq 1, the extent of this conformational rearrangement would involve about a 10-Å increase in the donor–acceptor distance.

The effects of PKI alone on the energy transfer of the heterochromatic holoenzyme and the production of fully dissociated protein kinase in the presence of cAMP are of particular interest. A considerable amount of information is now available about the mode of interaction of PKI with the protein kinase catalytic site. This is principally obtained from studies of substituted peptides (Glass *et al.*, 1989; Walsh *et al.*, 1990) and their solution conformation (Reed *et al.*, 1989), and from the resolution of the 3D structure of the binary catalytic subunit-PKI(5–24) complex (Knighton *et al.*, 1991a,b). Binding of PKI to C is derived primarily from two sites, the pseudo-substrate domain containing Arg-15, -18, and -19 and Ile-22 and also the α-helical domain containing Phe-10. Specific binding sites for each of these residues have been identified (Knighton *et al.*, 1991b). The binding site on C for R overlaps with that for PKI and includes the recognition determinants for the residues of the pseudosubstrate domain. It does not include the hydrophobic cleft that binds PKI-Phe-10 but does include an additional region composed of Lys-189, -213 and -217, Gly-193, Arg-194, and Trp-196 of the catalytic subunit that has been proposed as a unique recognition site for R (Gibbs *et al.*, 1992). As evidenced by the total elimination of energy transfer between the fluorophores and the full restoration of the fluorescence characteristics of free C (Table II) PKI, in the presence of cAMP, quite clearly promotes the full dissociation of the holoenzyme to free subunits. The binding of PKI to C promotes a conformational change in C (Mitchell *et al.*, 1990) and quite possibly it is this which eliminates the residual binding of R that is still occurring in the presence of cAMP alone. The binding of PKI to $R_2C_2cAMP_4$ that is responsible for these changes might involve either the pseudosubstrate binding site, the Phe-10 binding cleft, or both, and this remains to be resolved.

Also to be resolved is whether or not the $R_2C_2cAMP_4$ complex, as detected by these fluorescence energy-transfer experiments is catalytically active. Since the high-affinity substrate (Ser21)PKI(14–22)amide did not cause a change in energy transfer nor increase the conformational changes induced by cAMP and since these results were obtained with a maximal level of cAMP (thus reflecting the endpoint of cAMP action), then either the $R_2C_2cAMP_4$ complex is active or there is some important difference between the conditions of the fluorescence experiments and those under which protein kinase activity is assayed. Two important differences do, however, exist. First, the second substrate MgATP is present in the assay but cannot be present under the conditions where fluorescence energy transfer is measured since it would lead to the rapid removal of peptide and the formation of phosphopeptide. Although MgATP addition did not effect the changes observed with PKI alone or plus cAMP, it might still be required for a substrate-promoted event since even though (Ser21)PKI(14–22)amide is a very high affinity substrate, it still binds to the protein kinase with only a fraction of the binding energy as does PKI. The second major difference between the two sets of conditions is that the activity assay is conducted at dilutions of protein kinase 100-fold or higher than the measurements of fluorescence and that dilution would promote dissociation of weakly associated species.

A recent observation of Adams *et al.* (1991), is very suggestive that $R_2C_2cAMP_4$ is indeed active. In their experiments cells were microinjected with heterochromatic enzyme (fluorescein-labeled C–tetramethyl-rhodamine R_I and then subjected to repeated cycles of changes in intracellular cAMP. If activation of the protein kinase had been occurring via dissociation then the expected result would have been a scrambling between exogenous labeled and endogenous unlabeled subunits, leading to diminished energy transfer. This scrambling however did not occur, and although Adams *et al.* (1991) gave no explanation for their results, they are what would be predicted to occur if the end product of cAMP stimulation was the $R_2C_2cAMP_4$ complex rather than the free subunits. (The holoenzyme evaluated in these current studies was constituted with phospho-R_{II}. It remains to be determined whether similar results will be obtained with holoenzyme containing either dephospho-R_{II} or R_I.)

It would not be unreasonable for $R_2C_2cAMP_4$ to be active. The 10-Å change that occurs upon cAMP addition could have resulted in an opening up of the catalytic site, leading to active

enzyme without dissociation. Such an activation mechanism is similar (if not identical) to that envisioned for the activation of the cGMP-dependent protein kinase where dissociation into regulatory and catalytic subunits is not possible. If $R_2C_2cAMP_4$ is catalytically active this would better fit the proposal, for which there is now substantial evidence, that the R_{II} subunit serves to target specific substrates for phosphorylation (Scott *et al.*, 1990). Also of note, a tetrameric active cAMP-dependent holoenzyme has been obtained by mutation of the regulatory subunit in one of the regions of its binding to catalytic subunit (Wang *et al.*, 1991). An important aspect of the fluorescence experiments reported here is that the lifetime experiments (Table II) were undertaken at the lower end of the concentrations of the cAMP-dependent protein kinase found physiologically (Hofmann *et al.*, 1977) and the steady-state measurements at one-fifth to one-tenth lower concentrations (Figure 4). If active $R_2C_2cAMP_4$ occurs under these conditions, then it would be highly likely that it would also occur physiologically.

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