Effects of cAMP-binding Site Mutations on Intradomain Cross-communication in the Regulatory Subunit of cAMP-dependent Protein Kinase I*

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Each protomer of the regulatory subunit dimer of cAMP-dependent protein kinase contains two tandem and homologous cAMP-binding domains, A and B, and cooperative cAMP binding to these two sites promotes holoenzyme dissociation. Several amino acid residues in the type I regulatory subunit, predicted to lie in close proximity to each bound cyclic nucleotide based on affinity labeling and model building, were replaced using recombinant techniques. The mutations included replacement of 1) Glu-200, predicted to hydrogen bond to the 2'-OH of cAMP bound to site A, with Asp, 2) Tyr-371, the site of affinity labeling with 8-N3-CAMP to the 2'-OH of CAMP bound to site A, with Asp, 2) Tyr-371, the site of affinity labeling with 8-N3-CAMP differed markedly for each mutant protein. Furthermore, these mutations affect the cAMP binding properties not only of the site containing the mutation, but of the adjacent nonmutated site as well, thus confirming that extensive cross-communication occurs between the two cAMP-binding domains. Photoaffinity labeling of the native R-subunit results in the covalent modification of two residues, Trp-260 and Tyr-371, by 8-N3-cAMP bound to sites A and B, respectively, with a stoichiometry of 1 mol of 8-N3-cAMP incorporated per mol of R-monomer (Bubis, J., and Taylor, S. S. (1987) Biochemistry 26, 3478-3486). A stoichiometry of 1 mol of 8-N3-cAMP incorporated per R-monomer was observed for each mutant regulatory subunit as well, when 2 mol of 8-N3-cAMP were bound per R-monomer; however, the major sites of covalent modification were altered as follows: R(Y371/W), Trp-371; R(E200/D), Tyr-371, and R(F247/Y), Tyr-371.

The regulatory subunits of cAMP-dependent protein kinase contain two tandem and homologous but nonequivalent cAMP-binding domains at the carboxyl terminus (1, 2). These sites can be distinguished on the basis of preference for binding different analogs of cAMP as well as kinetically by measuring off-rates of bound cAMP (3, 4). The first site in the linear sequence, site A, preferentially binds analogs of cAMP containing substitutions at the N6 position of the adenine ring, and cAMP dissociates very slowly from site B in the absence of catalytic subunit (3, 4). Binding of cAMP as well as activation of the catalytically inert holoenzyme complex by cAMP show positive cooperativity. This positive cooperativity results primarily from interactions between the two tandem cAMP-binding domains within each protomer of the regulatory subunit (5, 6). The dimeric form of the regulatory subunit is not essential for this positive cooperativity (7), whereas deletion of cAMP-binding site B abolishes positive cooperativity (8).

These two cAMP-binding domains found in all regulatory subunits share extensive sequence similarities not only with each other but also with the catabolite gene activator protein (CAP) of Escherichia coli. On this basis, Weber et al. (9) constructed a model of each cAMP-binding domain by building the amino acid sequences of the cAMP-binding domains for the type I and type II regulatory subunits into the CAP crystal structure. This model of the cAMP-binding domains of the regulatory subunits is consistent with photoaffinity labeling using 8-azido-cyclic adenosine 3',5'-monophosphate (8-N2-CAMP) that identified specific amino acid residues in proximity to the C-8 position of the adenine ring of bound cAMP for each cAMP-binding site (10, 11). Site-specific mutations in the regulatory subunit also confirm the validity of this CAP-based model as a general framework for the folding of the subpeptide chain in each CAMP-binding domain (12, 13).

Several of the amino acids that contribute directly to cAMP binding in CAP are highly conserved in all known regulatory subunit sequences. Each cAMP-binding domain contains, in particular, an invariant Arg and Glu. The conserved Arg in CAP is thought to ion pair with the exocyclic phosphate oxygens of bound cAMP while the conserved Glu is predicted to hydrogen bond with the 2'-OH of the ribose ring of bound cAMP (9). The corresponding residues in the type I regulatory subunit are Arg-209 and Glu-200 in site A and Arg-343 and Glu-334 in site B. Replacement of Arg-209 with Lys abolishes high affinity cAMP binding to site A thus confirming the prediction that this residue is essential for interaction with all cAMP analogs.

The abbreviations used are: CAP, catabolite gene activator protein; HPLC, high performance liquid chromatography; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; 8-N2-cAMP, 8-azido-cyclic adenosine 3',5'-monophosphate.

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bound CAMP (13). Whether or not Glu-200 is likewise essential has not been tested previously.

Three new point mutations in the R'-subunit are described here. Two of these mutations were designed as a result of photoaffinity labeling studies which revealed that Tyr-371 and Tyr-381 in the CAMP-binding site B of the R' and R''-subunits, respectively, were close to the C-8 position of the adenine ring of bound 8-N<sub>C</sub> CAMP (10, 11). One mutation replaced Tyr-371 in the B domain with a Thr. The other mutation replaced Phe-247 in site A with a Tyr. The position of Phe-247 in domain A of the R'-subunit is homologous to Tyr-371 in domain B. The third mutation replaced the conserved residue in site A, Glu-200, with an Asp in order to evaluate possible interactions between Glu-200 and the 2'-OH of bound CAMP. The effects of all three mutations on CAMP binding affinities, ATP binding properties, CAMP off-rates, and photoaffinity labeling were studied.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents were purchased as follows: CAMP, ATP, bovine serum albumin, 8-azido-cyclic adenosine 3':5'-monophosphate (8-N<sub>C</sub>CAMP), CM-Sepharose CL-6B, Sigma; [2-8-3H]CAMP (27 Ci/mmol), [32-3H]adenosine 3':5'-monophosphate (30 Ci/mmol), Ecolte, ICN; 8-<sup>N</sub>C cyclic adenosine 3'-5'-monophosphate (50 Ci/mmol), Du Pont-New England Nuclear Research Products; trfluoroacetic acid, sequential grade, Pierce Chemical Co.; filters type HA (0.45 μm), Millipore Corporation; QNTPs, P-L Biochemicals; L-1-tyosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin, Sequenase Kit, U. S. Biochemical Corp.; Luria broth (L-broth) and acetonitrile (HPLC grade). Fisher Enzymes used in DNA manipulations were from either Bethesda Research Laboratories or Boehringer Mannheim and were used according to the manufacturer's specifications. All other reagents were analytical grade. Catalytic subunit was purified from porcine heart according to Nelson and Taylor (14).

**Mutagenesis—**The bacterial expression vector for the type I regulatory subunit, pLST2, was described previously (15). The procedure used for mutagenesis was described by Durgerian and Taylor (16).

The oligonucleotide probes used to introduce the point mutations are shown in Fig. 1. The oligonucleotides were synthesized on an Applied Biosystems, Inc. (Foster City, CA) DNA synthesizer at the Peptide and Oligonucleotide Synthesis Facility at the University of California, San Diego. Photoaffinity labeling was accomplished with 12.5% acrylamide as described by Laemmli (23). After electrophoresis on SDS-polyacrylamide gels followed by visualization after staining with Coomassie Brilliant Blue R 250. The plasmids isolated from these cells were released as pLST5a for the Tyr-311 to Trp mutation, pLST1b for the Phe-247 to Tyr mutation, and pLST5c for the Glu-200 to Asp mutation. The proteins expressed by these mutant constructs were electrophoresed on SDS-polyacrylamide gels followed by autoradiography. R(dy51/W), R(PR247/Y), and R(E200/D), respectively. The first amino acid symbol before the number refers to the residue found in the native R'-subunit and the following amino acid symbol represents the residue that has replaced it. The r indicates that the regulatory subunit has recombinant origin.

**Purification of the Mutant and Native R'-subunits—**E. coli 222, transformed with the appropriate plasmid, was grown in L-broth containing ampicillin 50 μg/ml as described previously (8). After rupturing in a French pressure cell, the regulatory subunit was purified by affinity chromatography using CAMP Sepharose (N<sub>4</sub> ethane spacer) as described earlier (8). Native R'-subunit and the deletion mutants, rR(AA-390), rR(AA-390) rR(ΔA-90, Δ260-379), were purified as described previously (8, 15).

Protein concentration was measured by the method of Bradford (18) and verified by comparison to the CAMP binding capacity as described below.

**Holoenzyme Formation—**Holoenzyme was formed as described by First and Taylor (19) by incubating the purified R'-subunits (0.5-1.0 mg/ml) with a 10% molar excess of catalytically active R'-monomer and dialyzing for 48 h against 20 mM potassium phosphate (pH 6.5), 5 mM β-mercaptoethanol, 10-4 M ATP, and 5×10-4 M MgCl<sub>2</sub>. The ATP and MgCl<sub>2</sub> were then removed by dialysis for 48 h against 40 mM potassium phosphate (pH 6.1) containing 2 mM EDTA, 5% glycerol, and 8 mM β-mercaptoethanol (buffer 1). Excess free catalytic subunit was removed by CM-Sepharose CL-6B (1 ml of resin/5 mg of excess catalytic subunit).

**Measurement of CAMP Binding and Exchange Rates—**The binding of CAMP was measured by ammonium sulfate precipitation followed by filtration through Millipore filter discs according to the method of Drisko and Ørsted (20). The CAMP off-rates for bound CAMP was carried out under both high (3.5 mM NaCl) and low (0.15 mM NaCl) salt conditions (8). After saturating the R'-subunit with [3H]CAMP, the solution was incubated for the designated times at 20 °C with a 200-fold excess of nonlabeled CAMP. All measurements were carried out twice and repeated for at least two different batches of each protein. Variability was typically ±5%. The CAMP off-rate, t<sub>1/2</sub>, is expressed as the time required for 50% of the [3H]CAMP to be released.

**Activation Assays—**Protein kinase activity was assayed spectrophotometrically (21) using the synthetic peptide, L-R-R-W-S-V-A-Trp.

**Photoaffinity Labeling—**Aliquots (100 μl) of the wild type and mutant holoenzymes (20 nm) were incubated with 1 μM 8-N<sub>C</sub>CAMP on ice for 10 min as indicated previously (10, 11, 22). To measure total binding, duplicate aliquots of 20 μl were removed, precipitated with 95% ammonium sulfate, filtered, and counted in a liquid scintillation counter as described above. The remaining samples were photolyzed for 10 min using a short wave UV 811 lamp (254 nm) at a distance of 5 cm. In order to determine the amount of nonlabeled CAMP that was bound to a holoenzyme after incubation with nonradioactive CAMP, and the mixture was incubated at 37 °C for 3 h, prior to ammonium sulfate precipitation and filtration. As a control for maximum binding and to confirm that the conditions for the chase procedure were sufficient to remove all noncovalently bound nucleotide, an aliquot of each sample was also incubated with [3H]CAMP as described above and assayed for CAMP binding after the chase incubation with nonradioactive CAMP.

**Identification of the Photolabeled Residues—**Residues 2 mg of each holoenzyme in 1 ml were dialyzed against buffer I, incubated with a 1.5 molar excess of 8-N<sub>C</sub>CAMP over CAMP-binding sites, and photolabeled as described above. After photolabeling exhaustively against 10 mM potassium phosphate (pH 8.0), the samples were digested with TPCK trypsin and the resulting tryptic peptides resolved by high performance liquid chromatography (HPLC) under the conditions described previously (22). After carrying out the first I1PLC elution at pH 6.8 and eluting with a gradient of 0-60% acetonitrile, each pooled radioactive peak was reinjected on a second identical reversed phase column using a 0.1% trifluoroacetic acid (pH 2.13) buffer and a gradient of 0-15% acetonitrile in 60 min. Radioactive peaks were collected, lyophilized, and resuspended in 0.1% trifluoroacetic acid (pH 2.13), and sequenced using an Applied Biosystems model 470A gas phase protein sequencer with an on-line HPLC system for detection of the PTH-derivative. Typical yields of radioactivity after the first HPLC column were 60-70%.

**Glycolylase Gel Electrophoresis—**Slab gels were prepared with 12.5% acrylamide as described by McPherson (22). After electrophoresis, the gels were stained with 10% glacial acetic acid, 25% isopropanol, and 0.25% Coomassie Blue R 250.
RESULTS

Construction of the Mutant Plasmids—Three point mutations in the cAMP-binding domains of the R'-subunit were introduced using the synthetic oligonucleotides described in Fig. 1. The three mutant proteins produced were: 1) rR(Y371/W), where the Tyr in the cAMP binding domain B known to be in the vicinity of the C-8 position of the adenine ring of bound cAMP (10, 11) was converted to a Trp; 2) rR(F247/Y), where the residue in cAMP-binding domain A homologous to Tyr-371 (9) was converted to Tyr; and 3) rR(E200/D), where the Glu-200 residue in the A domain, thought to form a hydrogen bond with the 2'-OH of bound cAMP (9), was converted to Asp. The location of each of these sites of mutation with respect to the overall domain structure of the regulatory subunit is shown in Fig. 2. The mutagenesis was carried out as described under "Experimental Procedures."

Expression and Purification of Mutant Proteins—Following transformation of E. coli 222, several colonies expressing large amounts of a protein migrating with an apparent M, of approximately 49,000 following polyacrylamide gel electrophoresis were identified. The mutant proteins expressed in these cells were then purified to homogeneity by affinity chromatography using CAMP-Sepharose. A typical yield was approximately 15-20 mg per liter of culture.

General Structural Features—All the mutant R'-subunits retained many of the general properties of the native R'-subunit. The expressed proteins did not appear to be significantly more susceptible to proteolysis than the native protein, since no major degradation products were observed. A slight amount of proteolysis was observed on storage with both the mutant rR-subunits and the wild type rR-subunit. Sequence analysis revealed the proteolytic fragment to be a mixture of two fragments beginning at either Arg-92 or Arg-93. This endogenous cleavage site lies in the hinge region known to be susceptible to proteolysis in the native protein (24-26).

Holoenzyme Formation—All three mutant R'-subunits reassociated with the catalytic subunit yielding an enzymatically inactive holoenzyme complex in vitro. Based on staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the rR-subunit and the catalytic subunit were present in a molar ratio of approximately 1:1 in each holoenzyme. Activation of each holoenzyme complex was achieved by the addition of cAMP.

cAMP Binding—As seen in Fig. 3, each holoenzyme bound 2 mol of cAMP per regulatory subunit monomer. The apparent Kd for the wild type holoenzyme was 17 nM with a Hill coefficient of 1.3. The apparent Kd values for the rR(Y371/W), rR(F247/Y), and rR(E200/D) holoenzyme complexes were each 34 nM, with Hill coefficients of 1.4. cAMP binding was measured only in the absence of MgATP.

cAMP Exchange Rates—The off-rate of bound [3H]cAMP was measured for the wild type and mutant R'-subunits under both low and high salt conditions, and the results are summarized in Table I and Fig. 4. For the wild type R'-subunit, the cAMP off-rates in low salt were 4.5 and 55 min for sites A and B, respectively. Under high salt conditions, the off-rate for cAMP from site A was increased to 1.8 min while the off-rate for cAMP bound to site B was reduced to 140 min.

All three mutant proteins had faster cAMP exchange rates than the wild type protein. The rR(F247/Y) subunit most resembled the wild type rR-subunit. The low salt off-rates were 1.5 and 12.6 min, respectively, for site A, while the high salt off-rates were 1.3 and 45 min, respectively. The cAMP off-rates for the rR(Y371/W)-subunit were much faster. The off-rates in low salt were 1 and 4.2 min for sites A and B, respectively. Under high salt conditions, the off-rate for cAMP from site A was increased to 1.8 min while the off-rate for cAMP bound to site B was reduced to 140 min.

The relative rates for sites A and B compared to the wild type are indicated in parentheses.

### Table 1

| Protein | Low salt | High salt |
|---------|----------|-----------|
|         | Site A | Site B | Site A | Site B |
| rR | 4.5' (1.0) | 55' (1.0) | 1.8' (1.0) | 140' (1.0) |
| rR(F247/Y) | 1.5' (3.0) | 12.6' (4.4) | 1.3' (1.4) | 45' (3.1) |
| rR(Y371/W) | 1.0' (4.5) | 4.2' (13.1) | 23' (4.7) | 2.2' (63.6) |
| rR(E200/D) | 15' (18) | 5' (11) | ND* |

* ND, not determined.
had off-rates of 15s and 5 min for sites A and B, respectively, under low salt conditions.

**ATP Binding**—The holoenzymes formed with all of the mutant R-subunits retained the high affinity MgATP-binding site observed in the wild type holoenzyme. As seen in Fig. 5, the wild type and mutant holoenzyme complexes each bound 2 mol of MgATP per holoenzyme complex, had identical $K_d$ values of 15–20 nM, and had identical Hill coefficients of 1.6. Thus, although these three mutations exhibited altered CAMP binding properties to varying degrees, each showed no alterations in the MgATP-binding site in contrast to two deletion mutants where slight differences were observed (8). The effect of MgATP on the apparent $K_d$ for CAMP and on the induced off-rate for CAMP in the presence of the catalytic subunit has not been determined yet for these mutants.

**Photolabeling of the Mutant Proteins**—The stoichiometry of 8-N3-[3H]cAMP incorporation revealed that the R-subunit, rR(Y371/W), rR(Y247/Y), and rR(E200/D) all bound 2 mol of label per mol of subunit but covalently incorporated only 1 mol of label per mol of subunit (Table II). Typically, no radioactivity was incorporated in the presence of an excess (100 μM) of cold cAMP. After removing the noncovalently bound 8-N3-[3H]cAMP, the remaining unlabelled sites were saturated readily with [3H]cAMP. The stoichiometry for labeling two deletion mutants, rR(Δ260–379) and rR(Δ1–92; Δ260–379), was also indicated in Table II. These two proteins still incorporate 0.9 mol of 8-N3-[3H]cAMP per mol of R-subunit, but only a single residue is modified, Tyr-244. Thus, in these two mutants binding and covalent modification are similar.

In order to analyze photoaffinity labeling of the mutant subunits more precisely, 2 mg of each protein were photolyzed in solution with 8-N3-[3H]cAMP, digested with TPCK-trypsin, and the resulting tryptic peptides resolved by HPLC as indicated in Fig. 6. Major fractions containing radioactivity were pooled and rechromatographed using the same column but a different gradient. Each of the radioactive peptides from this second gradient were lyophilized, resuspended in 0.1% trifluoroacetic acid, and sequenced. The radioactivity remained bound to the filter during sequencing, and hence it was not possible to directly correlate the radioactivity with the step missing in the sequence. Instead, at the position that is photolabeled, no residue was identified in contrast to a clearly resolved PTH-derivative at the corresponding position in the unmodified peptide. The missing residue at these steps can thus be identified unambiguously as the site of covalent modification.

The major sites of modification were identified as: Trp-371 in rR(Y371/W), Tyr-371 in rR(F247/Y), and Tyr-371 in rR(E200/D). The mutation in rR(Y371/W) eliminates most of the photolabeling of the A domain. In this case, the tryptophan in the B domain that replaced Tyr-371, normally labeled in the wild type R-subunit, was the primary site of photolabeling. The two conservative point mutations in rR(F247/Y) and rR(E200/D) also were sufficient to eliminate most photolabeling of the A domain. In both of these mutants, Tyr-371 in domain B was the dominant site of photolabeling. The reason why this peptide containing Tyr-371 elutes in various positions is not clear but may be due to different addition products or to rearrangement products once a covalent adduct was formed. In each case the sequence was identical and the site of labeling was the Tyr; hence, the variability does not appear to be due to incomplete digestion with trypsin. Since all of the minor peaks were not sequenced, we cannot rule out unambiguously that no labeling of Trp-260 occurs; however, in comparison with labeling of the wild type R-subunit (11), labeling of Trp-260, if it occurs at all, is very low.

**DISCUSSION**

Three different point mutations, summarized in Fig. 2, were introduced into the R-subunit of cAMP-dependent protein kinase (cAPK). These mutations were designed to alter residues in the CAMP-binding site. The primary site of labeling in wild type R-subunits was identified as Tyr-244. This residue is unique to the CAMP binding site. Two conservative point mutations in Tyr-244 were made to examine the extent to which this residue could be replaced. The photolytic labeling of the A domain and the results of sequencing of this photolabeled peptide indicated that both of these simple mutations were sufficient to eliminate most photolabeling of the A domain. The mutation in Tyr-371 in domain B was the dominant site of photolabeling. The one conservative point mutation adjacent to Tyr-371 in domain B was sufficient to eliminate most photolabeling of the A domain. In both of these mutants, Tyr-371 in domain B was the dominant site of photolabeling. The reason why this peptide containing Tyr-371 elutes in various positions is not clear but may be due to different addition products or to rearrangement products once a covalent adduct was formed. In each case the sequence was identical and the site of labeling was the Tyr; hence, the variability does not appear to be due to incomplete digestion with trypsin. Since all of the minor peaks were not sequenced, we cannot rule out unambiguously that no labeling of Trp-260 occurs; however, in comparison with labeling of the wild type R-subunit (11), labeling of Trp-260, if it occurs at all, is very low.
kinase in order to further probe the two tandem cAMP-binding sites. The \( K_a(\text{cAMP}) \) and \( K_c(\text{cAMP}) \) were increased approximately 2-fold for holoenzyme formed with each of the mutant R-subunits. In contrast, the off-rates were increased by as much as 60-fold and also varied significantly for each mutant. As a general rule, measurement of the cAMP off-rates was more sensitive for detecting differences between the wild type and mutant proteins than the measurement of \( K_a(\text{cAMP}) \) or \( K_c(\text{cAMP}) \). Each mutation not only perturbed the CAMP-binding properties of the site that was altered but also affected the CAMP-binding properties of the adjacent site. Furthermore, this cross-communication can occur in both directions.

The potential importance of Glu-200 is based on several independent criteria. The general importance of hydrogen bonding of the 2'-OH of CAMP to the protein was indicated initially by mapping the CAMP-binding sites with analogs of CAMP. In this case, replacing the 2'-OH with hydrogen (2'-deoxy-CAMP) or methylating the 2'-OH were sufficient to increase the \( K_a \) by 170- and 500-fold, respectively (27). The crystal structure of CAP identified Glu-70 as the most likely acceptor group (28). Glu 70 in CAP corresponds to Glu 200 in site A of the R'-subunit (9). Finally, the invariance of this Glu in all of the R-subunits is consistent with its playing an important functional role (9, 28). The role of this Glu in the cyclic nucleotide-free structure is not known since the crystal structure has not been solved for CAP in the absence of cAMP. Since Glu and Asp differ by only a single methylene, this conservative replacement should maintain the overall charge environment while potentially disrupting hydrogen bond interactions. Replacement of Glu-200 with Asp results in only a 2-fold increase in the \( K_c(\text{cAMP}) \). Thus, Glu-200 cannot be considered as essential in spite of its conservation throughout evolution. This is in contrast to the invariant residue, Arg-209, where the guanidinium group, not just a positive charge, does appear to be necessary for high affinity binding of cAMP (13). Although rR(E200D) still supports high affinity binding of cAMP, the off-rate for cAMP from site A is enhanced nearly 18-fold. This rapid off-rate may reflect weakened hydrogen bonding. This site A mutation also increased the observed cAMP exchange rate for site B by 11-fold, demonstrating significant intrasubunit communication between sites A and B. As indicated in Fig. 7, Glu-200 is thought to lie in a loop between \( \beta \) strands 6 and 7. The potential flexibility of this loop might minimize the consequences of replacing Glu with Asp thus enabling the mutant R-subunit to retain high affinity binding for cAMP.

The other two mutations probe the region surrounding the adenine ring of cAMP. Tyr-371 in site B, located near the C8 position of bound \( 8-N_3\text{CAMP} \) (11, 12), lies on the inward facing surface of the long C-helix according to the CAP-based model (9, 28). This mutation reduced the affinity for CAMP by 2-fold relative to the wild type R-subunit, and increased the off-rate for CAMP from site B 13-fold. The off-rate for cAMP from site A also was enhanced, once again emphasizing that these two sites communicate closely. However, unlike an earlier mutation replacing Tyr 371 with Phe (12), replacement with Trp had little effect on the cooperative interaction between the cAMP-binding sites. One feature that Tyr and Trp share, in contrast to Phe, is the capacity to provide \( \pi \)-donor interactions. Based on kinetic arguments, cAMP binds first to site B which causes a conformational change that makes site A, otherwise shielded, more accessible to CAMP (30, 31). A highly favorable stacking interaction between the aromatic side chain of residue 371 and the adenine ring of cAMP, coupled with movement of the long C-helix, may be required for inducing the conformational changes that lead to dissociation of the holoenzyme. Though Phe can provide hydrophobic interactions, Tyr and particu
The corresponding residues in site A are Glu-200 and Arg-209. The region surrounding the bound cAMP is shown below. The model is based on the crystal structure of CAP (9).

A second mutation replacing Phe-247 in site A with Tyr also was constructed based on the role of Tyr-371 and the long C-helix in site B. The model proposed by Weber et al. (9) suggests that the C-helix lining the cAMP-binding pocket is shorter in site A than in site B, and the position corresponding to Tyr-371 in site A is Phe-247. Based on sequence similarities, the two C-helices in sites A and B are not as highly conserved as other regions of the cAMP-binding domains (Figs. 7 and 8). However, both R\textsuperscript{I} and R\textsuperscript{II} subunits have conserved Phe at positions 247 and 251, respectively, in site A and a conserved tyrosine, 371 (discussed above), at the same position in site B (3, 4). If one considers this to be an amphipathic helix (Fig. 8), the residues lining the surface that faces the adenine ring of cAMP are hydrophobic in both sites and are highly conserved in R\textsuperscript{I} and R\textsuperscript{II} while those on the outer surface, presumably in contact with another domain or with solvent, tend to be hydrophilic and variable.

Replacing Phe-247 with Tyr is a relatively conservative change that would not be expected to greatly disrupt the protein structure. Indeed only slight differences were observed in the binding of cAMP and in the off-rates for cAMP, and cAMP binding still showed cooperativity. While the 2-fold increase in \( K_d(cAMP) \) was similar to R(Y371/W), only a 1.4-fold increase in the off-rates for cAMP was seen for site A. This site A mutation also increased the cAMP off-rate from site B consistent with previous studies showing that cAMP bound to site A alters the cAMP off-rate of site B for the wild type R\textsuperscript{II}-subunit (5, 6, 33).

Phe-247 in the A domain is located in the same position of the C-helix as Tyr-371 in the B domain (9). The possibility existed that the photoaffinity labeling of Trp-260 in site A of the wild type R\textsuperscript{I}-subunit was due to the relatively nonnucleophilic nature of Phe-247. Therefore, changing this residue to a potentially more reactive tyrosine might result in labeling at position 247. In the two deletion mutants indicated in Table II, site B, as well as Trp-260, are missing. In this case Tyr-244 gets photolabeled stoichiometrically so clearly several aromatic rings are in relatively close proximity to the adenine ring. Tyr-247 did not get photolabeled to any significant extent; however, neither did Trp-260, the normal site of modification in the wild type protein (11), or Tyr-244, the site of modification in the site B deletion mutant (8). Labeling of rR(F247Y) occurred predominantly at Tyr-371, in contrast to the native R\textsuperscript{II}-subunit where labeling occurs at both Trp-260 (40%) and Tyr-371 (60%) (11). This conservative point mutation apparently altered somewhat the location of Trp-260 with respect to the C-8 position of the adenine ring of 8-N\textsuperscript{3}-CAMP but did not compete effectively for photoincorporation.

In general, photoaffinity labeling proved to be a sensitive method for detecting changes, perhaps subtle ones, in the two cAMP-binding sites. When all of the photoaffinity labeling results are considered (Table II), including labeling of the native type I and II R-subunits (10, 11), a proteolytic fragment of the R\textsuperscript{II}-subunit (34), and various mutant forms of the R\textsuperscript{II}-subunit (12, 13, 22), several general observations are apparent. First, and perhaps most striking, is that the stoichiometry for covalent modification typically does not exceed 1 mol of 8-N\textsuperscript{3}-CAMP incorporated per mol of R-monomer even though one or both sites can be labeled. This is true even though in most cases 8-N\textsuperscript{3}-CAMP binds with a high affinity to both

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**Fig. 7.** The model for cAMP-binding site B, showing the locations of Tyr-371, Glu-323, and Arg-333. The corresponding residues in site A are Glu-200 and Arg-209. The region surrounding the bound cAMP is shown below. The model is based on the crystal structure of CAP (9).

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**Fig. 8.** The proposed C-helix of sites A and B in the bovine type I regulatory subunit. The residues on the inward surface that face the adenine ring of cAMP are indicated. The sites of affinity labeling are indicated by open arrows: Tyr-371 and Trp-260 in the wild type R\textsuperscript{II}-subunit and Tyr-244 in a truncated mutant that terminates at residue 250 (8, 11). The mutated residues, Tyr-371 to Trp and Phe, and Phe-247 to Tyr, are indicated by closed arrows. The conserved hydrophilic residues are indicated by the filled circles. The residues that are invariant in the bovine type I and type II R-subunits are designated in capitols. The nonconserved residues are in lower case with the corresponding residue in the R\textsuperscript{II}-subunit indicated below.
cAMP-binding sites. Furthermore, even after one site is covalently modified, the remaining site is still functional and can still bind 8-Ns-cAMP (11), there is just no stable photoaffinity incorporation. The second conclusion is that slight perturbations of cAMP-binding site A or B, either by proteolysis or mutagenesis, can alter the overall pattern of photoaffinity labeling even though the total stoichiometry remains relatively unchanged. It is as though covalent modification at one site precludes covalent modification of the adjacent site. Whichever site competes most efficiently will be the dominant site for covalent modification. In the case of the mutant proteins, the mutation could either change the geometry of the CAMP-binding site or increase the off-rate sufficiently so that the relative opportunity for covalent labeling is reduced. We see here examples where a mutation at one site can improve photoaffinity labeling at the mutated site as in the case of R(Y371W). On the other hand, both R(E200D) and R(F247Y) show reduced labeling of the mutated site in favor of the nonmutated site.

The results described here confirm earlier kinetic results (33, 35) indicating that considerable communication occurs between the two tandem CAMP-binding sites in each R monomer. A mutation at one site consistently perturbs the cAMP binding properties of the adjacent site. Measurement of cAMP off-rates and of sites of photoaffinity labeling are two very sensitive methods for detecting changes in each CAMP-binding site. Clearly further structural studies are required before the detailed molecular events that are associated with the cooperative communication between the two CAMP-binding sites can be elucidated.

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