Tyrosine-371 Contributes to the Positive Cooperativity between the Two cAMP Binding Sites in the Regulatory Subunit of cAMP-Dependent Protein Kinase I†

José Bubis,‡ Lakshmi D. Saraswat,§ and Susan S. Taylor*
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: The regulatory (R) subunit of cAMP-dependent protein kinase I has been expressed in *Escherichia coli*, and oligonucleotide-directed mutagenesis was initiated in order to better understand structural changes that are induced as a consequence of cAMP-binding. Photoaffinity labeling of the type I holoenzyme with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) leads to the covalent modification of two residues, Trp-260 and Tyr-371 [Bubis, J., & Taylor, S. S. (1987) *Biochemistry 26*, 3478–3486]. The site that was targeted for mutagenesis was Tyr-371. The intention was to establish whether the interactions between the tyrosine ring and the adenine ring of cAMP are primarily hydrophobic in nature or whether the hydroxyl group is critical for cAMP binding and/or for inducing conformational changes. A single base change converted Tyr-371 to Phe. This yielded an R subunit that reassociated with the catalytic subunit to form holoenzyme and bound 2 mol of cAMP/mol of R monomer. The cAMP binding properties of the holoenzyme that was formed with this mutant R subunit, however, were altered: (a) the apparent K_d(cAMP) was shifted from 16 to 60 nM; (b) Scatchard plots showed no cooperativity between the cAMP binding sites in the mutant in contrast to the positive cooperativity that is observed for the wild-type holoenzyme; (c) the Hill coefficient of 1.6 for the wild-type holoenzyme was reduced to 0.99. The K_a's for activation by cAMP were altered in the mutant holoenzyme in a manner that was proportional to the shift in K_d(cAMP). Finally, photolabeling of the mutant holoenzyme and of the R_2 dimer with 8-N_3[^{32}P]cAMP led to the covalent modification of only Trp-260; photolabeling of the second cAMP binding domain was abolished.

A major mechanism for regulation in eukaryotic cells involves protein phosphorylation (Krebs & Beavo, 1979), and one of the best understood protein kinases is the kinase that is activated in response to cAMP (Walsh et al., 1968). cAMP-dependent protein kinase is a tetramer containing both regulatory (R) and catalytic (C) subunits. In the absence of cAMP, the R subunit binds to the C subunit with a high affinity and keeps the enzyme in an inactive state. The amino acid sequences of two R subunits have been elucidated (Titani et al., 1984; Takio et al., 1984), and considerable information about the cAMP binding sites has emerged from mapping with a wide range of substituted analogues of cAMP (Corbin et al., 1982; Doskeland et al., 1983). Our knowledge of specific residues associated with the functional sites has derived primarily from affinity labeling and group-specific labeling (Kerlavage & Taylor, 1980; Bubis & Taylor, 1987; Nelson & Taylor, 1983). With the recent isolation of several cDNA clones for the kinase R subunits (Lee et al., 1983; Jahnsen et al., 1986), it has become possible to probe the functional sites of this protein by the selective mutagenesis of defined sites.

The cAMP binding sites of the R subunit have been characterized by a variety of methods. Each R subunit contains two tandem cAMP binding sites, designated as A and B, which account for the carboxy-terminal two-thirds of the polypeptide chain. Mapping with numerous analogues of cAMP has established that the two sites are nonequivalent in several regards. The dissociation rates of cAMP differ for each site (Rannels & Corbin, 1980; Doskeland, 1978), and the sites also differ in their preference for substituted analogues of cAMP (Corbin et al., 1982). The first site in the linear sequence, site A, has been correlated with the fast dissociation site and, in general, shows a preference for N^6- or C-substituted analogues of cAMP. Dissociation of cAMP from site B is much slower, and this site shows a general preference for C-8- or C-2-substituted analogues of cAMP. Binding of cAMP shows positive cooperativity with Hill coefficients in the range of 1.4–1.6 (Ogreid & Doskeland, 1982; Bubis & Taylor, 1985, 1987).

The cAMP binding sites also have been probed by affinity labeling. In the case of R^I, photolabeling with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) leads to the stoichiometric modification of two residues, Trp-260 and Tyr-371 (Bubis & Taylor, 1985, 1987). Trp-260 is labeled by 8-N_3cAMP bound to domain A, whereas Tyr-371 is labeled by 8-N_3cAMP that is bound to domain B (Bubis & Taylor, 1987). A single residue is labeled by 8-N_3cAMP in R^{II}, Tyr-381, which is homologous to Tyr-371 in R^I (Kerlavage & Taylor, 1980). Our ability to interpret these photoaffinity labeling results has been facilitated by the homologies that the R subunits share with the *Escherichia coli* catabolite gene activator protein, CAP, for which the crystal structure is known (Steitz & Weber, 1985). When the R^I sequences were actually built into the coordinates of the CAP crystal structure, the side chain of Tyr-371 does indeed come in close proximity to the C-8 position of the adenine ring, which is consistent with the photoaffinity labeling (Weber et al., 1987). However, on the basis of this model, it is not possible to distinguish whether simple hydrophobic interactions between the aromatic rings of Tyr-371 and cAMP are contributing to binding or whether dipole–dipole forces or hydrogen bonding also is involved.

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* Author to whom correspondence should be addressed.
‡ Supported in part by the Consejo Nacional de Investigaciones Cientificas y Tecnológicas (CONICIT), Caracas, Venezuela. Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.
§ Supported in part by U.S. Public Health Service Training Grant AM-07233. Present address: Department of Biochemistry, Brandeis University, Waltham, MA 02154.

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Consequently, selected mutagenesis of this tyrosine has been used in a preliminary effort to distinguish these possibilities.

A full-length cDNA clone for bovine R^I was isolated by Lee et al. (1983), and an expression vector for this cDNA was constructed subsequently in pUC7 (Saraswat et al., 1986). Maximum expression of R^I was achieved in *E. coli* 222, a strain that lacks active adenylate cyclase and that also contains a mutant form of CAP that binds to DNA with a high affinity in the absence of cAMP. The cDNA coding for this R subunit has been selectively modified by converting Tyr-371, which is photoaffinity labeled so readily, to Phe. The mutant protein containing Phe instead of Tyr at position 371 has been purified to homogeneity and compared to the wild-type R subunit. A preliminary report of some of this work has been described (Bubis et al., 1987).

Experimental Procedures

Materials. Reagents were purchased from the following companies: [2,8-^{3}H]cAMP (27 Ci/mmol), 8-N_3[^{32}P]cAMP (67.7 Ci/mmol), and Biotrans nylon membranes, ICN; 8-N_3[2-^{3}H]cAMP (17.0 Ci/mmol), New England Nuclear; [2-^{3}H]ATP (25.5 Ci/mmol) and [8-^{32}P]ATP (3000 Ci/mmol), Amersham; cAMP, 8-N_3cAMP, ATP, bovine serum albumin, histone IIA, phenylmethanesulfonyl fluoride (PMSF), isopropyl β-D-thiogalactopyranoside (IPTG), benzamidine, soybean trypsin inhibitor, 3,3′-diaminobenzidine tetrahydrochloride (DAB), and CM-Sepharose CL-6B, Sigma; dNTP's, P-L Biochemicals, Inc.; N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK), leupeptin, and TPCK-treated trypsin, United States Biochemical Corp.; trifluoroacetic acid (TFA), sequenal grade, Pierce; acetonitrile (HPLC grade), Fisher Scientific; type HA filters (0.45 μm), Millipore Corp.; Cytoscint, West Chem; nitrocellulose (0.45 μm), Bio-Rad. Enzymes used in DNA manipulations were obtained from either Bethesda Research Laboratories or Boehringer Mannheim and were used according to the manufacturer's specifications. All other reagents were of analytical grade.

Native Proteins. C subunit was purified from porcine heart (Nelson & Taylor, 1981) and R^I subunit from porcine skeletal muscle (Zick & Taylor, 1982). Native holoenzyme was reconstituted as described previously (Bubis & Taylor, 1985).

Site-Directed Mutagenesis. A pUC7 derivative, pLST-1, containing the full-length clone for bovine R^I has been constructed, and R subunit isolated from *E. coli* transformed with pLST-1 behaved as the native R^I isolated from porcine skeletal muscle (Saraswat et al., 1986). A conservative oligonucleotide-directed mutation was introduced into the R^I gene to remove the internal *Eco*RI site (Saraswat et al., 1988), and this plasmid, pLST-2, was used for the mutagenesis described here.

Oligonucleotide-Directed Mutagenesis of R^I. The 19-base oligonucleotide indicated in Figure 1 was synthesized on an Applied Biosystems DNA synthesizer, purified on a preparative 20% polyacrylamide gel, and phosphorylated for use as a hybridization probe and DNA primer as described by Zoller and Smith (1982). After digestion of pLST-2 with *Eco*RI, two fragments (1155 and 2700 bp) were isolated from agarose gels. The 1155-bp fragment containing the full-length R^I clone was ligated into M13 mp 9 that had been linearized with *Eco*RI. *E. coli* JM 101 cells were transformed with this ligation mixture, and colonies containing the 1155-bp fragment, identified as clear plaques, were purified. Single-stranded DNA was prepared as described by Schreier and Cortese (1979), and oligonucleotide-directed mutagenesis was performed by the protocol of Nisbet and Beilharz (1985). Briefly, the single-stranded template DNA (0.5 pmol) was annealed to the 19-base primer (2.5 pmol) in a 10-μL solution of 15 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 15 mM $MgCl_2$, 75 mM NaCl, and 1.4 mM dithiothreitol (DTT) by first heating the mixture at 80 °C for 5 min and then allowing it to cool to room temperature over a period of 15 min. To this was added 30 μL of a solution containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 4 mM DTT, 0.5 mM ATP, 80 μM dNTP mix, 2 units of T4 DNA ligase, and 2.5 units of the Klenow fragment of DNA polymerase I. The mixture then was incubated at 30 °C for 1 h. A solution (10 μL) of 190 mM dNTP, 2 units of T4 DNA ligase, and 2.5 units of Klenow fragment was then added, and incubation was continued for 3 h. After confirming that ligation had occurred by electrophoresis of a 10% aliquot on a 1% agarose minigel, *E. coli* JM 101 was transformed with the remainder of the reaction mixture. Colonies were transferred to nylon membranes and screened with the ^{32}P 5′-end labeled 19-base oligonucleotide. After the initial hybridization at 37 °C, washes were carried out at 55 °C, conditions which were capable of discriminating between wild-type and mutant plasmids. Positive colonies were selected and purified, and double-stranded DNA was prepared from the cell pellets according to the alkaline lysis method described by Maniatis (1982). After digestion with *Eco*RI, the resulting 1155-bp fragment containing the mutation site was isolated and ligated back with the 2700-bp fragment of pLST-2.

Screening for Expression of R^I. *E. coli* strain 222 was transformed with this ligation mix, and the colonies were plated and then lifted onto nylon membranes and screened with the labeled oligonucleotide probe as described above. The positive colonies could be screened for expression of R subunit by three different methods. (1) Since *E. coli* 222 lacks adenylate cyclase, any R subunit that is expressed will be free of endogenous cyclic nucleotide. Therefore, cAMP binding can be measured directly in the total cell extracts. (2) The expression of functional R subunit could also be followed by photolabeling with 8-N_3[^{32}P]cAMP following electrotransfer from sodium dodecyl sulfate (SDS)–polyacrylamide gels to nitrocellulose. (3) Finally, the R subunit could be detected by immunoblotting with serum antibodies to porcine muscle R^I. Representative colonies that hybridized with the oligonucleotide were picked and grown in 5–10-mL cultures. After an overnight incubation at 37 °C, the cells were pelleted, resuspended in gel sample buffer (2.5% SDS, 6% sucrose, 2 M β-mercaptoethanol, and 0.01% phenyl red) by repeated vortexing, and then placed in a boiling water bath for 2–5 min. After centrifugation, an aliquot was electrophoresed on 10% polyacrylamide gels containing SDS according to Laemmli (1970). Following electrophoresis, the proteins were electrotransferred (50 V, 4 °C, overnight) to nitrocellulose in 20 mM Tris (pH 8.3), 154 mM glycine, and 20% methanol on an electroblotting apparatus (Hoefer Scientific Instruments). The nitrocellulose was incubated for 1 h at room temperature with buffer A [0.05% Tween 20, 150 mM sodium chloride, and 10 mM Tris (pH 7.4)] to block excess binding sites. At this point, either antibodies or photolabeling could be used for detection.

For immunodetection, the filters were incubated for 1 h, at room temperature, with 20 mL of buffer A containing 50 μL of serum antibodies raised in rabbits against porcine skeletal muscle R^I. The antigenically cross-reacting protein was then reacted with biotinylated secondary antibody raised against rabbit IgG and the ABC kit (Vector Laboratories, Inc), containing avidin and biotinylated horseradish peroxidase. The protein bands were visualized by the addition of 10 mL of 0.5%

DAB, 0.04% $NiCl_2$, and 0.015% hydrogen peroxide in 10 mM Tris (pH 7.4).

For photolabeling, the nitrocellulose filter was washed at room temperature with buffer B (same as buffer A except that Tween 20 was omitted). The filter was then incubated with 20 nM 8-N_3[^{32}P]cAMP in buffer B for 60 min in the dark at room temperature. After being washed with ice-cold buffer B, the filter was irradiated with a UVS-11 lamp (254 nm) for 5 min, washed again, blotted dry, and subjected to autoradiography on Kodak X-O mat film. The construction of the vector and the presence of the mutation were confirmed by restriction mapping and DNA sequence analysis, respectively. This vector is subsequently referred to as pLST-2:Y371/F.

Purification of Wild-Type and Mutant Regulatory Subunits. L broth (20 mL) containing 50 μg/mL ampicillin was inoculated with either pLST-2 or pLST-2:Y371/F and grown at 37 °C until the cell density at 550 nm reached 0.1–0.5. L broth (4 L) with ampicillin was then inoculated with 5 mL/L of the culture and incubated for approximately 16 h. All subsequent procedures were carried out at 4 °C. The cells were centrifuged at 5000*g* for 30 min, and the resulting pellet was resuspended in 50 mL of buffer I [20 mM potassium phosphate, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM β-mercaptoethanol, 10 mM benzamidine, 10 mg/L trypsin inhibitor from soybean, 15 mg/L PMSF, 10 mg/L TLCK, 10 mg/L TPCK, and 0.5 mg/L leupeptin, pH 6.4]. This suspension was passed twice through a French pressure cell and then centrifuged at 5000*g* for 30 min. After the pellet was reextracted with 50 mL of buffer I, the supernatant fractions were pooled, diluted to 300 mL, and added to cAMP–agarose (N^6-ethane spacer) prepared as described by Dills et al. (1975). The slurry was rotated gently overnight. The resin was washed with buffer II (20 mM potassium phosphate, 5 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 10 mM benzamidine, 15 mg/L PMSF, 10 mg/L TLCK, 10 mg/L TPCK, 0.5 mg/L leupeptin, and 2 M sodium chloride, pH 6.4) and then with buffer II minus NaCl. R subunit was eluted overnight at 4 °C with 50 mM cAMP in buffer II without NaCl.

Wild-type and mutant holoenzymes were reconstituted by dialyzing the R subunits with a 5% excess of C subunit as described by Bubis and Taylor (1985). Excess free C subunit was removed by ion-exchange chromatography.

Measurement of cAMP and ATP Binding. cAMP binding was measured by equilibrium dialysis and Millipore filtration methods A and B (Bubis & Taylor, 1985). The binding mixtures contained 20–30 nM holoenzyme and various concentrations of [^{3}H]cAMP. ATP binding was measured by equilibrium dialysis and Millipore filtration with holoenzyme (20 nM) that was incubated for 16 h at 4 °C with various concentrations of [^{3}H]ATP in 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 10 mM magnesium acetate, and 1 mg/mL BSA, pH 6.5.

Assay. Protein kinase activity was assayed spectrophotometrically (Cook et al., 1982) with the synthetic peptide L-R-R-W-S-V-A-E-L as substrate. The peptide was synthesized on an Applied Biosystems peptide synthesizer at the University of California Peptide and Oligonucleotide Facility.

Photolabeling of Wild-Type and Mutant Holoenzymes with 8-N_3cAMP. Holoenzymes were dialyzed against 25 mM potassium phosphate–2 mM EDTA, pH 6.5, to remove β-mercaptoethanol prior to modification by 8-N_3cAMP. Wild-type or mutant holoenzymes (3.0 nmol in 2 mL) were incubated with a 2.5-fold molar excess of 8-N_3[^{32}P]cAMP over

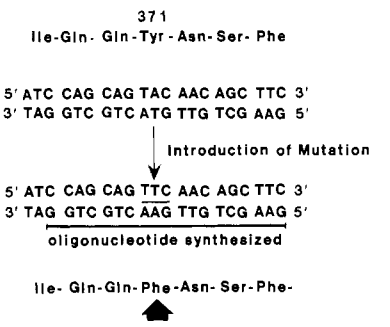

FIGURE 1: Oligonucleotide used for mutating Tyr-371 to Phe. Underlined is the 19-base oligonucleotide that was used to introduce a single base change into the bovine R^1 cDNA clone. The oligonucleotide was synthesized by the Peptide and Oligonucleotide Synthesis Facility at the University of California, San Diego.

cAMP binding sites, for 16 h at 4 °C. Samples were irradiated on ice with a UVS-11 lamp (254 nm) for 10 min at a distance of approximately 5 cm with occasional mixingg. Covalent incorporation was determined by TCA precipitation and by ammonium sulfate precipitation following incubation with cAMP as described earlier (Bubis & Taylor, 1987).

Isolation of Covalently Modified Peptides. After photolabeling, proteins were digested with TPCK-trypsin (1:50 w/w). The resultant peptides were resolved by high-performance liquid chromatography (HPLC) on an Altex 3200 system with a Vydac C18 column (0.46 × 25 cm) as described by Bubis and Taylor (1987).

Amino Acid Analyses and Peptide Sequencing. Analyses were performed on a LKB Biochrom Model 4400 automated amino acid analyzer. Samples were hydrolyzed in vacuo at 110 °C in 6 M HCl for 24 h. Sequencing was carried out on an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH) amino acids were identified by HPLC (Hunkapillar & Hood, 1983).

DNA Sequencing. DNA sequencing was carried out according to the dideoxy method of Sanger et al. (1977) as described by Schreier and Cortese (1979).

RESULTS

Construction and Expression of the Mutant Plasmid. Tyr-371 of the R subunit was selectively changed to Phe with the synthetic oligonucleotide probe shown in Figure 1. After the M13 mp9 clones that contained the mutation were identified by hybridization, the region coding for the R subunit was excised and religated into pUC7 as described under Experimental Procedures. *E. coli* 222 was then transformed with this ligation mixture, and transformants were screened with the ^{32}P-labeled oligomer. Those colonies that gave positive signals were screened subsequently for expression of R subunit by photolabeling with 8-N_3[^{32}P]cAMP following electrotransfer of colonies to nitrocellulose. The orientation of the insert was confirmed by both restriction mapping and by photolabeling of total cell extracts following SDS–polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose. Clones that expressed R subunit also were identified by immunoblotting with anti-porcine R^1 antibodies. DNA from the mutant clone was sequenced in its entirity to confirm that only the correct mutation had been introduced. The expression of the mutant R subunit was independent of IPTG, and the protein accumulated in large amounts in the stationary phase of growth as in the case of the wild-type R subunit (Saraswat et al., 1986). An expression of 12 to 16 mg of R subunit/L of culture was estimated on the basis of cAMP binding assays.

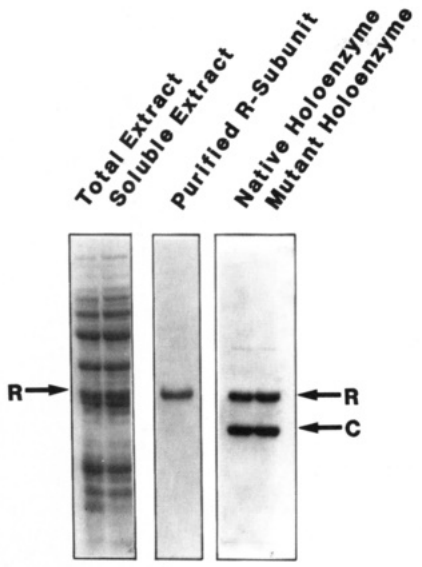

FIGURE 2: Expression and purification of mutant R subunit. The total cell extract and the soluble cell extract are shown in the left lanes, and the R subunit following purification by affinity chromatography is in the middle. Electrophoresis of R and C subunits following formation of holoenzyme with both native and mutant R subunit is shown on the far right.

Purification and Characterization of the Expressed Mutant Protein. In order to more fully characterize the mutant protein, the expressed mutant R subunit was purified to homogeneity as described under Experimental Procedures. Following disruption in a French pressure cell, greater than 90% of the expressed R subunit was soluble and was subsequently purified to homogeneity by affinity chromatography (Figure 2). From 4 L of culture, 30 mg of R subunit was isolated. As in the case of the wild-type R subunit, the expressed protein migrated as a dimer when it was electrophoresed in the absence of β-mercaptoethanol. Amino acid analyses confirmed the conversion of Tyr to Phe. The mutant R subunit contained 10.9 ($\simeq$11) mol of tyrosine and 16.9 ($\simeq$17) mol of phenylalanine per mole of R monomer, in contrast to 11.8 ($\simeq$12) Tyr's and 16.1 ($\simeq$16) Phe's in native R^I.

In order to form holoenzyme, the purified mutant R subunit was incubated with a slight excess of C subunit. When excess C subunit was removed by ion-exchange chromatography, the remaining protein was totally dependent on added cAMP for enzymatic activity. In addition, SDS–polyacrylamide gel electrophoresis showed equivalent amounts of R and C subunits, confirming the reassociation of stoichiometric amounts of both subunits to form holoenzyme (Figure 2).

The cAMP binding properties of the reconstituted mutant holoenzymes were characterized by several techniques and compared to those of the wild-type holoenzyme. Equilibrium dialysis indicated that the mutant protein bound 2 mol of cAMP/mol of R monomer as did the wild-type protein, but the apparent K_d(cAMP) was shifted from 16 nM in the wild type to 60 nM in the mutant (Figure 3). Scatchard plots showed no cooperativity between the cAMP binding sites in the mutant, in contrast to positive cooperativity that is observed for the wild-type holoenzyme (Figure 4), and the Hill coefficient of 1.56 for the wild type was reduced to 0.99 in the case of the mutant holoenzyme. Millipore filtration assays A and B also were used to determine cAMP binding to both holoenzymes. The wild-type protein behaved like native type I holoenzyme, and both filter disk assays showed apparent K_d(cAMP)'s of approximately 10–15 nM (Figure 5). Assay A is carried out in the presence of low salt and measures

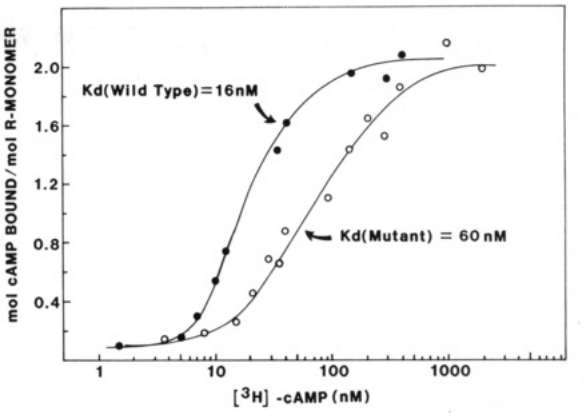

FIGURE 3: cAMP binding to the wild-type and mutant holoenzymes. cAMP binding was measured by equilibrium dialysis with 20 nM holoenzyme as described previously (Bubis & Taylor, 1985). Closed and open circles represent wild-type and mutant holoenzymes, respectively.

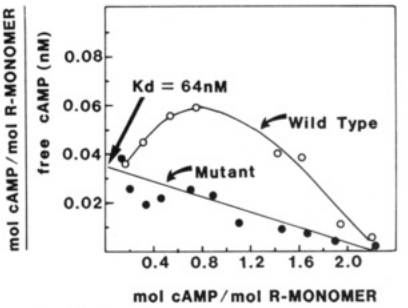

FIGURE 4: Scatchard plot of cAMP binding to wild-type and mutant holoenzymes. Open circles represent wild-type holoenzyme; closed circles represent mutant holoenzyme prepared with the mutated R subunit.

binding to site A only whereas assay B is carried out in the presence of high salt and histone and measures binding to both sites (Bubis & Taylor, 1985, 1987). On the other hand, a striking difference in the Millipore filtration assays were observed for the mutant protein. Nonsaturating cAMP binding curves were observed (Figure 5, top panel), even though equilibrium dialysis showed a saturating behavior (Figure 3). Maximum bindings of 0.5 mol of cAMP/mol of R monomer and 1.7 mol of cAMP/mol of R monomer with the filter disk assays A and B, respectively, were obtained at high concentrations of cAMP (2 μM), and the apparent K_d(cAMP)'s were shifted to the right.

When the cAMP activation of both holoenzymes was measured, the apparent K_a was shifted from 8 nM in the wild type to 30 nM in the mutant (Figure 5, bottom panel). The Scatchard plots showed no cooperativity between the cAMP binding sites in the mutant instead of the positive cooperativity that is observed for the wild-type R subunit, and Hill coefficients of 0.92 and 1.4 were observed for the mutant and wild-type holoenzyme, respectively.

Finally, the Mg-ATP binding properties of the mutant holoenzyme were determined. Equilibrium dialysis and Millipore filtration showed 2 mol of ATP bound/mol of holoenzyme with an apparent K_d(ATP) of approximately 5–6 nM for both the wild-type and the mutant protein (Figure 6).

Photoaffinity Labeling of Mutant Holoenzyme and R_2 Dimer. Mutant holoenzyme bound 1.9 mol of 8-N_3[^{32}P]-cAMP/mol of R monomer, but only 0.3 mol of 8-N_3cAMP/mol of R monomer remained covalently bound after photolysis in contrast to 1.0 mol in the native R subunit. Total 8-N_3[^{32}P]cAMP binding and covalent incorporation were assayed by ammonium sulfate and TCA precipitations, re-

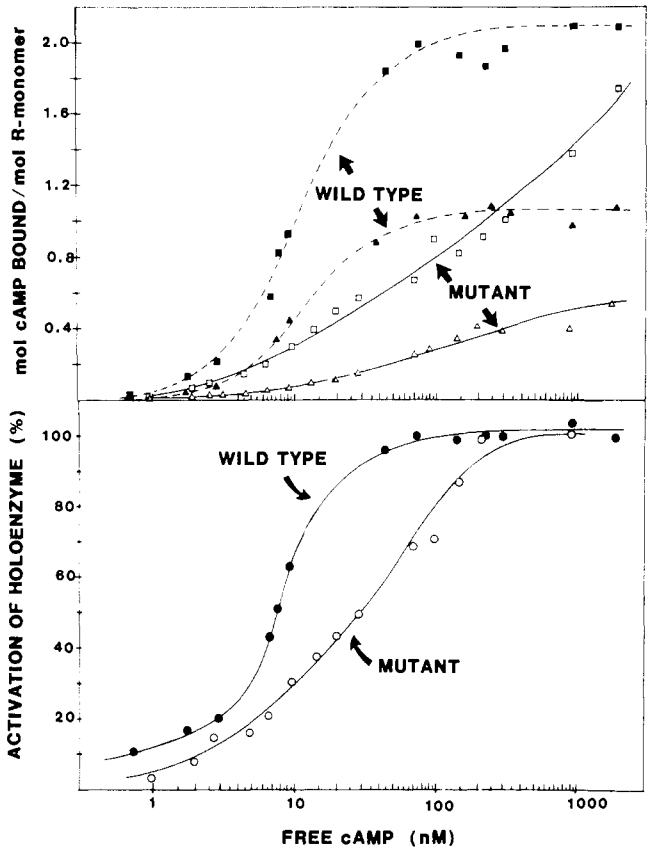

FIGURE 5: Measurement of cAMP binding by filter disk assays and percentage of activation of wild-type and mutant holoenzymes. Holoenzyme (30 nM) was incubated with various concentrations of [^{3}H]cAMP (0.0–2.0 μM). Closed symbols and open symbols show wild-type and mutant holoenzymes, respectively. (Top) Dashed curves and solid curves show filter disk assays measuring cAMP binding to wild-type and mutant holoenzymes, respectively. Conventional Millipore filtration assay (method A) (▲, △) and filtration assay in the presence of 2 M NaCl and 0.5 mg/mL histone IIA (method B) (■, □). (Bottom) Activation as a function of the concentration of cAMP for the wild-type (●) and the mutant (○) holoenzymes.

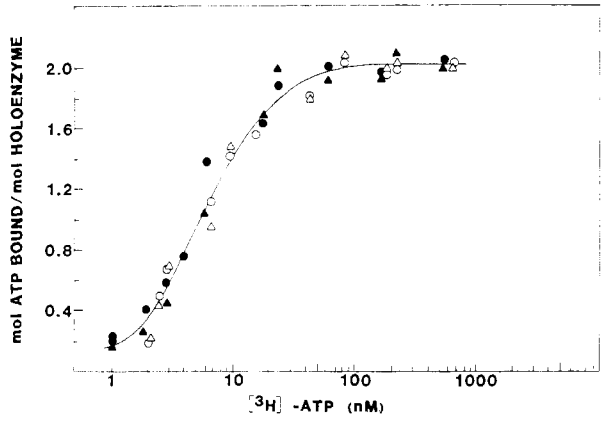

FIGURE 6: Measurement of Mg-ATP binding to wild-type and mutant holoenzymes. Holoenzymes (20 nM) were incubated with various concentrations of [^{3}H]ATP (0.0–1.0 μM). Nucleotide binding was measured by filter disk assay (▲, △) and by equilibrium dialysis (●, ○). Closed symbols and open symbols show wild-type and mutant holoenzymes, respectively.

spectively. The specificity of the photodependent covalent modification was assayed by digesting the modified protein with trypsin and then resolving the peptides by HPLC. As seen in Figure 7, a single major peak of radioactivity was obtained. When this peak was then rechromatographed on a second C_{18} column using a different gradient, one peptide was found to be specifically photolabeled (Figure 7, inset).

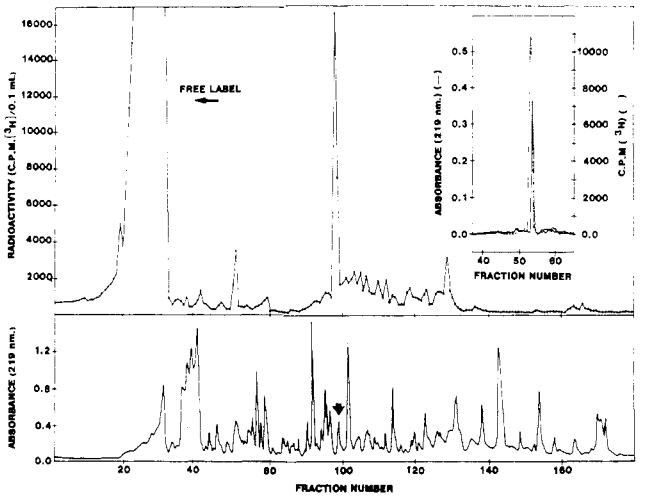

FIGURE 7: HPLC resolution of tryptic peptides from 8-N_3cAMP-labeled mutant R subunit. The buffers employed were (a) 10 nM sodium phosphate (pH 6.8) and (b) CH_3CN. The tryptic peptides were eluted with a 120-min linear gradient from 0 to 30% b and a 60-min linerar gradient from 30 to 60% b. (Top) Radioactivity (cpm ^{3}H); (bottom) absorbance at 219 nm. The inset shows the rechromatogram of the major radioactive peak on a second C18 column in a gradient of 0.1% TFA (pH 2.15) to CH_3CN (0–40%) in 80 min.

Dissociated mutant R subunit also was labeled with 8-N_3-[^{3}H]cAMP. The total stoichiometry of binding was 2.0 mol of 8-N_3cAMP bound/mol of R monomer on the basis of ammonium sulfate precipitation, and 0.45 mol/mol of R monomer remained covalently bound after a TCA precipitation. The specificity of this photoincorporation was determined as described above, and only one major peak of radioactivity was found that was identical with the peptide that was photolabeled in the holoenzyme (data not shown).

The purified labeled peptides from the mutant holoenzyme and from the mutant R dimer both gave the sequence. Val-Ser-Ile-Leu-Glu-Ser-Leu-Asp-Lys-X-Glu-Arg, which corresponds to residues 251–262. The unidentified residue at step 10 corresponds to Trp-260 in the unmodified peptide. As seen before, the labeled residue at step 10 stayed bound to the filter of the gas-phase sequencer, and no radioactivity or PTH-amino acid was detected (Bubis & Taylor, 1987).

Discussion

Photoaffinity labeling of cAMP-dependent protein kinase with 8-N_3CAMP has identified amino acid residues that are in close proximity to the cAMP binding sites. The focus of this study has been the tyrosine that is photolabeled with a stoichiometry of close to 1.0 in the R^I subunit (Tyr-371) and in the R^{II} subunit (Tyr-381) (Bubis & Taylor, 1985, 1987; Kerlavage & Taylor, 1980, 1982). There are two specific questions to be addressed. How does each tyrosine contribute to cAMP binding, and why is each photolabeled so efficiently?

The functional importance of this tyrosine in the binding of cAMP has been addressed here by generating a mutant R^I subunit containing Phe instead of Tyr at position 371. Introduction of this change yields a stable protein that reassociates with the C subunit to form holoenzyme. However, the properties of the mutant holoenzyme differ significantly in several important regards from the native protein. This single substitution alters three properties: (1) The K_d(cAMP) is increased from 16 to 60 nM, (2) photoaffinity labeling at that site is abolished, and (3) the positive cooperativity that is characteristic of the two cAMP binding domains in the wild-type holoenzyme is abolished. Although stacking types of interactions between the adenine ring of cAMP and the

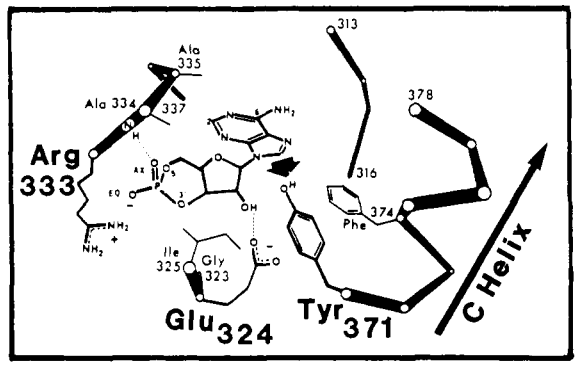

FIGURE 8: Model of cAMP binding domain B in R^I. This model for the cAMP binding site in domain B is based on the crystal structure of CAP (Weber et al., 1987). The arrow indicates the proposed position of Tyr-371, which was mutated to Phe.

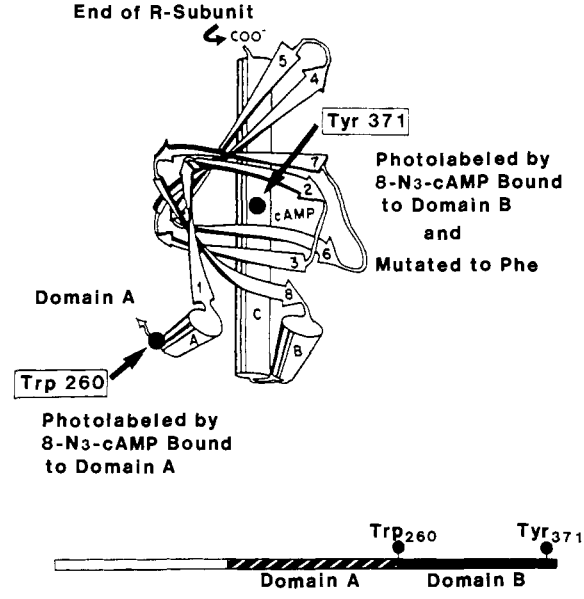

FIGURE 9: Model of cAMP binding domain B in R^I. This model was predicted by Weber et al. (1987) on the basis of the homologies of R^I with CAP. The location of the two residues that are photolabeled in R^I, Tyr-371 and Trp-260, are indicated. The location of this domain with respect to the rest of the molecule is indicated below.

phenol ring of Tyr-371 might be expected given the hydrophobic nature of both groups, the results shown here suggest that Tyr-371 contributes to the structure in ways which cannot be explained simply by hydrophobic interactions. Although further mutations at this site and in the surrounding region clearly are required for a more comprehensive understanding of these effects, several observations can be made at this point.

The analogies between CAP and the R subunits of cAMP-dependent protein kinase have provided a framework for modeling each cAMP binding domain. The model of cAMP binding site B in the R^I subunit, summarized in Figures 8 and 9, places the side chain of Tyr-371 in the middle of a long α-helix and in close proximity to the adenine ring of cAMP (Weber et al., 1987). Thus, the model is very consistent with the photolabeling results. Nevertheless, the detailed environment surrounding the bound cAMP in CAP and R must have important differences, since the affinities for cAMP differ by at least 3 orders of magnitude in the two proteins (Wu et al., 1974).

Although the mutation does increase the K_d(cAMP), the effect is relatively small and indicates that Tyr 371 contributes only marginally to the high-affinity binding properties of that site. It may, however, explain why certain analogues of cAMP show preferential binding to site B. The cumulative evidence that has emerged by extensive mapping of the cAMP binding sites with analogues of cAMP consistently indicates that the adenine ring of cAMP is not hydrogen bonded to the protein (de Wit et al., 1984). The preferential binding of various cAMP analogues to site B could be explained, however, by dipole–dipole interactions between the tyrosine ring and the adenine ring. For example, several analogues of cAMP bind to the R subunit with a higher affinity than cAMP itself. Analogues that have a high selectivity for site B fall into two classes: those which have electron-donating groups at the C-8 position and those which have electron-withdrawing groups at the C-2 position (Doskeland et al., 1983). Both kinds of substitutions will increase the dipole moment of the cyclic nucleotide in the direction of the C-8 position of the adenine ring. Thus, dipole–dipole interactions may play an important role. cAMP binding site B also has a slow dissociation rate for cAMP in contrast to cyclic nucleotide bound to domain A. The enhanced stability of this slow dissociation site also may be conferred in part by the interaction of the adenine ring with Tyr-371.

Figure 8 provides a closer look at the model of cAMP binding site B in the R^I subunit. The Arg and Glu that interact with the ribose phosphate are invariant in each cAMP binding domain; however, the primary sequence in the C helices of each cAMP binding domain differs. For example, the position corresponding to Tyr-371 in domain B is a Phe in domain A, and this Phe is not photolabeled by 8-N_3cAMP. In the case of CAP, a methionine residue is located at that position, and photolabeling of CAP with 8-N_3cAMP does not show stoichiometric labeling of a single residue (Aiba & Krakow, 1980). Thus, the hydroxyl group is critical for photolabeling. The photolabeling of Tyr-371 also is very efficient, in contrast to most photolabeling reactions, which may indicate that the hydroxyl group is partially ionized. Hydrogen bonding would enhance the nucleophilic character of the oxygen on the tyrosine ring, thus making it a better candidate for photolabeling by the reactive electron-deficient nitrene. Assuming that the Tyr is not hydrogen bonded to the adenine ring, it is most likely that the proton acceptor in such a hydrogen-bonded pair would be another part of the protein that is in close proximity such as Glu-324.

If one considers the overall location of Tyr-371 in the proposed structure of the B domain, it is potentially in a good position to mediate conformational changes. The β-roll structure that constitutes the cAMP binding site in CAP is distinct from other known adenine nucleotide binding structures and does not conform to the more typical $\beta 6\alpha 4$ nucleotide fold that is found in other kinases and dehydrogenases (Rossmann et al., 1974). CAP contains a long C helix that extends the entire length of the cAMP binding domain, and it is the inward-facing surface of this helix that provides major points of contact between the adenine ring of cAMP and the protein (Steitz & Weber, 1985). A movement of this C helix could potentially transmit conformational changes to other parts of the molecule. For example, the NH_2-terminal end of the C helix in CAP is adjacent to the short B helix, which in turn is close to the A helix. It is known also that the proposed A helix in domain B of R^I begins with Trp-260, which is photolabeled by 8-N_3cAMP that is bound to domain A (Figure 8). Thus, it is plausible that the binding of cAMP to domain B could induce a conformational change in the C helix that might be propagated to a region that is in close proximity to the A domain. The kinetic data of Ogreid et al. (1983), which predict that cAMP binds first to domain B in the holoenzyme, would be consistent with this model.

The structure of the apoprotein obviously is a critical piece of information that is missing. The two invariant charged residues in the cAMP binding site, Arg-82 and Glu-72, clearly make direct contact with the cyclic nucleotide ring in the binary cAMP–CAP structure. The position of these two residues in the apoprotein can only be speculated at this point since no crystal structure of the apoprotein is available. Obviously there is the potential for making and breaking hydrogen and electrostatic bonds in going from the apoprotein to the binary complex, and such changes could conceivably lead to a movement of the C helix. Additional structural data for both CAP and R^1 are required as well as additional mutagenesis studies before these questions can be adequately resolved.

Registry No. cAMP, 60-92-4; 8-N_3cAMP, 31966-52-6; Mg-ATP, 1476-84-2; L-Tyr, 60-18-4; CCAGCAGTTCAACAGCTTC·GGTCGTCAAGTTGTCGAAG, 112398-27-3; protein kinase, 9026-43-1.

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