Crosstalk between Domains in the Regulatory Subunit of cAMP-Dependent Protein Kinase: Influence of Amino Terminus on cAMP Binding and Holoenzyme Formation†

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ABSTRACT: The regulatory (R) subunit of cAMP-dependent protein kinase is an asymmetric multidomain protein with a dimerization domain at the N-terminus, an autoinhibitor site, and two cAMP binding domains at the C-terminus. Activation of the tetrameric holoenzyme is mediated by the cooperative binding of cAMP to the two cAMP binding sites. To better understand how the various domains influence each other, the N-terminus (Δ1–91) up to the autoinhibitor site was deleted. Not only did this monomeric deletion mutant, purified from *Escherichia coli*, still bind cAMP and the catalytic (C) subunit with high affinity, holoenzyme formation was actually accelerated by at least 50-fold. MgATP also was not required for rapid reassociation of (Δ1–91)R(cAMP)$_2$ and C. The K_d(cAMP) and the K_a(cAMP) were similar to those for holoenzyme formed with full-length R; however, cooperativity was lost. Thus the N-terminus, either by inter- or intraprotomer contacts, not only impedes holoenzyme formation but also influences the cooperative binding of cAMP. The 1–91 deletion also renders the remaining fragment resistant to proteolytic degradation. Finally, unlike full-length R, the mutant protein can migrate freely into the nucleus. Surface plasmon resonance studies for the first time enabled direct measurements of the association and dissociation rate constants both for the intact R and for (Δ1–91)R. Both displayed very fast on-rates (1×10^{-5} M^{-1} s^{-1} and 1.1×10^{-5} M^{-1} s^{-1}, respectively) and extremely slow off-rates (2.3×10^5 M^{-1} and 4.3×10^5 M^{-1}, respectively). Thus, unlike the heat-stable protein kinase inhibitor, the region preceding the autoinhibitor site in R does not contribute in a quantitatively significant way to the high-affinity binding of C.

cAMP-dependent protein kinase (cAPK)[1] exists as an inactive holoenzyme complex composed of two monomeric catalytic (C) subunits and a dimeric regulatory (R) subunit in the absence of cAMP. In response to nanomolar concentrations of cAMP this heterotetramer dissociates into an R subunit dimer and two active C subunits. The R subunits of cAPK can be divided into two major groups, type I and type II, distinguished by the order of elution of their corresponding holoenzymes from DEAE-cellulose (Corbin et al., 1975), amino acid sequence (Takio et al., 1984; Titani et al., 1984), and antigenicity (Kapoor et al., 1979; Mumby et al., 1985). Two genes have been identified in each category of R subunit (Clegg et al., 1988; Jahnsen et al., 1986; Lee et al., 1983; Scott et al., 1987).

Despite these distinguishing features, both R subunits have similar domain structures that include two tandem cAMP binding domains at the C-terminus, referred to as site A, showing a fast dissociation site for cAMP and a preference for N^6-substituted analogs of cAMP, and site B, showing a slower dissociation rate and a preference for C^8- and C^2-substituted analogs of cAMP (Øgreid et al., 1989). In both cases the N-terminus of the R subunits contains a site of dimer interaction, with the two protomers of the type I R subunit linked by two interchain disulfide bonds (Bubis et al., 1987; Zick & Taylor, 1982). It was recently demonstrated that the $R^I\beta$ even forms disulfide-bonded heterodimers with $R^I\alpha$ *in vivo* (Tasken et al., 1993). All of the R subunits also contain a hinge region displaying the consensus recognition sequence for cAPK between residues 90 and 100 (Figure 1). In the free R subunit this region is very sensitive to proteolysis, but in the holoenzyme it is protected (Potter et al., 1978; Rannels et al., 1985). There are two conserved arginines in the hinge region. On the basis of site-specific mutagenesis of both the R^I and the R^{II} subunits at least one is essential for tight binding to the C subunit (Buechler et al., 1993; Wang et al., 1991). Proteolysis N-terminal to these two arginines yields a protein capable of recombining with the C subunit to form holoenzyme, but cleavage after the arginines renders the enzyme incapable of forming a high-affinity complex with the C subunit (Mumby et al., 1985; Weber & Hilz, 1979; Weldon et al., 1983).

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[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; C, catalytic subunit of cAPK; cAMP, adenosine 3′,5′- cyclic monophosphate; cAPK, cAMP-dependent protein kinase; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; FITC, fluorescein 5-isothiocyanate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PKI, heat-stable protein kinase inhibitor; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MW, molecular weight; NH, *N*-hydroxysuccinimide; R, regulatory subunit of cAPK; RU, response units (=1000 ng/mm^2); SDS, sodium dodecyl sulfate.

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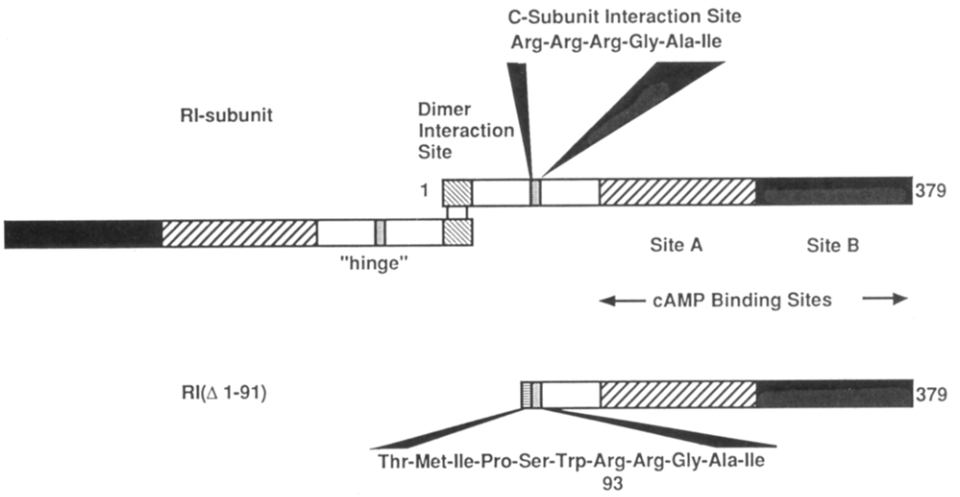

FIGURE 1: General domain structure of the R^I/R^{II} subunits. Cartoon of the dimeric R subunit of cAMP-dependent protein kinase. The deletion of the first 91 amino acids causes a monomeric but biologically fully functional R subunit with a calculated molecular weight of 33 040.

In order to study the functional importance of the residues N-terminal to the consensus recognition sequence, the biochemical properties of a single-deletion mutant (Δ1–91) of the R^I subunit was compared to those of the wild-type R subunit. Particular attention focused on interactions with the C subunit in the absence and presence of MgATP and on changes in cAMP binding. A novel technology using surface plasmon resonance (SPR) was used for the characterization of R/C interactions. In this method the binding of an analyte to a ligand immobilized to a (carboxymethyl)dextran surface is measured in real time. SPR, the optical technique utilized in the used Biacore instrument, measures changes in refractive index in the vicinity of the dextran surface and allows us to correlate these changes directly to the association and dissociation rates of the analyte (Karlsson et al., 1991).

EXPERIMENTAL PROCEDURES

Reagents. The peptide substrate LRRASLG was obtained from the UCSD Peptide and Oligonucleotide Facility and purified by reverse-phase HPLC. ATP was purchased from Sigma, [γ-^{32}P]ATP(3000 Ci/mmol) was purchased from Amersham, and [^{3}H]cAMP was purchased from New England Nuclear. Before use, [^{3}H]cAMP was purified on poly(ethyleneimine)–cellulose (Hofmann, 1985). PMSF was purchased from Boehringer Mannheim. Nitrocellulose filters (BA 85, 0.45 μm) were from Schleicher & Schuell. Materials used in cloning were molecular biology grade. Enzymes used in DNA manipulations were obtained from either Boehringer Mannheim or New England Biolabs.

Mutagenesis. The 1–91 deletion was introduced into the N-terminus of the R^I subunit according to Ringheim and Taylor (1990) using a pUC 19 cloning vector. The coding sequence for the first 91 amino acids was removed from the 5′-end of the R^I cDNA by digestion with *Nae*I and *Hin*dIII. The ends were filled in with Klenow fragment and then subjected to blunt-end ligation. As a result of the construct design, Arg92 was replaced with Trp. The resulting vector coded for an in-frame β-galactosidase fusion protein, (Δ1–91)rR,[2] with the sequence Thr-Met-Ile-Thr-Pro-Ser-Trp at the N-terminus of the R^I sequence starting at Arg93.

Purification. Following overexpression in *Escherichia coli* (Slice & Taylor, 1989), the rC subunit was first purified by phosphocellulose chromatography (P11 Whatman) and then resolved into discrete isoelectric variants on a Mono S HR10/10 column using FPLC (Pharmacia/LKB) (Herberg et al., 1993). Isozymes I and II were used for these experiments. The purity of the protein was checked by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), isoelectric focusing (Righetti, 1983), and analytical gel filtration (see below). The specific activity of the rC subunit was 25 μmol/(min·mg) as measured by the coupled spectrophotometric method of Cook et al. (1982) using the heptapeptide, LRRASLG, as a substrate. The purified rC subunit was stored at 4 °C in 20 mM potassium phosphate, 110 mM KCl, and 5 mM 2-mercaptoethanol, pH 7.0.

The rR^I subunits were overexpressed in *E. coli* E222 and purified as described previously (Saraswat et al., 1986). The R^I subunits [wild-type and (Δ1–91)] were purified by ion-exchange chromatography on DEAE-cellulose (Buechler & Taylor, 1991). The R^I subunits were frozen in 20 mM potassium phosphate, 2 mM EDTA, 30% glycerol, and 5 mM 2-mercaptoethanol, pH 6.5, and stored at −20 °C. The deletion mutant was extremely stable against proteolysis, showing a single band in SDS–PAGE after storage for 1 year at 4 °C. In contrast, the full-lenght rR subunit breaks down readily within days if not kept frozen under the conditions described above.

To obtain cAMP-free R subunit, the R subunits were unfolded with 8 M urea as described by Buechler et al. (1993). While the rR subunit can be denatured in 8 M urea and then refolded readily at room temperature, attempts to refold the deletion R subunit under these conditions yielded a predominantly insoluble protein. Precipitation occurred when dialyzed against urea concentrations less than 5 M. To strip cAMP from the mutant R subunit, denaturation was carried out at 8 M urea at a protein concentration of 1 mg/mL or less, and refolding was done at 4 °C. Under these conditions less than 5% of the protein aggregated, as shown by analytical gel filtration. However, this stripped protein tended to precipitate again when stored at room temperature at concentrations higher than approximately 1–2 mg/mL. Addition of cAMP stabilized the deletion R subunit, yielding a protein indistinguishable in its solubility from wild-type protein.

[2] rR refers to the recombinant R subunit expressed in *E. coli* from the pLST vector (Saraswat et al., 1986); (Δ1–91)rR is the deletion mutant described here.

Sequencing. Amino acid sequencing was performed on a gas-phase sequencer (Applied Biosystems 470A) with an on-line PTH analyzer (Applied Biosystems 120A).

Sucrose Gradients. The $s_{w,20}$ values were determined on 5–15% sucrose density gradients. Centrifugation was carried out at 65 000 rpm for 108 min using a Beckman VTi 65.1 rotor. Proteins (25–250 mg) and sucrose solutions were in 20 mM potassium phosphate (pH 7.0), 2 mM benzamidine, and 2 mM EGTA. The gradients were fractionated into 27 aliquots, and protein concentrations were measured either by the Bradford assay or by cAMP binding. The following internal marker proteins were used: phosphorylase *b* ($s_{w,20}$ = 8.2), γ-globulin ($s_{w,20}$ = 6.36), bovine serum albumin ($s_{w,20}$ = 4.2), horseradish peroxidase ($s_{w,20}$ = 3.67). and lysozyme ($s_{w,20}$ = 2.1).

cAMP Exchange Rates. Wild-type and mutant R subunits were incubated for 30 min at 30 °C in 50 mM MES, pH 6.9, 0.5 mM EGTA, 1 mM Mg–acetate, 10 mM NaCl, 10 mM DTT, 600 nM [^{3}H]cAMP, and 0.5 mg/mL BSA at a concentration of approximately 100 nM. After addition of cold cAMP to a final concentration of 100 μM, aliquots were taken at various time points ranging from 10 s to 90 min. The protein was precipitated in 3 mL of ice-cold 95% ammonium sulfate (10 mM HEPES, pH 7.0, 2 mM EDTA) and filtered over 0.45-mm pore size nitrocellulose filters (Døskeland & Øgreid, 1988). The filters were washed twice with 3 mL of ice-cold 70% ammonium sulfate (10 mM HEPES, pH 7.0, 2 mM EDTA) and subsequently shaken in scintillation vials with 2 mL of water for 20 min. The filters were then dissolved in 10 mL of scintillation fluid prior to counting.

cAMP Equilibrium Binding and cAMP Exchange. Equilibrium binding was carried out in a total volume of 200 μL in 50 mM MES, pH 6.9, 0.5 mM EGTA, 1 mM Mg–acetate, 10 mM NaCl, 10 mM DTT, 0.5 mg/mL BSA, and concentrations of [^{3}H]cAMP varying from 2 nM to 2.5 μM. The binding reaction was allowed to reach equilibrium at 30 °C for 30 min, and an aliquot was then removed and added to 3 mL of ice-cold 95% ammonium sulfate. The protein was filtered over nitrocellulose filters and the retained [^{3}H]cAMP determined as described above. Total cAMP binding was calculated by this method. In an identical second reaction the mixture was treated with an excess of cold cAMP to allow for complete exchange of the fast dissociation site before precipitating an aliquot in ammonium sulfate. The incubation time could be estimated from the individual rate constants and was 8 min for wild-type and 6 min for the deletion mutant R subunits, respectively. The cold cAMP chase allowed the calculation of the fraction of cAMP that bound to site B. The binding to site A was determined by subtracting the fraction of cAMP that bound to site B from the total bound cAMP. The apparent K_d values for each binding site were calculated by Scatchard analysis.

The stoichiometry of cAMP binding was determined from the amount of bound cAMP and the amount of R subunit added to the assay. Protein concentrations were determined according to Bradford (1976).

Holoenzyme Formation. Two methods were used to obtain holoenzyme. In method A, holoenzyme was formed in the presence of MgATP. The C and R^I subunits in a molar ratio of 1.2:1 were dialyzed for 24 h at 4 °C against 20 mM potassium phosphate, 100 mM KCl, 5 mM 2-mercaptoethanol, 5% glycerol, 100 μM ATP, and 1 mM $MgCl_2$, pH 6.5 (=buffer B).

In method B, holoenzyme without MgATP was obtained by mixing FITC-labeled rC subunit and cAMP-free (urea-stripped) wild-type R^I subunit in a molar ratio of 1.2:1 followed by incubation for 30 min at 22 °C.

To obtain kinetic values for the holoenzyme formation, 2 μM rR subunit and wild-type rC subunit were dialyzed in a molar ratio of 1:1.2 at room temperature for 24 h in buffer B. Aliquots were taken at different time points, and the remaining phosphotransferase activity was determined in the spectrophotometric assay according to Cook et al. (1982).

Holoenzyme formation with the mutant $R^I(\Delta 1–91)$ protein was measured additionally by combining 14 nM wild-type rC subunit and varying amounts of deletion R subunit in assay mix containing 1 mM ATP and 10 mM $MgCl_2$ and determining the decrease of phosphotransferase activity. Wild-type holoenzyme was measured under the same conditions.

Apparent Activation Constants (K_a) for cAMP. Holoenzyme at a concentration of 30 nM rC subunit was incubated for 5 min at room temperature in the assay mix described by Cook with varying concentrations of cAMP ranging from 1 nM to 10 μM. The reaction was initiated by adding Kemptide (100 μM), and the activity of the free rC subunit was followed using the spectrophotometric assay.

ATP Off-Rates. Binding studies with [γ-^{32}P]ATP were performed using the method described by Døskeland et al. for measuring the binding of cAMP (Døskeland & Øgreid, 1988). The protocol was modified as described in Herberg and Taylor (1993). After saturating the holoenzyme with [γ-^{32}P]ATP, the off-rate was measured after adding an 100-fold excess of cold ATP.

Analytical Gel Filtration. Analytical gel filtration was carried out using a Superose 12 HR10/30 column or a Superdex 75 HR10/10 column with flow rates of 0.8 mL/min at 22 °C in buffer A as described elsewhere (Herberg & Taylor, 1993). The percentage of holoenzyme at various protein concentrations was calculated on the basis of the peak areas corresponding to both holoenzyme and rC subunit.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) was used to study the interaction between the rC subunit and rR subunits of cAPK using an BIAcore instrument (Pharmacia/Biosensor). SPR was used to detect changes in mass in real time on a sensor chip surface (Karlsson et al., 1991), which was prepared by direct amine coupling of the rC subunit via primary amines to the CM dextran (Biosensor Amine Coupling Kit). After activating the surface of the chip for 2 min, 15 mL of protein was injected at 7 μg/mL in 10 mM sodium phosphate (pH 6.2) containing 1 mM ATP and 2 mM $MgCl_2$, and 425 RU's were immobilized (1000 RU = 1 ng/mm^2). Residual NHS esters on the sensor chip surface were reacted with ethanolamine (1 M in water, pH 8.5). Noncovalently bound protein was washed off with 500 mM NaCl. All binding interactions were performed at 23 °C in 20 mM sodium phosphate, 150 mM NaCl, and 1 mM EDTA at pH 7.2 or 20 mM sodium phosphate, 150 mM NaCl, 100 μM ATP, and 1 mM $MgCl_2$. To determine unspecific binding and bulk refractive index changes, blank runs were performed with 500 nM wild-type and deletion rR subunit using a nonactivated sensor chip. After injections of the rR subunits the rC subunit surface was regenerated by injection of 10 μL of 10 μM cAMP.

To immobilize the rR subunit, a surface was activated for 6 min, and protein was injected at 10 μg/mL in 10 mM acetate buffer (pH 3.8). A total of 1500 RU's of dimer were immobilized. Surface activity was calculated using the equation: $S = MW_L R_A / MW_A R_L$, where S is the stoichiometry, subscript L and A signify ligand (immobilized protein) and

Table 1: Biophysical Properties of the Recombinant Proteins

protein	partition coeff (σ)	MW (calcd)	MW (SDS–PAGE)	Stokes radius (R_S) (Å)	$s_{w,20}$ (S)	f/f_0	p*I* (IEF)
rC subunit	0.452	40 800	38 000	27.4 ± 0.2	3.2[a]	1.18	6.4/7.2/8.2
rR^I subunit	0.286	87 800	48 000	46.3 ± 0.3	4.7[a]	1.55	5.0
holoenzyme [$rR^I_2C_2$]	0.277	169 400	38 000/48 000	47.4 ± 0.5	6.7	1.27	nd
(Δ1–91)rR^I subunit	0.452	33 040	37 000	27.5 ± 0.2	4.1	1.27	4.5/4.6
holoenzyme [(Δ1–91)rR^IC]	0.367	73 840	38 000	36.7 ± 0.6	nd	1.30	5.1/5.3

[a] Data taken from Zoller et al. (1979).

analyte (injected protein), respectively, R = response in RU's, and MW = molecular weight.

Kinetic constants were calculated by linear regression of data using the BIAcore pseudo-first-order rate equation, $dR/dt = k_aCR_{max} - (k_aC + k_d)Rt$, where k_a is the association rate, k_d is the dissociation rate, C is the concentration of the injected analyte, and R is the response. Plots of dR/dt vs Rt have a slope of k_s. When k_s is plotted against C the resulting slope is equal to the k_a. k_d was calculated by integrating the rate equation when $C = 0$, yielding $\ln(Rt_1/Rt_n = k_d(t_n - t_1)$. Affinity constants were calculated from the equation $K_d = k_d/k_a$.

Association rate calculations were performed using concentrations between 30 and 500 nM for each rR subunit. The first 10 s of every sensogram was subtracted, correcting for the bulk refractive index change. Nonlinear curve fittings were attempted with Inplot software using the double-exponential equation $Y = A \times [1 - e^{(-BX)}] + C \times [1 - e^{(-DX)}]$, where A and C stand for the maximum number of binding sites for the first exponential and the second exponential phase, respectively, and B and D for the rate constants of both phases. A double-exponential equation yielded an excellent fit with an average R^2 of 0.999 whereas single exponential fits did not fit the observed data well (O'Shannessy et al., 1993) . The first (fast) association rate gave a similar rate constant as linear first-order replots. Dissociation rate constants were determined over a longer time scale (2 h) at a concentration of 500 nM rR subunits and were calculated by linear analysis.

Fluorescence Labeling and Microinjection. The rC subunit (0.5–1 mg/mL) was labeled with fluorescein 5-isothiocyanate (FITC, Molecular Probes) using a 35-fold molar excess of FITC. The labeling was carried out in 100 mM HEPES (pH 8.0), 5 mM $MgCl_2$, and 5 mM ATP at 22 °C. The reaction was stopped after 30 min by passing the reaction mixture through a prepacked Sephadex G-25 column (NAP 10 column, Pharmacia/LKB) equilibrated with buffer A and stored at 4 °C. This FITC-labeled rC subunit retained more than 95% of its initial phosphotransferase activity and was able to form holoenzyme with the R^I subunit in a manner that was indistinguishable from that of the unlabeled rC subunit.

The (Δ1–91)rR subunit also was labeled on cysteine residues with 5-(iodoacetamido)fluorescein (Molecular Probes) at a protein concentration of 2 mg/mL in a 36-fold molar excess of fluorophor over protein. The reaction was performed overnight at room temperature using the same buffers as for the labeling of the rC subunit. The labeling reaction was quenched by the addition of 5 mM 2-mercaptoethanol, and the free label was removed by passing the sample twice over a PD 10 buffer-exchange column. Under these conditions, 0.95 mol of FITC was incorporated per mole of R monomer. Under similar conditions, less than 0.1 mol of FITC/mol of R monomer was incorporated into the native full-length rR subunit. Microinjection experiments were performed in rat fibroblasts described elsewhere (Meinkoth et al., 1990). Fluorescein-labeled holoenzyme was formed according to method A.

RESULTS

Physical Properties of the Deletion Mutant. A monomeric form of the R^I subunit of cAPK was constructed by deleting the first 91 amino acids at the N-terminus and then fusing the remaining residues to a short segment of β-galactosidase (Figure 1). The resulting monomeric protein still contained the consensus recognition site (residues 94–98) and both tandem cAMP binding sites, A and B. This deletion mutant, (Δ1–91)rR, was expressed in *E. coli* and purified as described in the Experimental Procedures using DEAE chromatography as a single purification step. The N-terminal sequence of the protein was confirmed by sequencing the first 10 amino acids. The purified protein had an apparent molecular weight (appMW) of 37 000 based on SDS gel electrophoresis (see Table 1). This appMW was similar to the molecular weight of the rC subunit, which has an appMW of 38 000 according to SDS–PAGE, although the calculated MW's of the (Δ1–91)rR and the wild-type rC subunit are 33 190 and 40 800, respectively (Table 1). Holoenzyme formed with (Δ1–91)rR bound to carboxymethyl cation-exchange resins (CM-Sepharose, Pharmacia LKB) like the rC subunit even though the isoelectric point of the holoenzyme was 5.1–5.3. Neither the wild-type rR subunit nor R_2C_2 bound to CM-Sepharose.

On the basis of analytical gel filtration, the Stokes radius of the (Δ1–91)rR subunit was 28.0 Å. The sedimentation coefficient ($s_{w,20}$) for (Δ1–91)rR was 4.1 S, similar to that for the wild-type rR subunit ($s_{w,20}$ = 4.7 S). On the basis of the Stokes radius and the $s_{w,20}$, the frictional coefficient (f/f_0) was calculated as a gauge for dimensional asymmetry (Erlichman et al., 1973). The catalytic subunit with f/f_0 of 1.12–1.18 is a rather spherical protein (Erlichman et al., 1973; Zoller et al., 1979). In contrast, the full-length rR subunit displayed a significant degree of dimensional asymmetry (Table 1). The (Δ1–91)rR still showed a significant degree of asymmetry in comparison to C. The full-length holoenzyme showed a loss of dimentional asymmetry, whereas no change was observed for the mutant holoenzyme.

Isoelectric focusing of the (Δ1–91)rR subunit showed a doublet with p*I*'s of 4.5 and 4.6. In comparison, the rR subunit focused at a p*I* value of 5.0. The monomeric mutant holoenzyme resolved into two bands under nondenaturing conditions with p*I*'s of 5.1 and 5.3.

When unfolded in urea, the (Δ1–91)rR mutant behaved like the intact rR subunit. The N-terminus does not affect the overall stability of the (Δ1–91)rR subunit. The deletion mutant does, however, have a greater tendency to aggregate. Unfolding studies also revealed that cAMP is still bound to the purified protein, since the intrinsic fluorescence is quenched significantly, in contrast to rR subunit that has been stripped of cAMP (Leon and Taylor, in preparation).

cAMP Dissociation Rate and cAMP Equilibrium Binding. Deletion of the N-terminus clearly changed the kinetic parameters of the two cAMP binding sites. In the wild-type rR subunit the cAMP binding sites could be readily distinguished by their dissociation kinetics. When measuring

Table 2: Binding Data of the Wild-Type and (Δ1–91) Protein

	wild-type (dimer)	(Δ1–91) (monomer)
appk_{assoc}(BIAcore) (M^{-1} s^{-1})	1.0×10^5	1.1×10^5
appk_{diss}(BIAcore) (s^{-1})	2.3×10^{-5}	4.3×10^{-5}
appK_d(BIAcore) (M)	2.3×10^{-10}	3.9×10^{-10}
K_d(cAMP) (nM)	site A: 60	site A: 50
	site B: 15	site B: 50
Hill coeff, n	1.3–1.5	1.1
off-rates (cAMP) (min^{-1})	site A: 0.6	site A: 1.5
	site B: 0.02	site B: 35
K_a(cAMP) (nM)	105	110
Hill coeff, n	1.5–1.7	0.9–1.1
off-rates (ATP) (h)	11	10 (estimated)
app$K_d(R_2C_2)$		
+MgATP	$<5 \times 10^{-11}$ M	$\ll 1 \times 10^{10}$ M
-MgATP	12.5×10^{-8} M	1.5×10^{-8} nM

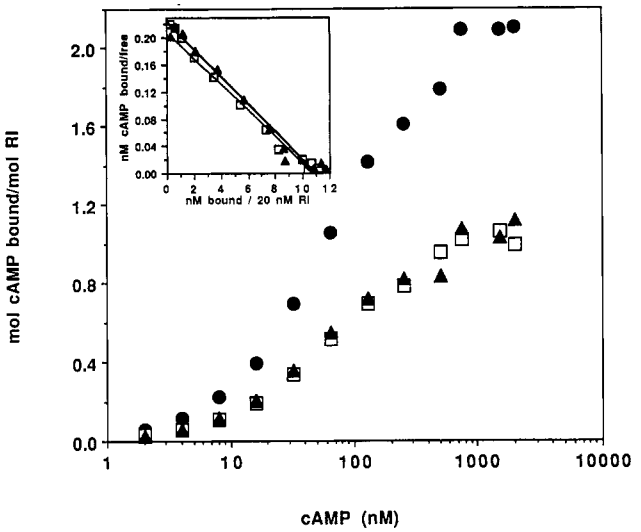

FIGURE 2: Equilibrium binding of cAMP to the (Δ1–91)rR-subunit. (●) cAMP binding site A+B; (□) cAMP binding site A; (▲) cAMP binding site B. The inset shows a Scatchard analysis of the data, yielding a single binding site with an affinity of 50 nM for both the A (□) and the B site (▲).

exchange kinetics in the presence of a 1000-fold excess of unlabeled cAMP at physiological salt conditions, a biphasic dissociation curve was observed for the rR subunit. The fast dissociation (K_{off} = 0.6 min^{-1}) was linked to site A while the slow site was identified as site B (K_{off} = 0.02 min^{-1}). The (Δ1–91)rR subunit still showed a biphasic off-rate similar to that of the wild-type protein, and this was indicative of two different and distinct sites (Table 2). However, the most notable difference compared to the full-length wild-type protein was observed when cAMP equilibrium binding to the mutant protein was measured. The appK_d values for the wild-type protein were determined as 60 nM and 15 nM for sites A and B, respectively. The native rR subunit and the (Δ1–91)rR subunit both bound approximately 2 mol of cAMP/mol of R monomer. However, when determining the K_d values for the mutant rR subunit, identical binding constants of 50 nM were found for both binding sites (Figure 2). Also, the Hill coefficients as a measure for the cooperativity of cAMP binding differed for both proteins. While cAMP binding to the wild-type rR subunit showed a positive cooperativity (n = 1.3–1.5), the (Δ1–91)rR subunit displayed noncooperativity for cAMP binding (n = 1.1).

Holoenzyme Formation. Holoenzyme formed with the (Δ1–91)rR subunit using method A (Experimental Procedures) yielded a stable, dimeric complex with a Stokes radius of 36.7 Å (Table 1).

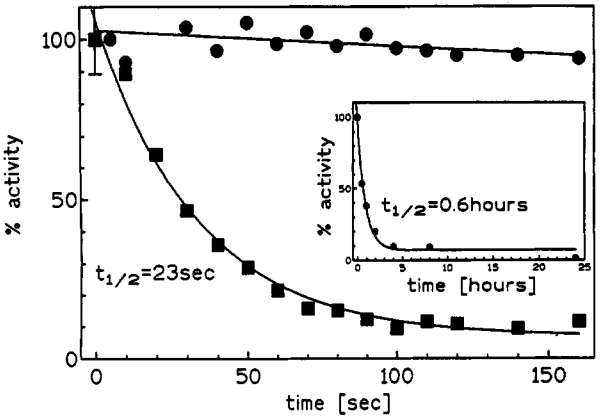

FIGURE 3: Holoenzyme formation with the mutant (Δ1–91)R^1 protein was too fast to measure using the dialysis method described in the Experimental Procedures. To determine the $t_{1/2}$ for the mutant holoenzyme, 14 nM wild-type rC subunit and 16 nM mutant rR subunit (■) were incubated in assay mix, and the decrease in phosphotransferase activity was monitored directly. The wild-type protein measured under the same conditions is shown as (●). In the case of the wild-type holoenzyme (inset) 2 μM rR subunit and rC subunit were combined in a molar ratio of 1:1.2 and dialyzed at room temperature. Aliquots were taken at time points as indicated in the inset.

To monitor the kinetics of holoenzyme formation, the reassociation of (Δ1–91)rR and the wild-type rR subunit with the wild-type rC subunit was measured using the spectrophotometric assay (Experimental Procedures). Figure 3 shows the formation of holoenzyme with (Δ1–91)rR in the presence of MgATP. Rapid inhibition of the activity of the rC subunit with a half-life of 20 ± 2.5 s was observed for the (Δ1–91)rR subunit in a 1:1 ratio of R subunit to C subunit. In a 2:1 ratio of rR subunit to rC subunit, a rate of 10 ± 1.5 s was observed. Within the duration of the assay (150 s) no more than 10% inhibition of the rC subunit was observed with the rR subunit. The inset of Figure 3 shows the holoenzyme formation of the wild-type rR subunit using method A. Under these conditions half maximal inhibition of the rC subunit was achieved after 36 min (Table 2).

To measure R/C complex formation in the absence of MgATP, analytical gel filtration with FPLC was used. FITC-labeled rC subunit (2 μM) was mixed with a 1.2 molar excess of (Δ1–91)rR subunit and immediately injected onto a Superdex 200 gel filtration as described previously (Herberg & Taylor, 1993). The column was equilibrated with buffer A containing 2 mM EDTA. A major fluorescence peak with a retention time of 19.05 min corresponding to holoenzyme and a shoulder at a retention time of 20.1 min corresponding to the free rC subunit could be detected (Figure 4, lower panel). Longer preincubation only marginally increased the amount of holoenzyme complex formed (data not shown). Under similar conditions the wild-type R, saturated with cAMP, and rC subunit did not form any holoenzyme complex (Figure 4, upper panel). Dialysis was required to remove the cAMP before a stable holoenzyme could be formed. When preformed wild-type holoenzyme was incubated with 100 μM cAMP, the complex dissociated completely, yielding a single fluorescent peak with the retention time of the free rC subunit as shown in Figure 4, upper panel. In contrast, when 100 μM cAMP was added to holoenzyme preformed with the deletion mutant prior to injection onto the Superdex 200 column, a majority of the fluorescence labeled protein was observed as holoenzyme, suggesting that holoenzyme re-formed very rapidly once cAMP was removed in the initial phases of the gel filtration procedure. Since the partition coefficients of

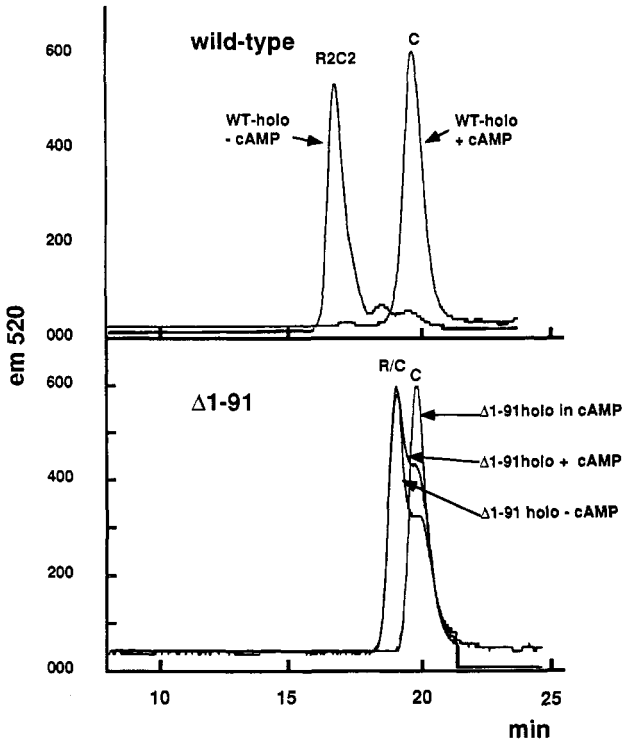

FIGURE 4: Gel filtration profiles of wild-type (WT) (upper panel) and mutant (lower panel) holoenzyme. 200 μL of holoenzyme (0.1 mg/mL) formed with FITC-labeled rC subunit were run on a Superdex 200 10/30 column in the absence (–cAMP) or presence (+cAMP) of 100 μM cAMP in the sample loop as indicated. The (Δ1–91) holoenzyme was additionally run in the same column equilibrated with 100 μM cAMP in the running buffer (in cAMP). The fluorescence profile shows a peak for the WT holoenzyme at 16.8 min, for the mutant holoenzyme at 19.05 min, and for the rC subunit at 20.10 min.

the (Δ1–91)rR subunit and the rC subunit were nearly identical, they could reassociate easily on the column once cAMP was retained. Only under conditions where the column was equilibrated with the same buffer containing 100 μM cAMP was the mutant holoenzyme complex was dissociated completely as shown in the lower panel of Figure 4. This was a strong indication that the R deletion mutant formed holoenzyme rather rapidly as soon as cAMP was removed from the buffer. It also suggested that cAMP was removed from the cAMP binding sites A and B and that, once removed, rC subunit competed effectively for the mutant R even in the absence of MgATP.

cAMP Activation. Activation of the wild-type and mutant holoenzyme was measured with proteins preformed by either method A or method B (Experimental Procedures). The appK_a for cAMP activation was not altered when holoenzyme was formed with (Δ1–91)rR subunit that was previously unfolded in urea and refolded (method B). The activation constant for the mutant holoenzyme was 110 nM [cAMP] and was similar to that for the wild-type holoenzyme (Figure 5). Although the K_a's were similar for both holoenzymes, the Hill coefficient showed that the holoenzyme formed with the (Δ1–91)rR subunit was significantly less cooperative, suggesting again that the kinetics of cAMP binding are influenced by the N-terminus (Table 2).

ATP Off-Rates. The off-rates of ATP from the holoenzyme complex were determined as described in Experimental Procedures using native and cAMP-stripped (Δ1–91)rR subunit. An extremely fast off-rate was monitored for the holoenzyme formed with the native (Δ1–91)rR subunit. This

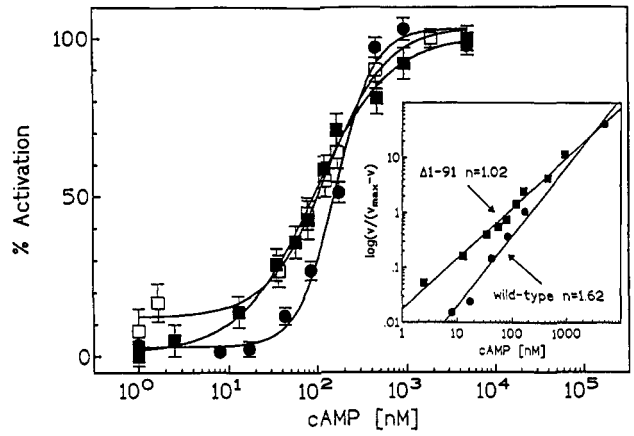

FIGURE 5: Activation of wild-type and mutant holoenzymes. Holoenzymes were preformed as described above [method A for WT protein (●), by combining untreated mutant rR subunit (■) or cAMP-free mutant protein (□) with rC subunit]. The holoenzymes (20 nM for the wild-type protein and 40 nM for the mutant holoenzyme) were then incubated with various concentrations of cAMP in the presence of MgATP, and the activity was measured as described by Cook et al. (1982). Hill plots derived from the data are shown as an inset.

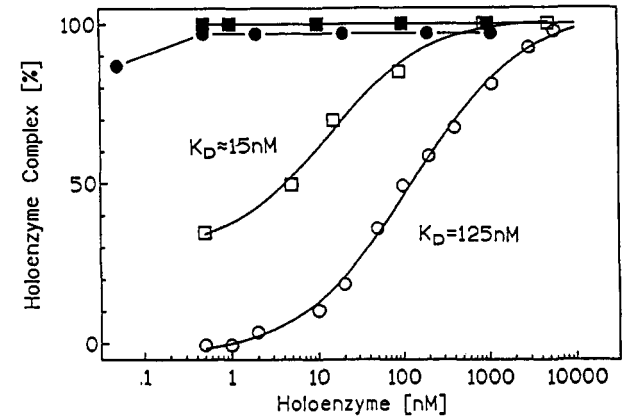

FIGURE 6: Determination of the appK_d of the wild-type and the mutant holoenzyme using gel filtration as described under Experimental Procedures. (■) (Δ1–91) holoenzyme (+MgATP); (●) wild-type holoenzyme (+MgATP); (□, ○) (–MgATP).

rate could not be accurately determined by the Millipore filtration assay, since ATP release was already 50% complete when the holoenzyme complex was precipitated with ammonium sulfate. In contrast, when the mutant protein was treated with urea to remove cAMP prior to forming holoenzyme, the off-rate for MgATP was extremely slow (Table 2), yielding a value comparable to that for the full-length wild-type protein.

Measurement of appK_d by Gel Filtration. Analytical gel filtration was used to determine the interaction between the (Δ1–91)rR subunit and the rC subunit as described for the wild-type protein (Herberg & Taylor, 1993). In the presence of 100 μM ATP and 1 mM $MgCl_2$ no dissociation of the preformed holoenzyme complex could be observed down to a concentration of 0.5 nM (Figure 6). In the absence of MgATP, the appK_d was approximately 15 nM, thus showing a substantial increase in affinity compared to that of the wild-type complex with the appK_d = 125 nM.

Surface Plasmon Resonance. Surface plasmon resonance (SPR) was used to investigate the interaction between the rC subunit, immobilized by amine coupling to a sensor chip, and the (Δ1–91) and wild-type rR subunit, respectively. To immobilize the rC subunit in a physiologically active con-

formation, the addition of MgATP during the immobilization process appeared to be important. Surface activity of the rC subunit was increased from 30% to 90% for the binding of R monomer in the absence or presence of 1 mM ATP and 2 mM $MgCl_2$, respectively. This may be due to blocking Lys72 (Zoller & Taylor, 1979) from coupling to the matrix. For both rR subunits the stoichiometry of binding to the immobilized rC subunit was determined. Injections of 500 nM R monomer bound 311 RU's (MW = 33.2K). This corresponds to 88% of the theoretical maximum for the rC subunit surface, assuming a stoichiometric relationship of 1:1. Injections of 2 μM R dimer bound 525 RU's. This corresponds to 55% of the calculated maximum for the surface, assuming 1:1 binding. If the dimer is binding monovalently so that only one R protomer is free to bind on immobilized rC subunit, then this may correspond to 100%.

The association rate constants (Table 2) obtained were very rapid and were similar for the wild-type and the (Δ1–91) protein (k_{ass} = 1 × 10^5 M^{-1} s^{-1}, Figure 7A–C). In contrast, the dissociation rate constant for the (Δ1–91) protein was twice as fast as that for the wild-type protein. The dissociation rates were calculated after the end of an injection, from time 500 to 7000 s (Figure 7D). Rates of 2.3 × 10^{-5} s^{-1} and 4.5 × 10^{-5} s^{-1} were determined for the wild-type and the (Δ1–91) protein, respectively. From the association and dissociation rate constants appK_d's of 0.39 nM and 0.23 nM for the (Δ1–91) and the wild-type protein were calculated. Immobilization of the rR subunit dimer and measurement of the association and dissociation of the rC subunit yielded different k_{assoc} and k_{diss}. From these data a K_d of about 0.7 nM was calculated. The difference might be due to steric hindrance on the surface since a higher amount of rR subunit was immobilized in this experiment.

Effect of the N-Terminus on Subcellular Localization. To determine the consequences of deleting the N-terminus on the subcellular localization of the rR subunit, the mutant rR subunit was fluorescent labeled and injected into rat fibroblasts. In contrast to the full-length rR subunit, which was restricted to the cytoplasm (Fantozzi et al., submitted 1993; Fantozzi et al., 1992), the deletion mutant migrated into the nucleus (Figure 8). The mutant holoenzyme, like the wild-type holoenzyme, was, however, still restricted to the cytoplasm. The microinjection studies thus demonstrated that the dimerization domain may be important for other aspects of subcellular localization in addition to anchoring cAPK to cytoplasmic proteins. The dimeric R not only served as an inhibitor of C, it could also serve as a cytoplasmic anchor.

DISCUSSION

The R subunits of cAPK have a well-defined domain structure, and the construction of various deletion mutants provides an opportunity to understand how each of these domains contributes to holoenzyme formation and activation. Of particular interest is understanding the role of the N-terminus. Limited proteolysis established that the N-terminus was required for dimerization (Bubis et al., 1987; Rannels et al., 1985; Reimann, 1986; Weber & Hilz, 1979). Scott et al. showed that, for the $R^{II}\alpha$ subunit, deletion of the first 14 residues was sufficient to prevent dimerization (Scott et al., 1990). The R subunit also functions as an anchoring or targeting subunit and serves to dock the enzyme at specific subcellular locations. These anchoring sites are located at the N-terminus, and dimerization is required for anchoring to other proteins (Carr et al., 1991; Scott et al., 1990). Most of the antigenic sites are located in this region of the R subunit,

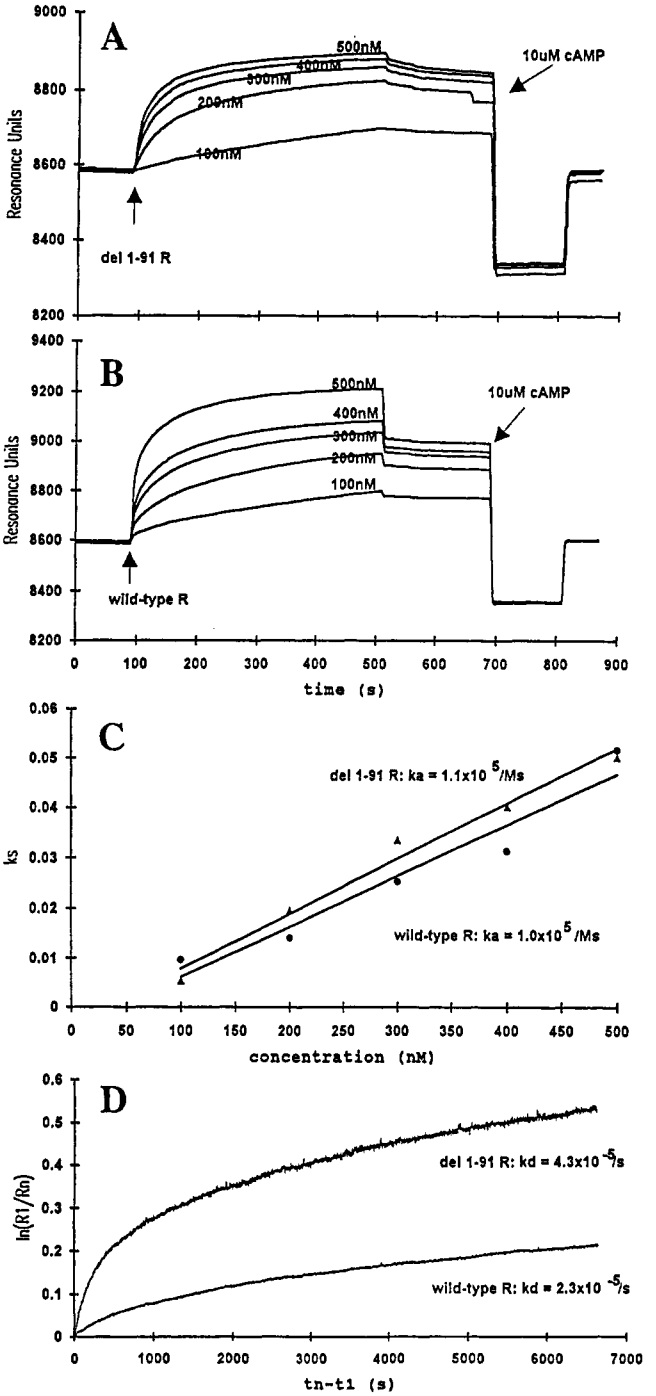

FIGURE 7: Surface plasmon resonance experiments on R/C interaction. Association phase of type I rR subunits binding to 424 RU's of immobilized rC subunit. Measurements of the (Δ1–91) monomer (A) and the wild-type rR subunit dimer (B) were taken over a concentration range of 100–500 nM. (C) First-order replots of the initial slopes of the association phases in (A) and (B). Dissociation plots of $\ln(R_1/R_n)$ versus $T_n - T_1$. Only the late portion of the dissociation phases, time 500–7000 s, was used to determine off-rates of the rR subunits from immobilized rC subunit. Values of the off-rates are indicated in the plots.

and these are exposed in the holoenzyme as well as in the free R subunit (Mumby et al., 1985; Weldon et al., 1983). As shown here, the dimeric R^I subunit is also required for localizing the rC subunit in the cytoplasm.

Although the N-terminus is essential for dimerization, for targeting, and for cytoplasmic localization, it is not essential for other functions. Limited proteolysis, for example, also showed that the N-terminus is not required for binding to the

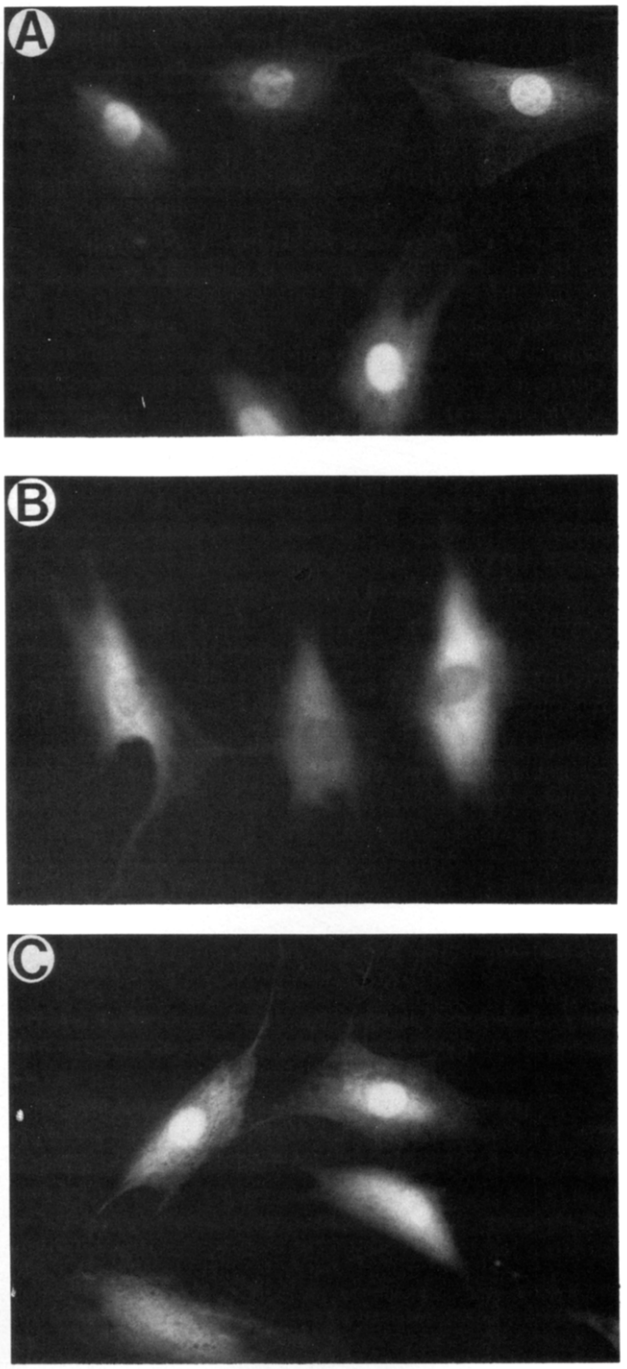

FIGURE 8: Microinjection of the FITC-labeled (Δ1–91)rR subunit into rat fibroblasts: (A) Cytosolic injection of the (Δ1–91)rR subunit showing mainly nuclear distribution. (B) Cytosolic injection of (Δ1–91) holoenzyme. Here most of the fluorescence is localized in the cytosol. (C) Addition of 100 μM 8-bromo-cAMP to cytosolic injected cells yielded in nuclear localization of the (Δ1–91)rR subunit. All cells were photographed 30–45 min after microinjection.

rC subunit (Potter et al., 1978; Rannels et al., 1985; Reimann, 1986; Weber & Hilz, 1979). Deletion of cAMP binding domain B and the N-terminal 91 residues established that the autoinhibitor site through domain A was sufficient to form a stable complex with the rC subunit (Ringheim & Taylor, 1990). By characterizing the effects of deleting only the first 91 residues of the $R^I\alpha$ subunit, we were able to quantitatively evaluate the role of the N-terminus with respect to cAMP binding, holoenzyme activation, and holoenzyme formation. Our results demonstrated that the N-terminus, although separated in the linear sequence, does nevertheless influence both R/C interaction and cAMP binding.

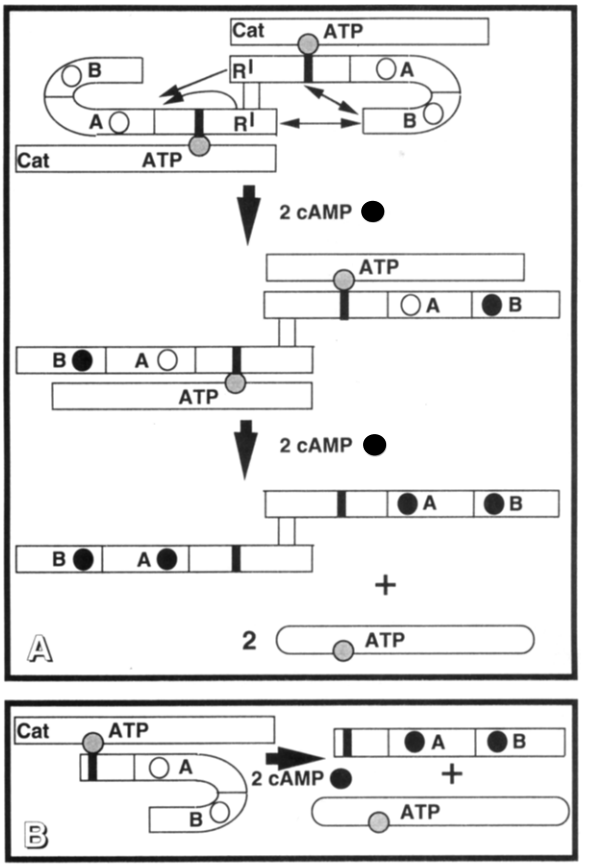

FIGURE 9: Model of the association of the subunits of cAMP-dependent protein kinase.

cAPK is a tightly regulated enzyme that shows strong positive cooperativity, both in its activation by cAMP and in the reassociation of its R and C subunits. Previous results based on the deletion of cAMP binding domain B established that both cAMP binding domains are required for this cooperativity (Ringheim et al., 1988). The results described here show for the first time that, for the type I holoenzyme, the N-terminal 91 residues are also required for cooperativity. Before understanding the role of the N-terminus, however, one must first consider the general mechanism of activation of the wild-type holoenzyme. Kinetic studies and an analysis of the two cAMP binding sites in the free R subunit and in the holoenzyme provide a basis for understanding the cooperative activation of the intact native holoenzyme (Øgreid & Døskeland, 1981a,b). As summarized in Figure 9A, the physiological activation of cAPK is mediated by the cooperative binding of cAMP to the two distinct cAMP binding sites in the holoenzyme. In the free R subunit, cAMP can bind freely to each site, and the most distinguishing feature of these two sites is their off-rates, with site A having a fast off-rate ($t_{1/2}$ = 2 min) and site B having a slow off-rate ($t_{1/2}$ = 45 min). In the holoenzyme, however, site A is masked; only site B is accessible. cAMP binding to site B leads to a conformational change that "opens" site A, and cAMP binding to site A then promotes dissociation of the complex. In the type I holoenzyme, ATP is an essential lock that stabilizes the holoenzyme complex (Neitzel et al., 1991). Without MgATP, the type I holoenzyme would begin to dissociate under physiological conditions (Herberg & Taylor, 1993). How is this model perturbed when the N-terminus is deleted?

Although the distinct kinetic properties of each cAMP binding site are slightly perturbed, more importantly, the activation of the kinase no longer showed positive cooperativity.

Since the K_d's for both sites were indistinguishable, it is unclear whether the obligatory order of binding to site B followed by site A is still maintained in the mutant holoenzyme (Figure 9B). In this complex formed with the truncated rR subunit, site A is obviously more accessible than in the native dimer. Whether dimerization per se or interactions involving a specific segment in the N-terminal 91 residues with more C-terminal regions in the same protomer are responsible remains to be established. In the case of the R^{II} subunit, deletion of the first 45 residues was not sufficient to destroy cooperativity (Rannels et al., 1985; Reimann, 1986). Thus there may be a difference between the type I and type II holoenzymes in terms of the N-terminus influencing the cAMP binding properties. The type I holoenzyme with its requirement for ATP may behave differently from the type II holoenzyme.

Photoaffinity labeling with 8-N_3cAMP of the R^{II} subunit gives additional evidence that the N-terminus influences the cAMP binding domains of the protein (Bubis & Taylor, 1987). Only a single site, Tyr381 in domain B, was photolabeled in the intact protein. When a chymotryptic fragment of the R^{II} subunit (residues 91–400) was photolabeled, a second site was covalently modified. This site was identified as Tyr196 in domain A.

In terms of reassociation, the monomer behaves very differently from the dimer. Not only is the N-terminus unnecessary for holoenzyme formation, subunit reassociation is actually enhanced when residues 1–91 are missing. As summarized in Figure 9A, in the type I holoenzyme that contains tightly bound MgATP, cAMP must compete with C·MgATP for the R subunit (Neitzel et al., 1991). The two physiologically relevant states of the type I R subunit are as follows:

$$R_2C_2(MgATP)_2 \underset{MgATP}{\overset{cAMP}{\rightleftharpoons}} R_2(cAMP)_4 + 2C$$

Unlike the full-length R subunit, the deletion mutant can reassociate readily in solution with the rC subunit even in the absence of ATP. Its behavior in some ways resembles that of R, that has been stripped of cAMP. However, on the basis of the urea denaturation (Leon and Taylor, in preparation) and binding stoichiometries, both cAMP binding sites are filled in the deletion mutant as they are in the native dimer after purification. In the native dimer, MgATP serves as a "lock" to stabilize the holoenzyme, and cAMP must somehow compete against this lock. For the full-length R subunit, C alone does not compete effectively for $R_2(cAMP)_4$ without ATP. The deletion mutant competes well with or without ATP. Even though ATP still binds with high affinity, as demonstrated here by the slow ATP off-rates, it is not required for the efficient formation of holoenzyme. Reassociation of $R_2(cAMP)_4$ with the C subunit occurs readily in the assay mix and is even observed when the dissociated complex is applied to a gel filtration column as long as cAMP is not included in the running buffer. If anything, the N-terminal sequence or the physical dimeric configuration exerts an inhibitory effect on holoenzyme formation. This may be due to a specific sequence in the N-terminus, or alternatively, it could result from steric hindrance that blocks access of the consensus sequence to the active site of the C subunit.

Surface plasmon resonance allowed us for the first time to readily and directly measure association and dissociation rates for the R and C subunits. The rC subunit can be immobilized on the sensor chip containing (carboxymethyl)dextran and regenerated rapidly by adding cAMP to the wash buffer. In this way, kinetic rates were measured for both the native dimer and the mutant monomeric rR subunits. The dissociation rate for the native dimer is very consistent with previous results and is also consistent with the very slow off-rates for ATP. ATP binds with a high affinity (60 nM) to the type I holoenzyme and has a calculated $t_{1/2}$ of 11 h. This number is very close to the off rates for R and C measured with the BIAcore, indicating that release of ATP from the holoenzyme complex is coincident with subunit dissociation. The precision and accuracy of this method provide an excellent system for rapidly quantitating the effects of other mutations on the R/C interaction.

Surface plasmon resonance demonstrated quantitatively that the affinity of R for C is not significantly reduced by deleting the first 91 residues. This important finding is in strong contrast to the other family of physiological inhibitors of the C subunit, the heat-stable protein kinase inhibitors (PKI's). In the case of PKI, the region N-terminal to the consensus site is required for high-affinity binding. This was predicted on the basis of extensive analysis of peptide analogs (Walsh et al., 1990) and was confirmed by the crystal structure of C bound to PKI(5–24) (Knighton et al., 1991). We show here unambiguously that high-affinity binding of R to C requires only regions that are C-terminal to the consensus site. This is consistent with the finding of Gibbs et al. (1992) and Orellana (Orellana & McKnight, 1992), who showed that the mutations in the C subunit that lie on the surface C-terminal to the consensus site are critical for binding the R subunit. Point mutations in C have subsequently confirmed that the surface that is N-terminal to the consensus site can be mutated so that PKI binding, but not R binding, is blocked (Wen & Taylor, 1994). In contrast, mutations on the surface of the C subunit C-terminal to the consensus site selectively interfere with R binding but do not affect PKI binding (Cox & Taylor, 1994). These two important inhibitors thus share a common recognition site, but each uses different regions of the C subunit surface to achieve high-affinity binding.

Does the monomeric form of the R subunit have physiological relevance? With the exception of the *Dictyostelium* R subunit (Mutzel et al., 1987), every R subunit studied so far is dimeric. Whether the deletion of the N-terminus of the *Dictyostelium* R subunit has physiological significance remains to be established, but the results described here suggest it may be less tightly regulated and more mobile in the cell. Although the dissociated R subunit is quite prone to proteolytic cleavage at the hinge region *in vitro*, it is not known whether a monomer is an intermediate on the physiological degradation pathway.

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