Interaction of the Regulatory and Catalytic Subunits of cAMP-dependent Protein Kinase

ELECTROSTATIC SITES ON THE TYPE Ia REGULATORY SUBUNIT*

(Received for publication, December 19, 1996, and in revised form, April 17, 1997)

Robin M. Gibson, Ying Ji-Buechler, and Susan S. Taylor†

From the Department of Chemistry and Biochemistry, University of California, San Diego,
La Jolla, California 92093-0654

Since a basic surface on the catalytic (C) subunit of cAMP-dependent protein kinase is important for binding to the regulatory (R) subunit, acidic residues in R were sought that might contribute to R-C interaction. Using differential labeling by a water-soluble carbodiimide (Buechler, T. A., and Taylor, S. S. (1990) Biochemistry 29, 1937–1943), seven specific carboxylates in RIα were identified that were protected from chemical modification in the holoenzyme; each was then replaced with Ala. Of these, rRI(E15A/E106A/D107A), rRI(E105A), rRI(D158A), rRI(E143A), and rRI(D258A) all were defective in holoenzyme formation and define negative electrostatic surfaces on Rα. An additional conserved carboxylate, Glu101 in RIα and the equivalent, Glu99 in RIIα were mutated to Ala. Replacement of Glu101 had no effect while rRI(E99A) was very defective. RIa was very defective. RII(E99A) was very defective.

This paper is available on line at http://www.jbc.org

---

* This work was supported in part by National Institutes of Health Grant GM34921 (to S. S. T.) and National Institutes of Health Training Grant T32 CA095223-08 (to R. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: University of Washington, Dept. of Pharmacology, Box 357750, Seattle, WA 98195-7750.

‡ Present address: EG&G Corp., 3550 General Atomics Ct., 02-544, San Diego, CA 92121.

¶ To whom correspondence should be addressed: University of California, San Diego, 92093-0654, La Jolla, CA 92095-0654. Tel.: 619-534-3677; Fax: 619-534-8193; E-mail: staylor@ucsd.edu.

1 The abbreviations used are: cAPK, cAMP-dependent protein kinase; R, cAMP-dependent protein kinase regulatory subunit; RI, type Ia regulatory subunit of cAMP-dependent protein kinase; RII, type IIa regulatory subunit of cAMP-dependent protein kinase; C, cAMP-dependent protein kinase catalytic subunit; β-ME, β-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl; PKI, heat stable protein kinase inhibitor; PRS, peripheral recognition site; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.
To chemically identify potential sites on the RI-subunit involved in R-C interaction, type I holoenzyme was treated with the water-soluble carbodiimide, 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide·HCl (EDC), and [14C]glycine ethyl ester in the absence and presence of cAMP (22). Five carboxyl groups in C were identified, Glu170, Asp228, Asp229, Glu322, and Glu323, that were protected in the holoenzyme complex. The amino acids that were labeled in the free RI-subunit were: Glu77, Glu105, Glu106, Asp107, Asp140, Glu143, Glu255, Asp258, and Glu275. The reactivity of seven of these residues (Glu105, Glu106, Asp107, Asp140, Glu143, Glu255, Asp258, and Asp258) was significantly reduced in the holoenzyme complex. To clarify the potential importance of these residues for R-C interaction, each was replaced with Ala. The mutant R-subunits were then purified and tested for their ability to inhibit C.

Several additional sites were tested. The highly conserved glutamic acid residue 150 (Glu101 in RI), when mutated to Ala in BCY1, the yeast regulatory subunit, led to defective recognition of C (TPK1). This site was therefore mutated to Ala in R. Sequence alignment of bovine RI and RII subunits with the yeast regulatory subunit, BCY1 is indicated for the segment that is required for high affinity binding to the C-subunit. Conserved residues, black boxes; autoinhibitor region, hatched bar; cAMP-binding domain A, solid line; acidic sites protected from chemical modification in the presence of C-subunit, *; additional acidic sites targeted by site-directed mutagenesis.}

**Experimental Procedures**

Materials—Reagents were obtained from the following sources: phosphono-pyruvate, magnesium chloride, reduced nicotinamide adenine dinucleotide (NADH), MES, MOPS, Tris, pyruvate kinase (rabbit muscle), and lactate dehydrogenase (bovine heart), Sigma; restriction endonucleases, T4 ligase, T7 polymerase, U. S. Biochemical Corp. or Life Technologies, Inc.; radioactive nucleotides, Amersham or NEN Life Science Products; and media supplies, Difco. The peptide substrate, LRRASLG, was synthesized at the Peptide Facility at the University of California, San Diego. Oligonucleotides were synthesized using an Applied Biosystem DNA synthesizer, Model 380B. The following bacterial strains were used: Escherichia coli DH5α, E. coli JM101 (ATCC), E. coli BL21-DE3 (gift from William Studier of Brookhaven National Laboratories, Upton, NY), and E. coli E222. The following media were used: phagemid pUC119 (ATCC), PLWS-3 (24), and PRSET-B (Invitrogen).

**Mutation of Charged Residues in RIα of cAPK**

**Fig. 1. Location of mutation sites on the type I regulatory subunit of the cAMP-dependent protein kinase.** Schematic diagram of the regulatory subunit: N-terminal dimerization domain (hatched box), autoinhibitor region (black box), cAMP-binding domains A and B (shaded boxes). Top, acidic sites protected from chemical modification in the holoenzyme complex. Bottom, additional charged residues that were mutated to Ala. Sequence alignment of bovine RI and RII subunits with the yeast regulatory subunit, BCY1 is indicated for the segment that is required for high affinity binding to the C-subunit. Conserved residues, black boxes; autoinhibitor region, hatched bar; CAMP-binding domain A, solid line; acidic sites protected from chemical modification in the presence of C-subunit, *; additional acidic sites targeted by site-directed mutagenesis.
BASES

TABLE I  
R-subunit mutations

| R-subunit | Mutation          | Reason for mutation |
|-----------|-------------------|---------------------|
| RI        | E105A/E106A/D107A | Sites protected from chemical modification in type I holoenzyme |
| RI        | E105A            |  | 
| RI        | E106A            |  | 
| RI        | D107A            |  | 
| RI        | D140A            |  | 
| RI        | E255A            |  | 
| RI        | D258A            |  | 
| RI        | E101A            | Conserved residue. Equivalent mutation in yeast R-subunit, BCY1, disrupted R/C interaction (Footnote 3). Not protected from chemical modification in type I holoenzyme |
| RI        | E99A             | Hydrogen bond with Arg^209 |
| RI        | D170A            | Conserved acidic residue in the A-domain of R. |
| RII       | E105A            | ( ) |
| RII       | E106A            | ( ) |
| RII       | RI(D170A)        | ( ) |
| RII       | RI(D107A)        | ( ) |

Con) prior to addition of glyceraldehyde. (Final concentration, 30%) and stored at −20 °C. To obtain cAMP-free R-subunit, the R-subunits were unfolded with 8 M urea, dialyzed, and then purified by gel filtration as described by Buechler et al. (10).

**Kinase Assay**—Kinase activity was measured spectrophotometrically using the peptidyl arginine substrate, LRRASLG (Kemptide), and a coupled enzyme assay (30). The concentration of C in the assay was between 200 and 60 μM. Holoenzyme samples were assayed first in the absence and then in the presence of 100 μM cAMP to obtain a ratio of activity representing the amount of free C in the mixture.

**Holoenzyme Formation**—cAMP-saturated native RI subunit (3 μM) and C-subunit (2 μM) were combined (0.8 ml) and dialyzed in a multiwell dialysis chamber against buffer B (25 mM potassium phosphate, 100 μM ATP, 500 μM MgCl2, 5 μM glycerol, 5 mM β-ME, pH 6.5) at 22 °C. Aliquots (30 μl) were removed at the indicated times and assayed for catalytic activity. The H6RII holoenzyme was formed at 4 °C in buffer C (25 mM potassium phosphate, pH 6.5, 5 μM glycerol, 5 mM β-ME, 0.1 mM EDTA, 0.1 mM EGTA) to minimize breakdown of the H6RII-subunit. Dialysis experiments were typically carried out in duplicate and were highly reproducible. As an alternative method for measuring holoenzyme formation, the activity of 20 nM C-subunit was titrated directly in the spectrophotometric assay by the addition of increasing amounts of R-subunit that had been stripped of cAMP by urea.

**Tryptophan Fluorescence**—R-subunit was dialyzed overnight against 1 liter of buffer D (5 mM MOPS, pH 7.0, 0.5 mM EDTA, 100 mM KCl, 5 mM β-ME) and against an additional 1 liter for 4 h to remove free CAMP. Fluorescence measurements were made using 1 ml of 0.2 μM R-subunit in a 1-cm quartz cuvette at 23 °C and a SLM Aminco SPF-500 spectrophotofluorimeter interfaced with a PC/AT computer. The excitation wavelength was 290 nm with a bandpass of 10 nm. The excited samples were scanned for tryptophan fluorescence from 300 to 450 nm using an emission bandpass of 5 nm. Spectra were analyzed using Spectra-Calc software. For the cAMP titrations, urea-ripped RI-subunit (50 μM) was incubated in buffer E (20 mM MOPS, 150 mM KCl, 5 mM β-ME, pH 7.0) with increasing concentrations of cAMP (0–100 μM) as described by León and Taylor.4 The excitation wavelength was 295 nm and the emission was measured at 347 nm.

**Apparent Activation Constants (K_a) for cAMP**—Apparent activation constants, K_a(cAMP), were determined according to Herberg et al. (32). Wild-type and mutant holoenzymes were prepared with 1.2 μM C-subunit and 1.0 μM R-subunit by dialysis overnight at room temperature against buffer F (20 mM potassium phosphate, 100 mM KCl, 5 mM β-ME, 5% glycerol, 100 μM ATP, 1 mM MgCl2, pH 6.5). Holoenzyme (30 nM) was incubated for 5 min at room temperature with cAMP (1–180 μM) in 1 ml of assay mixture prior to the addition of Kemptide substrate. Activity was then measured using the spectrophotometric assay. 

**Analytical Gel Filtration**—The Stokes radius was determined using an FPLC Superdex 200 column as described previously (28). The column was equilibrated with (20 mM MOPS, 150 mM KCl, 5 mM β-ME, pH 7.0) and run with a flow rate of 0.8 ml/min.

**Surface Plasmon Resonance**—Surface plasmon resonance was used to study the interaction between the R-subunit and the C-subunit of cAPK using a BIAcore instrument (Pharmacia/Biosensor) (33). The C-subunit was coupled to a sensor chip by direct amine coupling to the CM dextran surface (Biosensor Amine Coupling Kit) as described previously (32).

Kinetic constants were calculated using the BIAcore pseudo-first order rate equation,

\[
dR/dt = k_{ass}RC_{max} - (k_{ass}C + k_{dis})Rt \quad (Eq. 1)
\]

where \( k_{ass} \) is the association rate, \( k_{dis} \) is the dissociation rate, \( C \) is the concentration of the injected analyte, and \( R \) is the response. Plots of \( dR/dt \) versus \( Rt \) have a slope of \( k_{ass} \). When \( k_{dis} \) is plotted against \( C \) the resulting slope is equal to the \( k_{dis} \). The dissociation rate, \( k_{dis} \), was calculated by integrating the rate equation when \( C = 0 \), yielding \( \ln(Rt/R_0) = k_{dis}(t-t_0) \). Affinity constants were calculated from the equation

\[
K_a = k_{ass}/k_{dis}
\]

All binding interactions were performed at 23 °C in 20 mM MOPS, 150 mM KCl, 100 μM ATP, 1 mM MgCl2, 0.5 mM dithiothreitol, 0.005% P20, pH 7.0. After injections of the R-subunits the C-subunit surface was regenerated by injection of 10 μl of 10 μM cAMP. Association rate calculations were performed using concentrations between 30 and 500 nM for RI(WT), RI(D170A), and RI(K242A). All of the R-subunits were stripped of cAMP prior to injection.

**RESULTS**

To characterize the importance of various charged residues in the R-subunit for recognition of C and formation of holoenzyme, the following charge to Ala mutations were engineered: E101A, E105A/E106A/D107A, E105A, E106A, D107A, D140A, E143A, E255A, D258A, D170A, K242A in RI and E99A in RII (Table I). Each mutant RI-subunit was expressed in E. coli and purified as described under “Experimental Procedures.” The proteins were typically at least 80% pure following DE52 anion exchange chromatography and eluted as a sharp peak from the Superdex 200 gel filtration column. Typical yields were 40–50 mg of RI-subunit per 4 liters of cells. All of the purified RI-subunits were greater than 95% pure as judged by SDS-PAGE.

The poly-His-tagged RI-subunits, H6RII(WT) and H6RII(E99A), were purified from N2 resin by batch elution with imidazole. H6RII(E99A) was greater than 95% pure following FPLC Mono Q chromatography; however, degradation occurred upon long-term storage (>6 months) at −20 °C.

**Importance of the Acidic Patch Glu^105—Glu^106—Asp^107**—An
obtained in three separate experiments.

In addition, mutation of Glu 101 to Ala, generating the following mutants: rRI(D140A), rRI(E143A), and rRI(D258A) with Wild-type C—although Glu 101 was not modified by EDC in either the cAMP-bound form of RI or in the type I holoenzyme, it is highly conserved in all regulatory subunits. Furthermore, mutation of this site to Ala in BCY1, the yeast regulatory subunit, abolished the ability of BCY1 to inhibit the yeast C-subunit, TPK1. As shown in Fig. 3C, however, mutation of this site to Ala in the RI-subunit had no affect on the rate of holoenzyme formation compared with RI-WT.

The Role of Glu101 in Holoenzyme Formation—Although Glu101 was not modified by EDC in either the cAMP-bound form of RI or in the type I holoenzyme, it is highly conserved in all regulatory subunits. Furthermore, mutation of this site to Ala in BCY1, the yeast regulatory subunit, abolished the ability of BCY1 to inhibit the yeast C-subunit, TPK1. As shown in Fig. 3C, however, mutation of this site to Ala in the RI-subunit had no affect on the rate of holoenzyme formation compared with RI-WT.

The Effect of Mutating Asp79 in the A-Domain of RI—Another acidic residue that is conserved among the regulatory subunits of the cAPK family is Asp79. It lies in cAMP-binding domain A where it forms a hydrogen bond with Arg209, a residue that is absolutely conserved among the cAMP-binding domains of the regulatory subunits and the catabolite gene activator protein (16, 23). Although Asp79 lies on the surface of the A-domain, it also reaches through the domain to make contact with Arg209 in the cAMP-binding site. This hydrogen bonding between Asp79 and Arg209, which potentially helps to neutralize the charge on Asp79, would have prevented the identification of Asp170 in the holoenzyme protection study that identified Glu105, Glu106, Asp107, Asp140, Glu143, Glu255, and Asp258 as potential R-C interaction sites by chemical modification. Asp170 was mutated to Ala to test whether it played a role in holoenzyme formation or cAMP binding.

The wild-type RIα-subunit binds cAMP very tightly, so that the purified R-subunit is always fully saturated with cAMP. Thus, under the conditions used for holoenzyme formation where C, R, and cAMP are present at relative concentrations of 1:1:2, wild-type RI does not linearly titrate the activity of the C-subunit. Linear and stoichiometric titration is achieved only...
when cAMP is stripped from R prior to reassociation with C. Otherwise, dialysis is required to remove the cAMP. In contrast to wild-type RI, when rRI(D170A) was added to the C-subunit, inactivation was immediate; rRI(D170A) behaved similarly to R-subunit that had been stripped of cAMP with urea. rRI(D170A) was then tested for its ability to titrate the catalytic activity directly in the spectrophotometric assay. The inhibition assay measured the following reaction,

\[ \text{R}_2\text{cAMP}_4 + 2C \rightarrow \text{R}_2\text{C}_4 + 4\text{cAMP} \]  

(Eq. 2)

where loss of activity correlates with the formation of the R$_2$C$_4$ complex. As shown in Fig. 4, when rRI(D170A) was added directly to the assay mixture where the C-subunit concentration was 20 nm, inhibition of catalytic activity was linear and stoichiometric. This was in striking contrast to the inhibition of C with wild-type RI which must be stripped of cAMP before stoichiometric inhibition can be achieved under these conditions. The linear and stoichiometric inhibition of C with rRI(D170A) indicated not only that the $K_d$ for this mutant R and rC(WT) was well below 20 nm, but also, under these conditions, rRI(D170A) had a higher affinity for C than for cAMP, causing the equilibrium of reaction (2) to lie to the right. Several factors could account for this. The affinity for cAMP could be reduced so that the purified rRI(D170A) was not saturated causing the equilibrium of reaction (2) to lie to the right. The fluorescence spectrum of rRI(D170A) compared with rRI(R209K) because no cAMP is bound to site A in rRI(R209K). The fluorescence spectrum of rRI(D170A) compared with rRI(WT) and rRI(R209K) is shown in Fig. 5. The spectrum of rRI(D170A) was similar to that of the wild-type RI-subunit and showed significant quenching compared with rRI(R209K). Thus cAMP was bound to the A-domain of rRI(D170A), similar to rRI(WT) and in contrast to the rRI(R209K) where the A site is not occupied by cAMP. To confirm this, an apparent $K_d$ for cAMP binding was obtained by titrating the tryptophan fluorescence of the urea stripped proteins with increasing amounts of cAMP. As seen in Fig. 5 (inset), the apparent $K_d$ for cAMP binding to rRI(D170A) ($K_d = 45$ nm) was similar to that of rRI(WT) ($K_d = 45$ nm) and was comparable to the $K_d$ measured by equilibrium dialysis (36).

To further characterize the cAMP binding properties of rRI(D170A), the apparent $K_d$ for cAMP activation of the mutant holoenzyme was measured. The $K_a$ for cAMP activation of rRI(D170A) was 127 nm which correlates well with 88 nm for wild-type holoenzyme. This is also similar to the previously reported $K_a$ for wild-type holoenzyme (6). In contrast, the $K_a$ for cAMP activation of rRI(R209K) was 1700 nm (6).

Stokes Radius—The Stokes radius of rRI(D170A) was measured to determine whether the mutation had altered the global structure of RI. The native form of rRI(D170A) had a Stokes radius of 46.1 Å, similar to that of native RI (32). Furthermore, the urea-stripped cAMP-free form of rRI(D170A) had a Stokes radius that was 1.3 Å smaller than the native, cAMP-bound form. This is also similar to the wild-type protein which exhibited a 2 Å decrease in Stokes radius upon removal of cAMP (32). In contrast, RI(R209K), which is defective in binding cAMP to the A-domain, has a Stokes radius that is similar to the cAMP-free form of rRI(WT) (32).

The Effect of Mutating Lys$^{242}$—To establish whether Lys$^{242}$ in RI contributed to the high affinity binding between R and C, the mutant protein, rRI(K242A), was tested for its ability to inhibit wild-type C. Surprisingly, like the D170 mutant, inhibition of rC(WT) with rRI(K242A) did not require dialysis. rRI(K242A) also inhibited 20 nm rC(WT) in the spectrophotometric assay in a linear and stoichiometric fashion without being stripped of cAMP. This mutation also did not appear to affect cAMP binding, since the apparent $K_a$ for cAMP activation of the rRI(K242A) mutant holoenzyme was similar to that of wild-type holoenzyme.
Table II

Quantitation of interaction between regulatory and catalytic subunits using surface plasmon resonance

| RIstripped | $K_d$ | $k_{on}$ | $k_{off}$ |
|------------|------|---------|---------|
| nm         | $M^{-1} s^{-1} \times 10^4$ | s$^{-1}$ | s$^{-1}$ |
| WT         | 0.1  | 8.1 ± 2.8 | 8.0 ± 2.7 |
| D170A      | 0.2  | 15.0 ± 6.0 | 23.3 ± 3.6 |
| K242A      | 0.1  | 11.2 ± 4.0 | 9.0 ± 0.9 |

**Surface Plasmon Resonance**—One explanation for the ability of rRI(D170A) and rRI(K242A) to stoichiometrically inhibit C without first removing cAMP is that the mutations increased the affinity of RI for C. Surface plasmon resonance was used to quantitate the affinity of these mutant R-subunits for C. Using a BIAcore instrument (Pharmacia), cAMP-free rRI(D170A) and rRI(K242A) were applied to a chip containing immobilized wild-type C-subunit. R/C binding was measured by monitoring changes in the refractive index on the surface of the chip, allowing real-time binding kinetics to be measured. The results using rRI(D170A) and rRI(K242A) are compared with rR(WT) in Fig. 6. Both the on- and off-rates are monophasic for all three proteins. As summarized in Table II, although the overall binding affinities of rRI(D170A) and rRI(K242A) were similar to rR(WT), rR(D170A) exhibited on- and off-rates that were 2- and 3-fold faster than the wild-type control.

**DISCUSSION**

Many lines of evidence indicate that high affinity binding of the R and C subunits of cAPK is a multistep process that requires more than just a linear sequence of R that occupies the active site cleft of the C-subunit. The inhibitor site in R resembles a peptide substrate and presumably binds to the active site of C in a manner similar to the consensus sequence of PKI (15, 17). This inhibitor site extends from the P–3 site to the P+1 site and will be similar for most substrates and inhibitors of cAPK. This site, however, is not sufficient to confer high affinity binding. In addition to the inhibitor recognition site, an additional large surface on C is apparently masked by the R-subunit. This peripheral site on C, referred to here as PRS2, is more complex and will almost certainly depend on the global conformation of the R-subunit. Neither site alone is sufficient for high affinity binding. From the crystal structure of the catalytic subunit we can define the inhibition recognition site very precisely, and, coupled with genetic and mutational mapping, the surface that comprises the peripheral recognition site (PRS2) can be mapped. In contrast to the acidic inhibitor recognition site on C, where the consensus site Arg’s dock, the PRS2 surface is very basic.

For the R-subunit, the crystal structure of a deletion mutant (Δ1–91)RI has defined at least some of the structural features that will be recognized by the PRS2 surface on C. Deletion mutants localized most of the essential docking requirements to the A-domain while chemical studies identified specific residues that may be important. Because the R-docking surface on C is very basic, acidic residues in the A-domain of R were specifically targeted. The location of most of these residues in (Δ1–91)RI are shown in Fig. 7A. Each of these residues was replaced with Ala. These mutants will be discussed as three groups. First are those that cluster near the inhibitor site. An additional set of acidic residues were identified by differential labeling with EDC. The third set were identified based on their position on the crystal structure of (Δ1–91)RI.

Glu-101, highly conserved in all R-subunits, lies in the P+4 position just following the inhibitor site. Although this site is not important for R-C interaction in the type I holoenzyme, mutation of this site in both the mammalian and yeast type II R-subunits seriously disrupted holoenzyme formation. The difference in the role of this conserved glutamate in RI and RII highlights that these two types of R-subunit may differ in significant ways in how they interact with C. The interaction of RI and C is potentially much simpler and does not require tight binding of MgATP as does RI and PKI (37).

The structure of the region containing the autoinhibitor region, Arg$^{84}$-Arg$^{90}$-Glu$^{105}$-Glu$^{106}$-Asp$^{107}$, is disordered in the crystal structure of (Δ1–91)RI and is therefore not seen in the crystal structure which begins with Arg$^{113}$. The position of Glu$^{105}$, Glu$^{106}$, and Asp$^{107}$ immediately following the autoinhibitor region, and the fact that these carboxylates were protected from chemical modification in the holoenzyme suggested that these residues interacted with the C-subunit. Based on sequence analysis of RI, RII, and BCTY1 (34), the region corresponding to Glu$^{105}$-Glu$^{106}$-Asp$^{107}$ in RI is only partially conserved (Fig. 1). While RII has six acidic residues (Asp$^{104}$-Glu$^{105}$-Glu$^{106}$-Glu$^{107}$-Asp$^{108}$-Asp$^{109}$), in RI the acidic patch is truncated to Glu$^{105}$-Glu$^{106}$-Asp$^{107}$.
Glu108-Asp109) in this region, BCY1 contains only two (Asp 150 and Asp151). The mutations described here indicated that an acidic patch, Glu 105-Glu106-Asp107, in RI was important for binding C; however, the defect exhibited by the triple mutant, rRI(EED/AAA), did not represent an additive effect of each of the single mutants. In fact, rRI(E105A) was the only single-site mutant that was defective in holoenzyme formation. Since the triple mutant resulted in a stronger defect than the single-site E105A mutation, it is likely that an extended negative surface is important for R-C interaction.

In addition to the acidic patch Glu 105-Glu106-Asp107, four other acidic residues in RI were identified which were protected from chemical modification by EDC in the presence of C. These were: Asp140, Glu143, Glu255, and Asp258. Of these sites, only Asp140 is conserved in RI, RII, and BCY1 (Fig. 1). As shown in Fig. 6A, Asp 140 and Glu143 lie on the N terminus of the A-helix at the beginning of the A-domain. Glu255 lies 5 residues beyond the C-helix of the A-domain on a turn that bends back toward the cAMP-binding site and ends at Trp260. Asp 258 is on the same turn and points toward the cAMP-binding site. Although removal of none of these residues abolished holoenzyme formation, replacing Asp140, Glu143, or Asp258 with an Ala did significantly reduce the binding efficiency between RI and C. Only mutation of Glu255 had no effect.

As shown in Fig. 7B, the hydrogen bond network in the cAMP-binding pocket of the A-domain is extensive. At one end of the extended network is Asp 170. Asp170, on β-strand 3, is on the surface of the A-domain and its side-chain forms a hydrogen bond with Arg209, another conserved residue that is an essential feature of the cAMP-binding pocket. When CAMP is removed from the A-domain, this hydrogen bonding network in the cAMP pocket must change significantly. This could change the position of Asp170, causing it to either interact more tightly to Arg209, or to be exposed on the surface where it could interact with another basic residue. Thus, Asp170 was mutated to Ala to see if this acidic site in the A-domain influenced R-C interactions. Although removal of Asp 170 did not significantly alter the overall affinity for R and C, the D170A mutation resulted in on- and off-rates that were both slightly faster than those of wild-type. The faster on-rate in the absence of Asp 170 indicates that Asp 170 may stabilize the cAMP-bound conformation. This constraint would be its interaction with Arg209 as seen in the crystal structure. Removal of Asp 170, however, did not alter the affinity for CAMP. Asp 170 may help to stabilize the final holoenzyme conformation as well since the dissociation rate for the mutant protein was also 3-fold faster.

The overall charge on the surface of the A-domain is acidic, however, there is a basic patch on the C-helix of the A-domain that is associated with Lys242. The adjacent residue, Arg241, is a critical residue for cooperativity between domain A and B (38). As shown in Fig. 7B, Arg241 forms hydrogen bonds with residues that reside both in the A-domain (Glu200) and in the B-domain (Asp267). Asp267 is the only residue in domain B that contributes directly to the hydrogen bond network associated with cAMP binding in site A. Both Arg241 and Lys242 lie on the C-helix of domain A, and the orientation of this helix relative to the β-barrel will very likely change when cAMP is released. When the structures of domains A and B of (Δ1–91)RI and the nucleotide-binding domain of CAP are superimposed, the most striking difference is the position of the C-helix (16). In the A-domain, the C-helix is longer, slightly bent and held away from the cAMP binding site. Furthermore, although Trp260, at the end of the loop that follows the C-helix was photoaffinity labeled with the cAMP analog, 8-azido-cAMP (8-N3-cAMP), in full-length RI, in a B-domain deletion mutant, RI(Δ260–379), Trp244 was labeled instead (31). Thus the orientation of the C-helix in the A-domain relative to the cAMP-binding pocket is capable of a large conformational change. The B-domain with

---

**Fig. 7. Location of mutation sites on the x-ray crystal structure of (Δ1–91)RI.** A, ribbon diagram. The N terminus, beginning with 113 and the cAMP-binding domain B are dark gray and the A-domain is white. CAMP is occupies each of the cAMP binding sites. B, hydrogen bond network in the cAMP-binding domain A. Asp170 is linked to the highly ordered hydrogen bond network surrounding cAMP via its interaction with NH2 of Arg209. Arg241 is located on the C-helix of domain A and Asp267 lies on the C terminus of the A-helix of domain B.

---

Mutation of Charged Residues in RIα of cAPK
its strong hydrophobic interactions with the C-helix exerts structural constraints on the conformation of the A-domain (16).

The surface of C that is thought to interact with R is mostly basic with the exception of the negative charge associated with the phosphorylated Thr197. Lys242 was thus targeted as a potential electrostatic interaction site for P-T197 in the holoenzyme complex. Like rRI(D170A), rRI(K242A) inhibited rC(WT) for cAMP activation. Why removal of this Lys actually facilitates R-C interaction is still unclear. To fully understand the precise molecular details of R-C interaction, a crystal structure of the holoenzyme complex is required.

Acknowledgments—We thank Dr. Sarah Cox and Dr. Fritz Herberg for valuable discussion. We also thank Lily Jun-shen Huang for useful discussions and assistance in measuring the surface plasmon resonance of the mutant R-subunits.

REFERENCES
1. Hofmann, F., Beavo, J. A., Bechtle, P. J., and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795–7801
2. Scott, J. D., Fischer, E. H., Takio, K., Demaillie, J. G., and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5732–5736
3. Titani, K., Sasagawa, T., Ericsson, L. H., Kumar, S., Smith, S. B., Krebs, E. G., and Walsh, K. A. (1984) Biochem. 23, 4193–4199
4. Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., and Titani, K. (1984) Biochemistry 23, 4200–4206
5. Hofmann, F. (1980) J. Biol. Chem. 255, 1559–1564
6. Herberg, F. W., Taylor, S. S., and Dostmann, W. R. G. (1995) Biochemistry 34, 2934–2942
7. Walsh, D. A., Angelos, K. L., Van Patten, S. M., Glass, D. B., and Garetto, L. P. (1990) in CRC Reviews (Kemp, B. E., ed) pp. 43–84, CRC Press, Inc., Boca Raton, FL
8. Zetterqvist, O. Z., Ragnarsson, U. and Engström, L. (1990) in CRC Reviews (Kemp, B. E., ed) pp. 171–187, CRC Press, Inc., Boca Raton, FL
9. Glass, D. B., Cheng, H.-C., Mende-Mueller, L., Reed, J., and Walsh, D. A. (1989) J. Biol. Chem. 264, 8802–8810
10. Buechler, Y. J., Herberg, F. W., and Taylor, S. S. (1993) J. Biol. Chem. 268, 16495–16503
11. Wang, Y. H., Scott, J. D., McKnight, G. S., and Krebs, E. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2446–2450
12. Weldon, S. L., and Taylor, S. S. (1985) J. Biol. Chem. 260, 4203–4209
13. Saraswat, L. D., Ringheim, G. E., Bubis, J., and Taylor, S. S. (1988) J. Biol. Chem. 263, 18241–18246
14. Ringheim, G. E., and Taylor, S. S. (1990) J. Biol. Chem. 265, 19472–19478
15. Knighton, D. R., Zheng, J., Ten Eyk, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 407–414
16. Su, Y., Dostmann, W. R. G., Herberg, F. W., Durick, K., Xuong, N.-h., Ten Eyk, L. Taylor, S. S., and Varughese, K. I. (1995) Science 269, 807–813
17. Knighton, D. R., Zheng, J., Ten Eyk, L. F., Xuong, N.-h., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 414–420
18. Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S., and Zoller, M. J. (1992) J. Biol. Chem. 267, 4806–4814
19. Levin, L. R., and Zoller, M. J. (1990) Mol. Cell. Biol. 10, 1066–1075
20. Orellana, S. A., and McKnight, G. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4726–4730
21. Orellana, S. A., Amieux, P. S., Zhao, X., and McKnight, G. S. (1993) J. Biol. Chem. 268, 6843–6846
22. Buechler, J. A., and Taylor, S. S. (1990) Biochemistry 29, 4937–4943
23. Bubis, J., Neitzel, J. J., Saraswat, L. D., and Taylor, S. S. (1988) J. Biol. Chem. 263, 9668–9673
24. Slice, L. W., and Taylor, S. S. (1989) J. Biol. Chem. 264, 20940–20946
25. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
26. Saraswat, L. D., Filutowics, M., and Taylor, S. S. (1988) Methods Enzymol. 159, 325–336
27. Saraswat, L. D., Filutowicz, M., and Taylor, S. S. (1986) J. Biol. Chem. 261, 11091–11096
28. Herberg, F. W., Bell, S. M., and Taylor, S. S. (1993) Protein Eng. 6, 771–777
29. Lein, D. A., Dostmann, W. R. G., and Taylor, S. S. (1991) Biochemistry 30, 3035–3040
30. Cook, P. F., Vrana, E. H., Carman, K. E., Hartl, F. T., and Roskoski, R., Jr. (1982) Biochemistry 21, 5794–5799
31. Ringheim, G. E., Saraswat, L. D., Bubis, J., and Taylor, S. S. (1988) J. Biol. Chem. 263, 18247–18252
32. Herberg, F. W., Dostmann, W. R. G., Zorn, M., Davis, S. J., and Taylor, S. S. (1994) Biochemistry 33, 7485–7494
33. Karlsson, R., Michaelsson, A., and Mattsson, L. (1991) J. Immunol. Methods 145, 229–240
34. Toda, T., Cameron, S., Sasso, P., Zoller, M. J., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G., and Wigler, M. (1987) Mol. Cell. Biol. 7, 1371–1377
35. Builder, S. E., Beavo, J. A., and Krebs, E. G. (1980) J. Biol. Chem. 255, 2350–2354
36. Ogreid, D., Ekanger, R., Suka, R. H., Miller, J. P., and Doskeland, S. O. (1989) Eur. J. Biochem. 181, 19–31
37. Herberg, F. W., and Taylor, S. S. (1993) Biochemistry 32, 14015–14022
38. Symons, M. M., Cauthron, R. D., Ogreid, D., and Steinberg, R. A. (1994) J. Biol. Chem. 269, 23925–23931