# Active Site Mutations Define the Pathway for the Cooperative Activation of cAMP-Dependent Protein Kinase†

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*Received July 18, 1995; Revised Manuscript Received November 8, 1995*⊗

ABSTRACT: cAMP-dependent protein kinase (cAPK) is a heterotetramer containing two regulatory (R) and two catalytic (C) subunits. Each R-subunit contains two tandem cAMP-binding domains, and activation of cAPK is mediated by the cooperative, high affinity binding of cAMP to these two domains. Mutant R-subunits containing one intact high affinity cAMP-binding site and one defective site were used to define the pathway for activation and to delineate the unique roles that each cAMP-binding domain plays. Two mutations were introduced by replacing the essential Arg in each cAMP-binding site with Lys (R209K in Site A and R333K in Site B). Also, the double mutant (R209/333K) was constructed. Analysis of cAMP binding and dissociation and the apparent constants for holoenzyme activation and R- and C-subunit interaction, measured by analytical gel filtration and surface plasmon resonance, established the following: (1) For rR(R209K), occupancy of Site B is not sufficient to activate the holoenzyme; the low affinity Site A must also be occupied. In rR(R333K), Site A retains its high affinity for cAMP, but Site A cannot bind until the low affinity Site B is occupied. Thus, both mutants, for different reasons, have similar $K_a$'s for activation that are approximately 20-fold higher than that of the wild-type holoenzyme. The double mutant with two defective sites is no worse than either single mutant. (2) Kinetic analysis of cAMP binding showed that the mutation in Site A or B abolishes high affinity cAMP binding to that site and slightly weakens the affinity of the adjacent site for cAMP. (3) In the presence of MgATP, both mutants rapidly form a stable holoenzyme even in the presence of cAMP in contrast to the wild-type R where holoenzyme forms slowly *in vitro* and requires dialysis. Regarding the mechanism of activation based on these and other mutants and from kinetic data, the following conclusions are reached: Site A provides the major contact site with the C-subunit; Site B is not essential for holoenzyme formation. Occupancy of Site A by cAMP mediates dissociation of the C-subunit. Site A is inaccessible to cAMP in the full length holoenzyme, while Site B is fully accessible. Access of cAMP to Site A is mediated by Site B. Thus Site B not only helps to shield Site A, it also provides the specific signal that "opens up" Site A. Finally, a nonfunctional Site A in the holoenzyme prevents stable binding of cAMP to Site B in the absence of subunit dissociation.

The regulatory subunits of cAMP-dependent protein kinase (cAPK)[1] serve as negative regulators of cAPK and maintain an inactive holoenzyme complex in the absence of cAMP. All R-subunits contain an inhibitory site that resembles either a substrate or an inhibitor followed by two contiguous cAMP-binding domains in each protomer (Takio et al., 1984; Taylor et al., 1990; Titani et al., 1984). The cooperative activation of the holoenzyme is mediated by the sequential binding of cAMP to these two sites, which then leads to the dissociation of the active C-subunit (Øgreid & Døskeland, 1981a). The two homologous cAMP-binding sites presumably arose by gene duplication and are related to the cAMP-binding domain of the catabolic gene activator protein (CAP) (Weber et al., 1987). Site B at the C-terminus is characterized by a higher affinity for cAMP, a slower off-rate for cAMP ($\tau_{1/2}$ = 30 min), and a preference for analogs containing substituents at the C8 position of the adenine ring. Site A shows a lower affinity, a faster off-rate ($\tau_{1/2}$ = 1 min), and a preference for N6 substituted analogs of cAMP (Øgreid et al., 1989). In the dissociated R-subunit, cAMP binds

† This work was supported by Grant GM34921 from the National Institutes of Health (to S.S.T.), by Grant Do329-2/2 from the Deutsche Forschungsgemeinschaft (to W.R.G.D.), and NATO Grant CRG 941235 (to W.R.G.D. and S.S.T.).

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⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; C, catalytic subunit of cAPK; cAMP, adenosine 3′,5′- cyclic monophosphate; CAP, catabolic gene activating protein; cAPK, cAMP-dependent protein kinase; DTT, dithiothreitol; EDC, *N*-ethyl-*N*′-(3-(diethylamino)propyl)carbodiimide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetracetic acid; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetracetic 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; NHS, *N*-hydroxysuccinimide; R, regulatory subunit of cAPK; $R^I$, $R^{II}$, type I and type II, respectively, of the R-subunit of cAPK; RU, response units (1000 RU = 1 ng/mm$^2$); SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance.

0006-2960/96/0435-2934$12.00/0 

preferentially to Site A, while in the holoenzyme complex the initial binding of cAMP is to Site B (Øgreid & Døskeland, 1981a,b).

The native regulatory subunit is a dimer with the two protomers interacting near the N-terminus. In the type I R-subunits the two protomers are linked by two interchain disulfide bonds (Bubis et al., 1987; Zick & Taylor, 1982). Deletion of cAMP-binding site B abolishes cooperativity of cAMP binding (Ringheim et al., 1988). However, when the N-terminus is deleted or removed by proteolysis, cooperativity is also no longer seen in the monomer, suggesting either that there are direct interactions between the N-terminal segment and the cAMP-binding sites within one protomer (intraprotomer contacts), or that interactions between the two protomers in the dimer are important for cooperativity (interprotomer contacts; Herberg et al., 1994).

To better understand the molecular basis for the cooperativity between the two sites, several mutants were studied. These mutants each contained a single replacement in the essential cAMP-binding sites A and B, respectively. Each binding site contains a conserved Arg that is predicted to interact with the exocyclic oxygens of cAMP based on homology modeling using the crystal structure of CAP as a template (Weber et al., 1987). In mutant A the essential Arg in Site A, R209, was replaced with Lys. This mutation, studied previously, showed an increase in $K_d$(cAMP) of about 10-fold (Bubis et al., 1988; Neitzel et al., 1991). In mutant B the essential Arg in Site B, R333, was replaced with Lys. Additionally, a double mutation, mutant AB R209/333K, was constructed. Finally, one deletion mutant was engineered, Δ1−91/R333K, by deleting the N-terminal dimerization domain. In addition to measuring cAMP binding and activation, the interactions of the mutant proteins with the catalytic subunit were characterized by analytical gel filtration and by surface plasmon resonance.

## 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, [$\gamma$-$^{32}$P]ATP(3000 Ci/mmol) from Amersham, and [$^3$H]cAMP from New England Nuclear. Before use the [$^3$H]cAMP was purified on poly(ethylenimine)−cellulose (Walsh et al., 1992). PMSF was purchased from Boehringer Mannheim. Nitrocellulose filters (BA 85, 0.45 mm) were from Schleicher & Schuell. Materials used in cloning were molecular biology grade. Media supplies were purchased from Difco. Restriction endonucleases and nucleic acid modifying enzymes were purchased from USB or Gibco/BLR, New England Biolabs, and Promega.

*Mutagenesis.* The R209K mutation was constructed as described previously (Bubis et al., 1988). The R333K mutation was made in the phagemid 118 as described earlier (Kunkel et al., 1991). The R209/333K double mutation was made by excising 150 base pairs (bp) with *Sac*II from the R333K/pUC118 vector and swapping it with a 150 bp piece that had been cut with *Sac*II from the R209K/pUC18 vector. The double mutation Δ1−91/R333K was constructed using the R333K/pUC118 construct described above. This construct was cut with *Nae*I and *Xma*I to generate a 900 bp fragment that deleted the first 91 amino acids of the RI subunit. The fragment then was cloned into pUC9 that had been previously cut with *Hinc*II and *Xma*I. The resulting vector coded for an in-frame $\beta$-galactosidase fusion protein (Δ1−91)rR, with the sequence Thr-Pro-Ser-Leu-Ala-Ala from $\beta$-galactosidase at the N-terminus fused to the RI sequence starting at Arg93.

The wild-type and mutant proteins are referred to as follows: rRIα, rR(R209K), rR(R333K), rR(R209K/R333K), rR(Δ1−91), and rR(Δ1−91/R333K), where r designates recombinant proteins. The construction and purification of the rR(Δ260−379) mutation were described previously (Ringheim et al., 1988).

*Purification of Proteins.* Following overexpression in *Escherichia coli* (Slice & Taylor, 1989), the C-subunit was purified and separated in distinct isozymes as previously described (Herberg et al., 1993). Isozyme II was 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 C-subunit was 25 $\mu$mol/(min·mg) as measured by the coupled spectrophotometric method of Cook et al. (1982) using the heptapeptide, LRRASLG, as a substrate. The purified C-subunit was stored at 4 °C in 20 mM potassium phosphate, 110 mM KCl, and 5 mM 2-mercaptoethanol, pH 7.0.

The recombinant RI-subunits were overexpressed in *E.* coli 222 and purified as described previously (Saraswat et al., 1986). The RI-subunits (RIα and rR(Δ1−91)) were purified by ion-exchange chromatography on DEAE-cellulose as previously described (Buechler & Taylor, 1991). The rRI-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 double mutation R209/333K was purified by co-lysis with a poly-His tagged C-subunit (Cox and Bell, manuscript in preparation). The pellets from cells (1 L) expressing poly-His C-subunit were co-lysed with cells (5 L) expressing the mutant R-subunit in lysis buffer (50 mM potassium phosphate, 300 mM NaCl, 5 mM 2-mercaptoethanol, pH 8.0). The lysate was clarified by centrifugation as described above, and holoenzyme formation was tested by assaying the extract in the absence and presence of 100 $\mu$M cAMP using the spectrophotometric assay. Nickel agarose (1 mL) (Quiagen) was added to the supernatant. After incubation for 30 min at 4 °C, the slurry was transferred to a column with a diameter of 10 mm and washed with 10 volumes of lysis buffer. The R-subunit was eluted 3 times with 1 mL of 1 mM cAMP in gel filtration buffer (see below).

To obtain cAMP-free R-subunit, the R-subunits were unfolded with 8 M urea and then dialyzed and purified by gel filtration as described by Buechler et al. (1993).

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

*cAMP Exchange Rates and cAMP Equilibrium Binding.* Wild type and mutant R-subunits were measured as previously described (Herberg et al., 1994). To detect low affinity binding sites for cAMP, the method for the cAMP exchange rates was varied by omitting the washing steps and subtracting the nonspecific binding.

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). cAMP bound to the purified proteins was estimated based on the 260/280 nm absorbance ratio.

*Holoenzyme Formation.* Holoenzyme was formed by dialyzing the rC- and rR-subunits in a molar ratio of 1.2:1 for 24 h at 4 °C against 20 mM potassium phosphate, 100 mM KCl, 5 mM 2-mercaptoethanol, 5% glycerol, 100 $\mu$M ATP, and 1 mM $MgCl_2$, pH 6.5.

Holoenzyme formation with the mutant proteins was measured by combining 20 nM wild-type rC-subunit and a 1.2 molar excess of R-subunit in assay mix containing 1 mM ATP, 10 mM $MgCl_2$, and 100 $\mu$M Kemptide and determining the decrease of phosphotransferase activity.

*Apparent Activation Constants ($K_a$) for cAMP.* Holoenzyme at a concentration of 20 nM C-subunit was incubated for 2 min at room temperature in the assay mix described by Cook et al. (1982) with varying concentrations of cAMP ranging from 1 nM to 100 $\mu$M. The reaction was initiated by adding Kemptide (100 $\mu$M), and the activity of the free C-subunit was followed using the spectrophotometric assay.

*Equilibrium Dialysis.* cAMP binding to holoenzyme and apparent activation constants for cAMP were also determined by equilibrium dialysis following the procedure of Neitzel et al. (1991) using a 250 $\mu$L eight chamber apparatus (Hoefer Scientific). After overnight incubation with rotation (16 h) two 20 $\mu$L aliquots were transferred into scintillation vials and counted in 6 mL of Cytoscint. The rest of each protein sample was added to 800 $\mu$L of assay mix (see above), and activity was determined before and after total activation with 100 $\mu$M cAMP in the spectrophotometric assay.

*Analytical Gel Filtration.* Analytical gel filtration was carried out using a Superdex 200 HR10/30 (all columns from Pharmacia LKB) column or a Superdex 75 HR10/10 column with flow rates of 0.8 mL/min at 22 °C in buffer A (20 mM MOPS, pH 7.0, 150 mM KCl, 1 mM DTT) as described previously (Herberg & Taylor, 1993). The percentage of holoenzyme at various protein concentrations was calculated based on the peak areas corresponding to both holoenzyme and C-subunit.

Analytical gel filtration was also used to determine [$^3$H]-cAMP binding to mutant and wild-type holoenzyme complexes. 250 nM of each holoenzyme was incubated with 600 nM [$^3$H]cAMP for 20 min, and 200 $\mu$L of this mixture was injected on a Superose 12 10/30 gel filtration column. Fractions of 500 $\mu$L were subsequently collected and mixed with 10 mL of Cytoscint scintillation fluid and determined in a scintillation counter. The specific activity of an aliquot of 50 $\mu$L was measured in the spectrophotometric assay.

*Surface Plasmon Resonance.* Surface plasmon resonance (SPR) was used to study the interaction between the C-subunit and R-subunits of cAPK using a BIAcore instrument (Pharmacia/Biosensor). SPR was used to detect changes in mass in real time on a sensor chip surface which was prepared by direct coupling of the C-subunit or the R-subunit by primary amines to the CM dextran (Biosensor Amine Coupling Kit) as previously described (Herberg et al., 1994). To determine unspecific binding, blank runs were performed with 500 nM wild-type R-subunit using a non-activated surface. No unspecific binding could be detected. About 400 RU's of the C-subunit and 500 RU's of the R-subunit were immobilized (1000 RU = 1 ng/mm$^2$). Surface activity was calculated using the equation: $S = (MM)_L R_A/(MM)_A R_L$, where $S$ is the stoichiometry, L = ligand (immobilized protein), A = analyte (injected protein), $R$ = response in RU's, and (MM) = molecular mass. All runs were performed using 100 $\mu$M ATP and 1 mM $MgCl_2$ in buffer A during the association and dissociation phase; regeneration of the surface was achieved by injecting 10 $\mu$L of 100 $\mu$M cAMP and 5 mM EDTA in buffer A.

Kinetic constants were calculated by linear regression of data using the BIAcore pseudo first order rate equation, $dR/dt = k_{assoc}CR_{max} - (k_{assoc}C + k_{dissoc})R_t$. The association rate is $k_{assoc}$, $k_{dissoc}$ is the dissociation rate, $C$ is the concentration of the injected analyte, and $R$ is the response. Plots of $dR/dt$ vs $R_t$ have a slope of $k_s$. When $k_s$ is plotted against $C$, the resulting slope is equal to the $k_{assoc}$. The $k_{dissoc}$ was calculated by integrating the rate equation when $C = 0$, yielding $\ln(R_{t1}/R_{tn}) = k_{dissoc}(t_n - t_1)$. Affinity constants were calculated from the equation $K_d = k_{dissoc}/k_{assoc}$.

Association rate calculations were performed using concentrations between 30 and 500 nM for each R-subunit. The first 10 s of every sensogram were subtracted correcting for bulk refractive index changes. Dissociation rate constants were determined over a longer time scale (2 h) at a concentration of 500 nM R-subunits and were calculated by linear analysis.

*ATP Off-Rates.* Binding studies with [$\gamma$-$^{32}$P]ATP were performed using the method described by Døskeland and Øgreid (1988) for measuring the binding of cAMP. The protocol was modified as described in Herberg and Taylor (1993).

## RESULTS

In order to investigate the role of a conserved Arg in the tandem cAMP-binding sites of the type I R-subunit of cAPK and also to better understand how communication between the domains is mediated, two single mutations (R209K and R333K) and two double mutations (R209/333K and Δ1−91/R333K) were engineered into the RIα subunit. All mutants were overexpressed in *E. coli*, and the proteins, except for the R209/333K mutant, were purified to homogeneity using DEAE chromatography as described in the Experimental Procedures. All purified full length recombinant R-subunits [rR(R209K), rR(R333K), and rR(R209/333K)] had a molecular mass based on SDS−polyacrylamide gel electrophoresis that was indistinguishable from that of the wild-type R-subunit (47 kDa). The deletion double mutant had a molecular mass of 38 kDa, identical to the Δ1−91 mutant alone (Herberg et al., 1994).

*Stokes' Radius.* As a general indicator of structural integrity, the Stokes' radius of each mutant was determined by gel filtration. rR(R333K), like the full length rRIα subunit, had a Stokes' radius of 43.0 ± 0.5 Å, similar to the mammalian RIα subunit. The frictional coefficient ($f/f_0$) of RIα is 1.55 (Zoller et al., 1979), indicating that this is a highly asymmetric molecule. The R209K mutant had a Stokes' radius of 41.5 ± 0.6 Å. This highly reproducible reduction in the Stokes' radius indicated that in the absence of cAMP bound to Site A the molecule was more compact. This effect correlated with Site A; no difference in Stokes' radius was observed for the Site B mutant. rRIα stripped of cAMP has a Stokes radius of 41 Å, comparable to that of the rR(R209K) mutant.

*cAMP Dissociation and Binding for Free Regulatory Subunits.* To investigate the activation mechanism of cAPK,

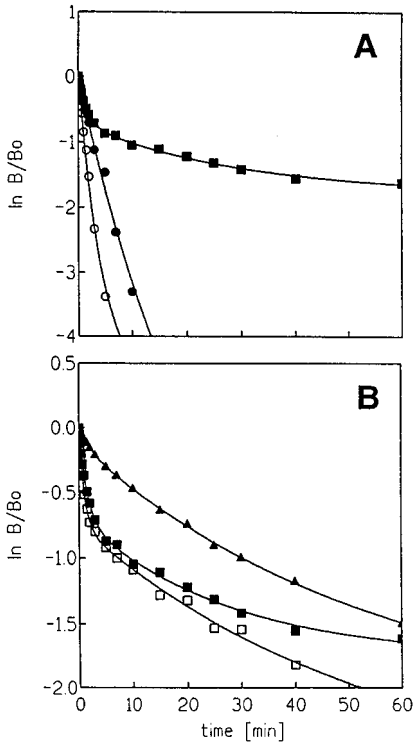


FIGURE 1: Determination of cAMP off-rates for various mutant regulatory subunits. The off rates for cAMP were determined using an ammonium sulfate/filtration method as described in the Experimental Procedures. Panel A shows rRIα (■), rR(R333K) (●), and rR(Δ1−91/R333K) (○). Panel B shows rRIα (■), rR(Δ1−91) (□), and rR(R209K) (▲).

the free mutant R-subunits were first characterized. The effects of these mutations on holoenzyme function and activation were then evaluated. The cAMP binding properties were determined as described in the Experimental Procedures. For wild-type R the two cAMP-binding sites can be distinguished readily by their corresponding cAMP exchange rates (Corbin et al., 1982; Døskeland et al., 1983). As demonstrated previously, exchange of cAMP is biphasic, with Site A having a faster exchange rate (1 min) and Site B a slower exchange rate (35 min) (Øgreid & Døskeland, 1980; Rannels & Corbin, 1980). As shown by Neitzel et al. (1991), the R209K mutation displayed only a single cAMP exchange rate with a half-life of 18 min (Figure 1A). This rate was closer to the value measured for Site B of the wild-type protein. The R333K mutation showed a single fast exchange rate of 6 min, a value that is closer to the cAMP exchange rate for Site A in the wild-type R-subunit (Figure 1B). The exchange rate for rR(Δ1−91/R333K) was intermediate between rR333K and rRIα (3 min, Figure 1).

Equilibrium binding revealed that cAMP binding to the nonmutated site was shifted to higher $K_d$ values for both mutant R-subunits (Table 3). The shifts (6−8-fold) were similar when binding was measured to the mutant holoenzymes in comparison to the wild-type protein. In addition, both mutants bound only 1 mol of cAMP/mol of monomeric R-subunit with high affinity, whereas the wild-type protein was fully capable of binding cAMP to both sites. Therefore, under these conditions the observed $K_d$'s for the mutant proteins reflected the binding constants to the nonmutated site. To clarify whether the mutated sites can still bind cAMP at micromolar concentrations even though this low affinity binding would not be measured with the assay

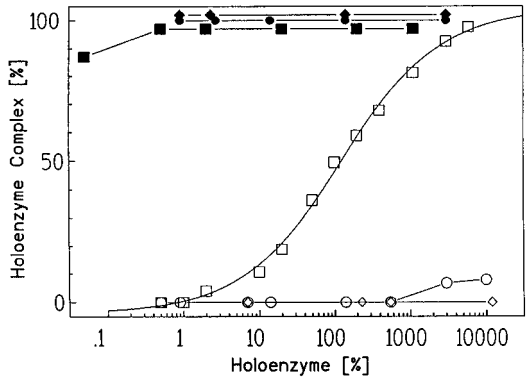


FIGURE 2: Holoenzyme formation of wild type and mutant R-subunits. Squares (□, ■) correspond to rRIα wild-type, diamonds (◇, ♦) to rR(R209K), and circles (○, ●) to rR(R333K). Open symbols refer to the absence and closed symbols to the presence of MgATP.

conditions as described under Experimental Procedures, the precipitated and filtered proteins were not washed with 70% ammonium sulfate. Using 4 $\mu$M [$^3$H]cAMP, the R209K and R333K mutants showed an increase in binding stoichiometry of 1.8- and 1.3-fold, respectively. However, the modified conditions did not allow the exact measurement of the equilibrium binding constants.

*Holoenzyme Formation.* Holoenzyme was formed by combining wild-type rC-subunit with varying amounts of mutant rR-subunit. All mutant proteins described above (rR(R209K), rR(R333K), rR(209K/R333K), rR(Δ1−91), and rR(Δ1−91/R333K) and a mutant lacking the B domain entirely, rR(Δ260−379), formed holoenzyme instantaneously in a 1:1 molar ratio in the assay mix as described in Experimental Procedures. The kinetics of holoenzyme formation were too fast to be followed in the spectrophotometric assay. In all cases, the $\tau_{1/2}$ for holoenzyme formation was faster than 10 s. The concentration of holoenzyme in the assay mixture was 20 nM. Because this process was so rapid, no differences in the kinetics of holoenzyme formation could be determined based on activity assays for these mutants.

Two new methods, independent from an activity assay, were used to evaluate holoenzyme formation and stability of the holoenzyme complexes formed with wild-type rC-subunit and the mutant rR-subunits. Using analytical gel filtration (Figure 2) FITC-labeled rC-subunit was mixed with a 1.2 molar excess of either rR(R209K) or rR(R333K) and immediately injected onto a Superdex 200 gel filtration column as described previously (Herberg & Taylor, 1993). The column was equilibrated with buffer A containing 2 mM EDTA or 100 $\mu$M ATP/1 mM $MgCl_2$. In the presence of MgATP, no dissociation of any mutant holoenzymes occurred down to a concentration of 0.1 nM, suggesting that the mutant holoenzyme complexes are at least as stable as the wild-type complex. This stable holoenzyme could be detected immediately after the C-subunit and the mutant R-subunits were mixed. In contrast, for the wild-type R-subunit, extensive dialysis was required before holoenzyme formation could be demonstrated by analytical gel filtration. When the column was equilibrated with EDTA, however, less than 5% holoenzyme complex was observed for the R209K and the R333K mutants at a concentration of 15 $\mu$M rR-subunit and 18 $\mu$M rC-subunit.

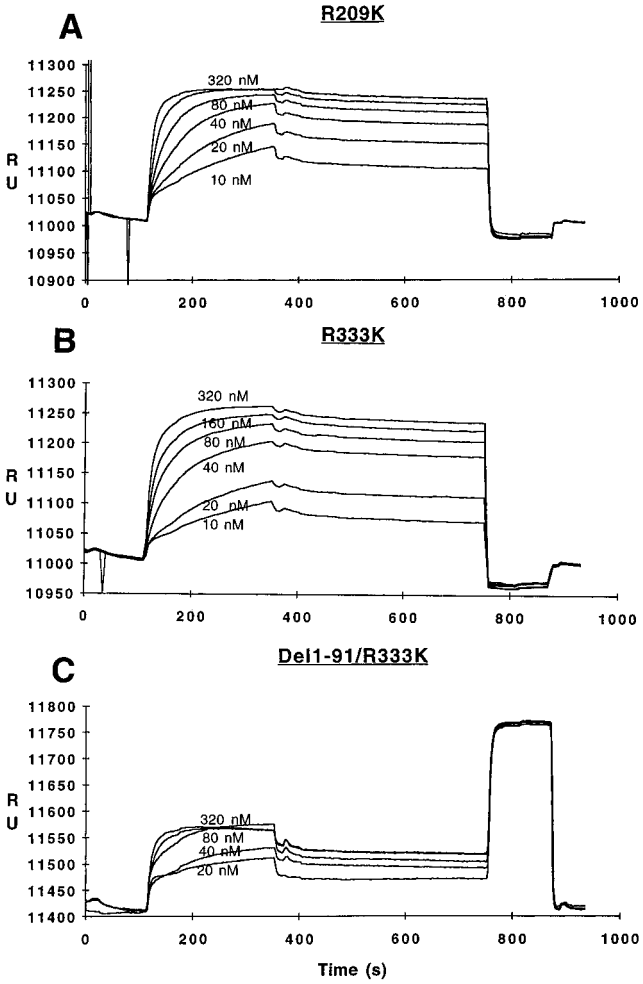


FIGURE 3: Measurement of association and dissociation rate constants for regulatory and catalytic subunits using surface plasmon resonance. For these experiments the C-subunit was immobilized and binding of R was monitored. Panel A: rR(R209K); panel B: rR(R333K); panel C: rR(Δ1−91/R333K).

Surface plasmon resonance was also used to evaluate holoenzyme stability. With this method, binding constants can be measured at subnanomolar concentrations. rC-subunit was immobilized by amine coupling onto the carboxymethyl surface of a sensorchip. The active site Lys72 of the C-subunit was protected with 1 mM ATP and 2 mM $MgCl_2$ during immobilization (Herberg et al., 1994). Apparent association ($k_{assoc}$) and dissociation ($k_{dissoc}$) constants were determined in the presence of 100 $\mu$M ATP and 1 mM $MgCl_2$ (Figure 3). In the case of the wild-type protein it was necessary to use urea stripped R-subunit to obtain accurate $K_d$ values, whereas for the mutant proteins that had one cAMP-binding site unoccupied this was not necessary. Presumably, in the cell the cAMP is removed rapidly by phosphodiesterases not present in the *in vitro* system. As seen in Table 2, when the wild-type R-subunit was not stripped of cAMP, the $K_d$ was extremely high (about 100-fold higher) and clearly not consistent with values obtained with other procedures. Surprisingly, the R209K mutation showed a significant increase in affinity (app $K_d$ = 0.036 nm) compared to the rRIα (app $K_d$ = 0.19 nm). This increase was due to a 1 order of magnitude faster on-rate of $1.4 \times 10^6$ $M^{-1}$ $s^{-1}$ versus $1.2 \times 10^5$ $M^{-1}$ $s^{-1}$ for the wild-type protein. All mutants tested showed a similar increase in $k_{assoc}$ compared to the wild-type protein. The differences

Table 1: cAMP and ATP Off-Rates

| | $k_{off}$(cAMP), site A (min) | $k_{off}$(cAMP), site B (min) | ATP off-rates (min) | ATP off-rates, cAMP stripped |
|---|---|---|---|---|
| wild-type rRIα | 1 | 45 | 74[a] | 11 h |
| rR(R209K) | | 18 | 2.8 ± 0.5 | 91 min |
| rR(R333K) | 6 | | 4.2 | 6 h |
| rR(Δ1−91/333K) | 3 | | nd | nd |
| rR(Δ1−91) | 1.5 | 40 | nd | 10 h |

[a] In the presence of 1.5% cAMP (mol of cAMP/mol of holoenzyme).

Table 2: Quantitation of Interaction between Regulatory and Catalytic Subunits Using BIAcore[a]

| | $K_d$ (nM) | $k_{assoc}$ ($M^{-1}$ $s^{-1}$) | $k_{dissoc}$ ($s^{-1}$) |
|---|---|---|---|
| wild type* | 0.19 | $1.2 \times 10^5$ | $2.3 \times 10^{-5}$ |
| rR(R209K) | 0.036 | $1.4 \times 10^6$ | $5.1 \times 10^{-5}$ |
| rR(R333K) | 0.15 | $1.9 \times 10^6$ | $2.9 \times 10^{-4}$ |
| rR(Δ1−91/R333K) | 0.089 | $1.9 \times 10^6$ | $1.7 \times 10^{-4}$ |
| rR(Δ261−379 | 2.6 | $6.9 \times 10^5$ | $1.8 \times 10^{-3}$ |
| wild type | 11 | $8 \times 10^4$ | $8.9 \times 10^{-4}$ |

[a] As indicated in the Experimental Procedures, the experiments were performed in the presence of MgATP. In the case of the WT R-subunit, accurate $K_d$ values required that endogenous bound cAMP be stripped with urea (wild type*). All other proteins were used directly and not treated with urea. *The values were obtained for R-subunit which was stripped of cAMP by urea treatment.

in $K_d$'s are attributed mainly to the different $k_{dissoc}$ values. The rR(R333K) mutant, in particular, showed a similar increase of 1 order of magnitude in $k_{assoc}$, but no increase in affinity was found, because the $k_{dissoc}$ was also increased by approximately an order of magnitude. To check whether the rate constants determined with the immobilized C-subunit were affected by steric constraints, the binding of the C-subunit was also measured after the wild-type and the R209K mutant had been immobilized to a sensorchip. A $K_d$ of 0.42 nM and 0.074 nM was determined for the wild-type and rR(R209K), respectively, under these conditions, close to what was obtained with the immobilized C-subunit.

*cAMP Activation.* Activation of the wild-type and mutant holoenzymes was measured as described in the Experimental Procedures. The app $K_a$ for cAMP activation was altered significantly for holoenzyme formed with any of these mutant R-subunits. The activation constants for the holoenzyme formed with rR(R209K) and rR(R333K) were 1.7 $\mu$M [cAMP] and 1.4 $\mu$M, respectively (Figure 4). These $K_a$'s were 20-fold higher than for the wild-type protein (Table 3).

The activation constant was also determined for the double mutant R209/333K. Except for this double mutant, all activation constants were determined using highly purified proteins. Since the double mutant was very labile and thus difficult to purify, the $K_a$ for this mutant was measured directly in the supernatant of the cell lysate after centrifugation. When the $K_a$ for each of the single mutants was measured in a similar way in the cell lysate, the values were the same as those measured with the purified proteins. The $K_a$ for the double mutant was 2 $\mu$M, similar to the holoenzymes formed with each of the R-subunits containing a single mutation.

*ATP Off-Rates.* The off-rates of ATP from the holoenzyme complex were determined as described in the Experimental Procedures. As discussed earlier, to measure the $k_{assoc}$ and $k_{dissoc}$ of the wild-type R subunits, it was necessary to

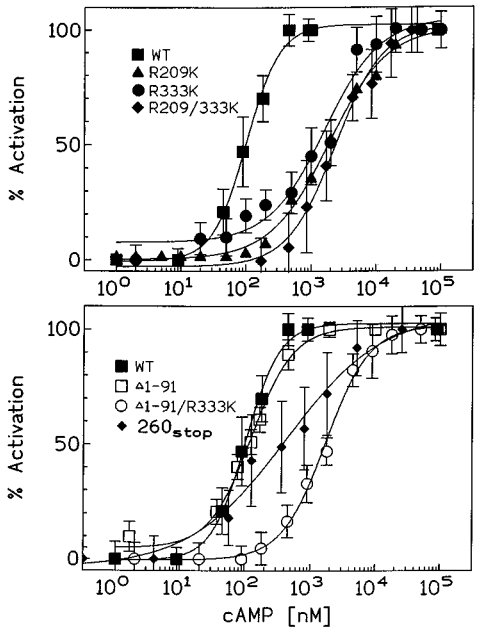


FIGURE 4: Activation of holoenzymes by cAMP. The app $K_a$'s for activation were measured as a function of cAMP as described in the Experimental Procedures. Upper panel shows holoenzyme formed with wild-type rRIα (■) and with the cAMP-defective R-mutants rR(R209K) ▲, rR(R333K) ●, and rR(R209K/R333K) ♦. The lower panel shows activation of holoenzyme formed with two Δ1−91 deletion mutants, rR(Δ1−91) □ and rR(Δ1−91/R333K) ○, and the 260 stop mutation ♦.

strip the cAMP from the protein. This stripping procedure, described previously, yields R-subunit that is comparable to the wild-type purified protein in terms of its cAMP binding properties. When cAMP is rebound, it also crystallizes in the same conformation, indicating that no major irreversible structural perturbations have occurred as a result of the stripping procedure (Y. Su and K. Varongese, unpublished results). Because holoenzyme re-forms so rapidly for the mutant R-subunits described here, it was not necessary to strip the mutant R-subunits. However, to measure the off-rates of MgATP, the mutant R-subunits were stripped of cAMP so that the results with the wild-type proteins were comparable. Thus for all R-subunits the ATP off-rates were compared for both stripped and unstripped proteins (Figure 5).

As seen in Table 1, the MgATP was locked firmly into the complex when the wild-type holoenzyme was formed with cAMP-free R-subunit. The $k_{dissoc}$ for R/C interaction is, in fact, identical to the off-rate for MgATP. The off-rate for holoenzyme formed with unstripped R-subunit was much faster. To determine whether this faster off-rate was due to a trace residual amount of cAMP bound to the R-subunit, cAMP was added to the holoenzyme formed with stripped R-subunit. cAMP sufficient to saturate only 1.5% of the cAMP-binding sites nevertheless led to an increase in the MgATP off-rate of over 10-fold (Figure 5).

The rate constants for the release of ATP from the two mutant holoenzymes were significantly accelerated in comparison to the wild-type protein. For the R209K mutant, the off-rate for holoenzyme formed with stripped R was 1.5 h and with nonstripped R 2.8 min. The rate was also accelerated for the rR(R333K) mutant (6 h for stripped R and 4.2 min for nonstripped, Figure 5). Thus, MgATP is not as tightly bound to these mutant holoenzymes.

*cAMP Binding to Holoenzymes.* As shown in Figure 4, activation of both mutant holoenzymes required high levels of cAMP. To determine whether cAMP was bound to the nonmutated site in either of the holoenzymes prior to activation, the binding of cAMP was determined using equilibrium dialysis conditions. In the presence of MgATP where stable holoenzyme is formed, no binding of cAMP was observed for the R209K mutant prior to activation (Table 3). When equilibrium dialysis was carried out in the absence of MgATP (Neitzel et al., 1991), cAMP binding reflects binding to the free R-subunit only, since, as shown by Herberg, et al. (1993), the complex under those conditions is dissociated.

For the wild-type holoenzyme, especially for the type I R-subunit, there is no evidence for a stable $R_2C_2cAMP_2$ intermediate. The R-subunit is either free of cAMP and bound to the C-subunit or the two cAMP-binding sites are occupied and R is dissociated. To further demonstrate whether a stable cAMP-bound form of holoenzyme exists for either of the mutants prior to dissociation, gel filtration was used. Holoenzyme (250 nM) and cAMP (600 nM) were incubated and immediately applied to a gel filtration column. This amount of holoenzyme corresponds to a total of 1000 nM cAMP-binding sites. Under these conditions, the concentration of cAMP was high enough to saturate the nonmutated site but not the mutated site. The concentration of cAMP also was only sufficient to saturate 60% of the total sites. When free R-subunit was subjected to similar gel filtration, cAMP remained firmly bound. However, when the mutant holoenzymes were separated on Superose 12 (Figure 6), no significant amounts of [$^3$H]cAMP were associated with the holoenzyme; essentially all of the [$^3$H]-cAMP eluted with the void volume. In a parallel experiment the percentage of dissociated holoenzyme was determined; no activation of the mutant holoenzyme was observed (Figure 6, inset). In contrast, the level of activation of the wild-type holoenzyme correlated with the amount of cAMP bound.

Thus, by the following criteria, no cAMP binds to either mutant holoenzyme prior to activation: (1) no high affinity binding to holoenzyme was measured by the ammonium sulfate precipitation assay, (2) no binding of cAMP to holoenzyme was measured by equilibrium dialysis, and (3) no cAMP binding to holoenzyme was measured by gel filtration.

## DISCUSSION

cAMP-dependent protein kinase is activated by the cooperative binding of cAMP to two tandem cAMP-binding domains in the regulatory subunit. This then promotes dissociation of the C-subunit. In an effort to better understand how each cAMP-binding site influences the adjacent site and how in molecular terms cooperativity is mediated, point mutations were generated in RI that contained one high affinity cAMP-binding site and one defective, low affinity binding site. The mutation in each site was the replacement of an essential Arg with Lys. In this way the contributions of Site A and Site B could be evaluated independently. After determining the cAMP binding properties of the free R-subunits, their binding properties were next evaluated in the respective holoenzyme complexes. In addition to holoenzyme activation, which is a gauge of the accessibility of each cAMP-binding site, the affinity of each holoenzyme complex

Table 3: cAMP Binding Properties

| | regulatory subunits | | holoenzymes | | |
|---|---|---|---|---|---|
| | $K_d$(cAMP) (nM) | cAMP bound (mol/mol) | $K_{d(cAMP)}$ (nM) | cAMP bound (mol/mol) | $K_a$(cAMP) + ATP (nM) |
| wild type | 22 ± 4 | 2.08 ± 0.19 | 166 ± 9 | 2.01 ± 0.04 | 101 ± 10 |
| rR(R209K) | 130 ± 14 | 1.17 ± 0.04 | 807 ± 150 | 1.06 ± 0.09 | 1700 ± 100 |
| rR(R333K) | 149 ± 26 | 0.93 ± 0.06 | >1500 | >0.6 | 1400 ± 200 |
| rR(Δ1−91/R333K) | 115 | nd | nd | nd | 1800 ± 200 |
| rR(R209/333K) | nd | nd | nd | nd | 2200 ± 300 |

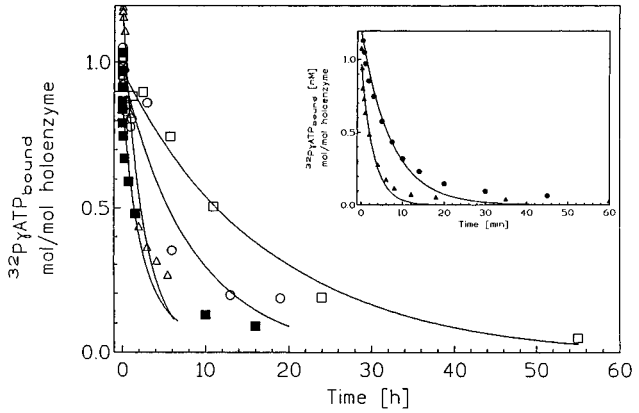


FIGURE 5: ATP off-rates for wild-type and mutant holoenzyme. The off-rates were determined for holoenzymes combined out of cAMP stripped (open symbols) and unstripped rR-subunits (closed symbols). □ rRIα, ○ rR(R209K), △ rR(R333K).

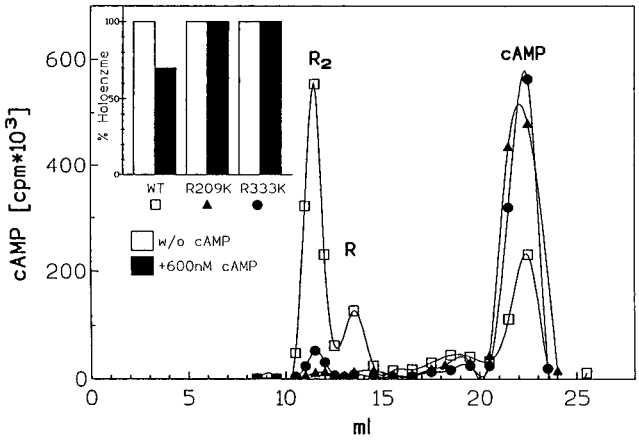


FIGURE 6: Gel filtration of holoenzymes with [$^3$H]-cAMP. 500 nM R- and C-subunits were labeled with 600 nM [$^3$H]cAMP as described under Experimental Procedures and injected on a Superose 12 gel filtration column. Only the wild-type rRIα showed cAMP binding at a retention time corresponding either to the dimeric R-subunit ($R_2$) or the holoenzyme. The inset shows the percentage of dissociated holoenzyme based on activity assays carried out in the presence and absence of cAMP.

for cAMP was determined, and the role of MgATP was evaluated.

In the native full length protein, Site A has a lower affinity (35 nM) than Site B (15 nM), and its off-rate for cAMP ($\tau_{1/2}$ = 1 min) is also faster than for Site B ($\tau_{1/2}$ = 45 min). When Arg209 in Domain A is replaced with Lys, cAMP is bound in the purified protein only to Site B with high affinity. The $K_d$ for Site B was increased to 130 nM, indicating that Site A exerts a positive influence (3-fold) on the binding affinity of Site B. In analogy, mutation of R333K also increased the $K_d$ for the nonmutated Site A to 149 nM. The off-rates for the high affinity site in rR(R209K) was 18 min faster than for Site B in wild-type R. The off-rate of cAMP for rR(R333K) was 6 min, slower than for Site A in wild-type R. Thus in these two mutants the remaining high affinity cAMP-binding sites were very similar in their cAMP binding properties. Figure 7 summarizes the various forms of free R in these three proteins and indicates also which form is measured by the ammonium sulfate assay. The $K_d$-(cAMP) for the mutated R209K Site A in rR(R209K) is greater than 1 μM, and the exchange rate is too fast to be measured by the standard ammonium sulfate precipitation assay. Based on quenching of the intrinsic fluorescence, due to cAMP binding to Site A, an app $K_d$ of 2 μM was measured for Site A in the R209 mutant (Leon and Taylor, unpublished results).

The $K_a$'s for activation of the mutant holoenzymes provide insights into the accessibility of each site when the R-subunit is complexed with the C-subunit. The $K_a$(cAMP) for holoenzyme formed with each of these mutant R-subunits was increased about 20-fold to approximately 1−2 μM even though each R-subunit retained one high affinity cAMP-binding site. In addition, the $K_d$(cAMP)'s for both mutants determined by the ammonium sulfate assay were also shifted to approximately 1 μM. The $K_d$(cAMP) for rR(R209K), measured directly by quenching of fluorescence, was very similar to the $K_a$(cAMP) of 1.7 μM. Thus, in each of these mutant holoenzymes activation was not achieved until both cAMP-binding sites are occupied. In the case of the R209K mutant, activation of the holoenzyme complex did not occur until Site A was occupied. Site B is functional and should be accessible, but occupancy of Site B in this mutant was not sufficient to activate the holoenzyme complex. In fact, no stable binding of cAMP to the holoenzyme could be demonstrated in the absence of dissociation. In the case of rR(R333K), Site A is normal, but it cannot be accessed until cAMP binds to Site B. Thus little cAMP was bound to the holoenzyme until cAMP concentrations were sufficient to saturate site B. Hence, the $K_a$ for activation of this mutant holoenzyme also reflects the $K_d$ of the mutated site but for different reasons. In this mutant only when cAMP bound to Site B did the high affinity binding site (Site A) become available.

In contrast to the free R-subunit, where both cAMP-binding sites are equally accessible, in the holoenzyme Site A is masked. Site B is preferentially accessible in the intact holoenzyme based on kinetic analysis (Øgreid & Døskeland, 1981a,b). Other studies with cAMP-binding site mutants (Zorn et al., 1995), deletion mutants (Ringheim et al., 1988; Saraswat et al., 1988), and cAMP analogs (Dostmann & Taylor, 1991), demonstrated that binding of cAMP to Site A is the critical step for dissociating the R−C complex. On the basis of the results described here, a clear model for the stepwise activation of the holoenzyme can be proposed (Figure 8). cAMP binds first to Site B, which then causes a conformational change that leads to the "opening up" of Site A. A second molecule of cAMP can then bind to Site

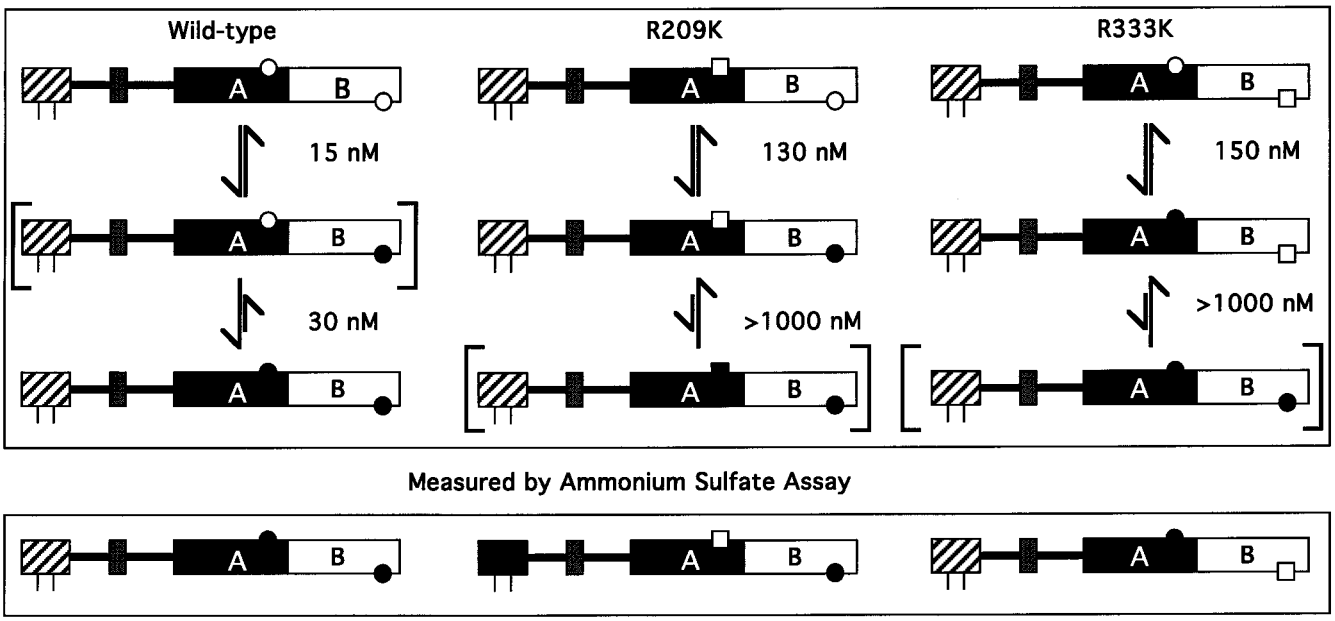


FIGURE 7: Model of the cAMP-binding sites in the free R-subunit. The functional sites, the dimerization domain, the inhibitor site, and cAMP-binding sites A and B are shown as boxes. Only one protomer is shown, with the lines showing the position of the two interchain disulfide bonds. High affinity (○, ●) and low affinity (□, ■) binding sites are indicated. Open and filled symbols correspond to empty and full cAMP-binding sites. Transient intermediates that cannot be detected are indicated in brackets. At the bottom in the box is indicated the form that is actually measured in the standard ammonium sulfate assay.

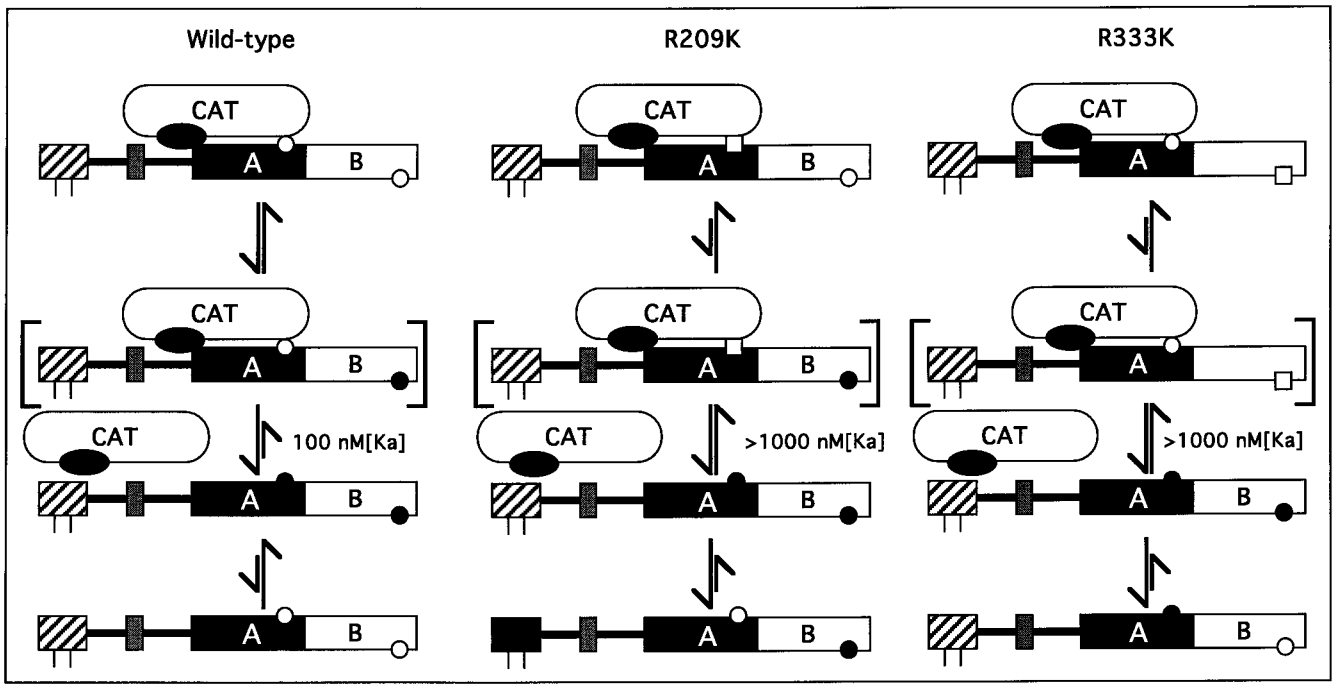


FIGURE 8: Model of the holoenzyme dissociation for wild-type and mutant holoenzyme. The symbols are the same as for Figure 7. The C-subunit, shown as an oblong, masks cAMP-binding site A in each complex. The closed oval corresponds to MgATP. Transient intermediates that cannot be detected are indicated in brackets. The form of the protein where cAMP binding can be measured readily is the same as in Figure 7.

A, and this causes the C-subunit to dissociate. In holoenzyme formed with rR(R209K) and rR(R333K), no activation takes place until the mutated low affinity site is occupied. This mechanism for activation is consistent with the proposed model for cAMP-binding site B (Weber et al., 1987) and the recently solved crystal structure of a mutant R-subunit (Su et al., in press, 1995). In this structure, each cAMP-binding site has an essential Arg that interacts with the exocyclic oxygens of cAMP. This Arg is at the C-terminus of a hydrophobic loop that bridges $\beta$-strands 6 and 7. At the beginning of this loop is a conserved Glu that binds to the 2′-OH of the ribose ring of cAMP. Cyclic AMP lies between the $\beta$ barrel and the C-helix. At the end of this helix is a loop that terminates with residue 259. Trp260 begins the A-helix of cAMP-binding domain B and is stacked against the adenine ring of cAMP bound to site A. This residue is also photoaffinity labeled by 8-$N_3$-cAMP bound to Site A and thus bridges the two cAMP-binding domains. Site B has a similar cAMP-binding motif, with Tyr371 stacking against the adenine ring of cAMP bound to Site B. Tyr371 is part of the C-helix in Domain B near at the C-terminus, while the N-terminus of this helix is close to

cAMP-binding site A. We predict that binding of cAMP to Site B will mediate conformational changes in Site A through movement of this C-helix and that these conformational changes lead to a more open Site A.

In addition to providing insights into the cooperative mechanism for holoenzyme formation, these mutations influenced interactions with the C-subunits. These effects were not apparent from the $K_a$'s for activation or from the $K_d$'s; however, they were clearly seen when the mutant R-subunits are added directly to C and when these differences in interaction were quantitated by surface plasmon resonance. Unlike native R-subunit, in the presence of MgATP both mutant R-subunits reassociate with C immediately even though one of the cAMP-binding sites is still saturated with cAMP. They titrate C just like the heat-stable protein kinase inhibitor, whereas for native R-subunit dialysis is required to remove cAMP before a stable holoenzyme complex forms. Surface plasmon resonance showed that the rates of association and dissociation were actually increased for each of the mutants by up to an order of magnitude. rR(R209K) actually bound more tightly to C than the wild-type R or rR(R333K) due to a selective increase in the rate of association. Without a crystal structure of the holoenzyme complex, it is still difficult to understand these ATP effects in molecular terms. Nevertheless, it is clear that extensive communication takes place between the various functional sites in this allosteric enzyme.

## ACKNOWLEDGMENT

We thank Michaela Zorn, Robin Chang, Iris Kühnert, Maria McGlone, Poopak Banky, and Sean Bell for technical support and for helpful advice.

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BI951647C