Limited Proteolysis Alters the Photoaffinity Labeling of Adenosine 3′,5′-Monophosphate Dependent Protein Kinase II with 8-Azidoadenosine 3′,5′-Monophosphate†

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ABSTRACT: Photoaffinity labeling of the regulatory subunits of cAMP-dependent protein kinase with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) has proved to be a very specific method for identifying amino acid residues that are in close proximity to the cAMP-binding sites. Each regulatory subunit contains two tandem cAMP-binding sites. The type II regulatory subunit (R^{II}) from porcine heart was modified at a single site, Tyr-381 [Kerlavage, A., & Taylor, S. S. (1980) *J. Biol. Chem. 255*, 8483–8488]. When a proteolytic fragment of this R^{II} subunit was photolabeled with 8-N_3cAMP, two sites were covalently modified. One site corresponded to Tyr-381 and, thus, was analogous to the native R^{II}. The other site of modification was identified as Tyr-196, which is not labeled in the native protein. Photoaffinity labeling was carried out in the presence of various analogues of cAMP that show a preference for one of the two tandem cAMP-binding sites. These studies established that the covalent modification of Tyr-381 was derived from 8-N_3cAMP that was bound to the second cAMP-binding site (domain B) and that covalent modification to Tyr-196 was due to 8-N_3cAMP that was bound to the first cAMP-binding site (domain A). These sites of covalent modification have been correlated with a model of each cAMP-binding site on the basis of the crystal structure of the catabolite gene activator protein (CAP), which is the major cAMP-binding protein in *Escherichia coli*.

The regulatory subunits (R^I and R^{II}) of cAMP-dependent protein kinase represent a family of homologous proteins that includes at least three unique gene products (Lee et al., 1983; Weldon et al., 1985; Stein & Rubin, 1985; Jahnsen et al., 1986). These regulatory subunits account for the different forms of cAMP-dependent protein kinase, which have been classified into two general groups referred to as I and II on the basis of the order of their elution from anion-exchange resins (Corbin et al., 1975; Corbin & Keely, 1977). There are additional properties such as autophosphorylation (Erlichman et al., 1974; Rosen & Erlichman, 1975) and immunochemical characteristics (Kapoor et al., 1979) that further distinguish these two classes. In spite of these structural differences, each R subunit retains many common features. Each R subunit is a dimer that in the absence of cAMP aggregates with two monomeric catalytic (C) subunits to form an inactive holoenzyme. It is only in the presence of cAMP that the inactive holoenzyme dissociates into its active form. Each R subunit also retains a common and well-defined domain structure which consists of three general segments: two in tandem gene-duplicated cAMP-binding domains confined within the carboxy-terminal two-thirds of the protein (Potter et al., 1978; Corbin et al., 1978; Potter & Taylor, 1979a; Takio et al., 1980, 1982) and an amino-terminal segment that accounts for one-third of the molecule and contains (a) an essential site for interaction with the C subunit (Weldon & Taylor, 1985), (b) the major site for interaction between subunits in the R dimer (Potter & Taylor, 1979a, 1980; Zick & Taylor, 1980; Reimann, 1986), and (c) a "hinge" region that in the presence of cAMP is very susceptible to proteolysis (Flockhart et al., 1980; Potter & Taylor, 1979b; Takio, et al., 1980). Limited proteolysis cleaves the R subunit into a carboxy-terminal fragment that retains the cAMP-binding properties of the native protein but that has lost the primary contacts between the two subunits and is, thus, monomeric.

Initial reports indicated that there was a single cAMP-binding site per R subunit monomer (Hofmann et al., 1975; Beavo et al., 1975), but the presence of two cAMP-binding sites has been confirmed later for both R^I and R^{II} by several techniques (Corbin et al., 1978; Doskeland, 1978; Weber et al., 1979; Builder et al., 1980; Ogreid & Doskeland, 1980), and this is consistent with their primary structures (Takio et al., 1984; Titani et al., 1984). Even the R subunit from *Dictyostelium discoideum*, which was cloned recently, has these two gene-duplicated sites (Mutzel et al., 1987). Kinetic evidence has shown that the two cAMP-binding sites of the R subunit are nonidentical with respect to the rate with which they exchange bound nucleotide (Rannels & Corbin, 1980; Ogreid & Doskeland, 1981) and with respect to their preference for various substituted analogues of cAMP (Rannels & Corbin, 1980; Corbin & Rannels, 1981). A comparison of the amino acid sequences of R^I and R^{II} with another cAMP binding protein, the *Escherichia coli* catabolite gene activator protein (CAP), has clearly established that the cAMP-binding domains of CAP, R^I, and R^{II} are homologous proteins that have evolved from a common ancestral precursor (Weber et al., 1982, 1987).

Photoaffinity labeling using 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) has proved to be a powerful analytical tool for covalently labeling the different types of R subunits (Haley, 1975; Pomerantz et al., 1975) and also has proved to be a highly specific tool for mapping the environment of the

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cAMP-binding sites. Photolabeling of R^{II} (Kerlavage & Taylor, 1980) or type II holoenzyme (Kerlavage & Taylor, 1982) leads to nearly stoichiometric modification of only one residue, Tyr-381, whereas two residues, Trp-260 and Tyr-371, are covalently modified in the type I holoenzyme (Bubis & Taylor, 1985, 1987). Interpretation of this photolabeling has been greatly facilitated by superimposing the cAMP-binding domains of R^{I} and R^{II} onto the crystallographic coordinates of the cAMP-binding domain of CAP (Weber et al., 1987). On the basis of this comparison, it was predicted that the modification of the homologous tyrosine residues, Tyr-371 in R^{I} and Tyr-381 in R^{II}, was due to 8-N_3cAMP bound to the second cAMP binding site (domain B) in each protein, whereas the photoincorporation into Trp-260 of R^{I} was due to 8-N_3cAMP bound to the first cAMP-binding site (domain A). This model was confirmed experimentally for the type I holoenzyme by photolabeling with 8-N_3cAMP in the presence of various analogues of cAMP that show a strong preference for one site (Bubis & Taylor, 1987).

In this study, photolabeling of proteolytic fragments of R^{II} was characterized in an effort to establish whether the environment of either cAMP-binding site was altered as a consequence of limited proteolysis. In addition, photolabeling of both the type II holoenzyme and the proteolytic fragments of R^{II} was carried out in the presence of various analogues of cAMP in order to establish whether photolabeling of Tyr-381 correlates with occupancy of the second cAMP-binding site in the linear sequence of the polypeptide chain. Photolabeling of a second site in the proteolytic fragments of R^{II} has been correlated with occupancy of the first cAMP-binding domain. Finally, the sites of covalent modification in the proteolytic fragments have been correlated with the overall domain structure of the protein and compared to the type I regulatory subunit.

Experimental Procedures

Materials. Reagents were purchased as follows: [2,8-^{3}H]cAMP (15.0 Ci/mmol) and 8-N_3[^{32}P]cAMP (67.7 Ci/mmol), ICN; 8-N_3[2-^{3}H]cAMP (17.0 Ci/mmol), New England Nuclear; [8-^{3}H]cGMP (15.8 Ci/mmol), Amersham; cAMP, 8-N_3cAMP, ATP, 8-(methylamino)-cAMP, cGMP, N^6-[[(6-aminohexyl)carbamoyl]methyl]-cAMP, N^6-monobutyryl-cAMP, bovine serum albumin, histone IIA, and CM-Sepharose, Sigma; Cytoscint, West Chem; L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, United States Biochemical Corp.; trifluoroacetic acid (sequanal grade), Pierce; acetonitrile (HPLC grade), Fisher Scientific; TPCK, Calbiochem.

Proteins. The R and C subunits of type II cAMP-dependent protein kinase were prepared from frozen porcine heart tissue as described previously (Nelson & Taylor, 1981), with the exception that the R subunit was eluted from cAMP–Sepharose with buffer I [25 mM potassium phosphate, 5 mM β-mercaptoethanol, and 2 mM ethylenediaminetetraacetic acid (EDTA) (pH 6.5)] containing 25 mM cGMP. The endogenous proteolytic fragment of R^{II}, COOH-R^{II}, was isolated in the same way from a preparation that was left at 4 °C overnight prior to the DE-52 purification step. R^{II} that was free of cyclic nucleotide was prepared by dialyzing 2 mg of cGMP-eluted R^{II} overnight in buffer I at 4 °C to remove most of the free cGMP. After addition of 0.1 mL of 1 mM [^{3}H]cGMP (50 000 cpm/μL), the mixture was incubated at room temperature for 30 min, which is sufficient time to exchange the bound nucleotide. The mixture was then dialyzed for 5 days with extensive changes of buffer I until no radioactivity was detectable. Another proteolytic fragment of R^{II}, Ch-R^{II}, was prepared by limited proteolysis with 1:1000 chymotrypsin:R^{II} subunit at 4 °C for 30 min and stopping the reaction with a 50-fold excess of TPCK. R^{II} was autophosphorylated by incubating 0.5 mg of the protein with 10 μg of C subunit for 1 h at 4 °C in buffer I containing 0.5 mM ATP and 5 mM magnesium acetate. Excess magnesium acetate and ATP were removed by dialysis against buffer I. Autophosphorylation was complete on the basis of polyacrylamide gel electrophoresis carried out in the presence of sodium dodecyl sulfate (SDS), which distinguishes the phospho and dephospho forms of heart R^{II} readily. Autophosphorylated Ch-R^{II} was prepared by limited chymotrypsin proteolysis of autophosphorylated R^{II}. Holoenzyme or Ch-R^{II}–C complex was reconstituted by dialysis of R^{II} or Ch-R^{II} with a 5% excess of C subunit in buffer I containing 5% glycerol for 5 days at 4 °C. To remove excess C subunit, 2 mL of CM-Sepharose was added per milligram of excess C subunit in the same buffer at pH 6.1. 2-Mercaptoethanol was removed by dialysis prior to 8-N_3cAMP binding.

Measurements of Cyclic Nucleotide Binding. Cyclic nucleotide binding was measured by Millipore filtration (methods A and B), equilibrium dialysis, and ammonium sulfate precipitation followed by Millipore filtration as described previously (Bubis & Taylor, 1985). The binding reaction mixture contained 40–95 nM cAMP-binding proteins and various concentrations of [^{3}H]cAMP (0–2 μM) or 8-N_3[^{3}H]cAMP (0–0.4 μM). Details are given in the figure legends.

Competition of Cyclic Nucleotide Binding by cAMP Analogues. Reaction mixtures contained 2 nM holoenzyme II or Ch-R^{II}–C complex, 30 nM [^{3}H]cAMP or 8-N_3[^{3}H]cAMP, and 0–50-fold excess derivative of cAMP over radioactive cyclic nucleotide in 50 mM potassium phosphate, 2 mM EDTA, and 1 mg/mL bovine serum albumin (BSA), pH 6.8. After an incubation at 4 °C for 16 h, ^{3}H-labeled cyclic nucleotide binding was measured as described above.

Isolation of Covalently Modified Peptides from 8-N_3cAMP-Labeled Proteins. Type II holoenzyme (8 nmol), R^{II} dimer (8 nmol), native COOH-R^{II} (41 nmol), and Ch-R^{II} (40 nmol) were incubated in volumes of 1–2 mL with 120 nmol of 8-N_3[^{3}H]cAMP for 1 h at 30 °C and then for 16 h at 4 °C. The samples were irradiated on ice with a UVS-11 lamp (254 nm) for 10 min at a distance of 5 cm with occasional mixing. The proteins were dialyzed against 50 mM NH_4HCO_3 (pH 8.3), digested with 50:1 TPCK-treated trypsin for 18 h at 37 °C, and then separated by high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis as outlined below.

Photoaffinity Labeling in the Presence of cAMP Analogues. Holoenzyme II (2.9 nmol) or Ch-R^{II} (6.5 nmol) was incubated for 1 h at 30 °C followed by an overnight incubation at 4 °C with 30–34 nmol of nonradioactive analogue and 0.074–0.09 nmol of 8-N_3[^{32}P]cAMP in 25 mM potassium phosphate (pH 6.5) containing 2 mM EDTA. Photolabeling was performed as described above. The covalent incorporation of 8-N_3cAMP was measured by the following criteria: (1) polyacrylamide gel electrophoresis; (2) trichloroacetic acid precipitation with a 10% aqueous solution, followed by filtration through GF/C glass fiber filters; (3) peptide mapping by HPLC or polyacrylamide gel electrophoresis following tryptic digestion.

High-Performance Liquid Chromatography (HPLC). HPLC was carried out on an Altex 3200 system with a Vydac C_{18} column. The buffers employed were (a) 0.1% trifluoroacetic acid (TFA) (pH 2.15) and (b) CH_3CN. Further purification of 8-N_3cAMP-modified peptides was achieved by rerunning the peptides on a second Vydac C_{18} column in a

different buffer system: (a) 0.01 N ammonium phosphate (pH 6.9) and (b) CH_3CN. Prior to sequencing, the peptides were chromatographed again on a third Vydac C_{18} column with a 20-min linear gradient from 0.1% TFA to CH_3CN (0–100%) to remove ammonium phosphate, which frequently interfered with the sequencing. Absorbance was monitored at 219 mm on a SF 769 Kratos spectrophotometer equipped with a flow-through cell. Details of the gradient elution are given in the figure legends.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with 10% acrylamide in the lower gel. The tryptic peptides of 8-N_3cAMP-modified proteins were separated according to the procedure of Pantazis and Bonner (1981) on 1.5-mm slabs containing 40% acrylamide. The gels were electrophoresed at 75 V for 2 days, dried on Whatman No. 3MM filter paper, and autoradiographed on Kodak X-Omat film.

Protein Kinase Assay. Protein kinase was assayed spectrophotometrically according to the procedure described by Cook et al. (1982), with the synthetic peptide L-R-R-W-S-V-A-E-L as substrate.

Amino Acid Analyses. Analyses were performed on an LKB Biochrom Model 4400 automated amino acid analyzer. Samples were hydrolyzed in vacuo at 110 °C in 6 M HCl for 24 h or at 150 °C in same solution for 1 h.

Sequencing. Gas-phase sequencing was carried out on an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH) amino acids were identified by HPLC as described by Hunkapillar and Hood (1983) on an IBM Cyano column. Manual sequencing was carried out by dansyl Edman degradation according to the method of Hartley (1970). The *tert*-butyl acetate washes generated after each step in the peptide degradation were transferred to scintillation vials and counted for radioactivity with 6 mL of Cytoscint. For solid-phase sequencing, peptides were coupled to DITC–glass by incubation overnight at room temperature in 0.1 M $NaHCO_3$ and 10% 1-propanol, pH 8.5. Sequencing was carried out on a sequencer modeled after the instrument described by Doolittle et al. (1975). Thioazolinone amino acids were converted to their PTH derivatives by incubation with 0.2 mL of 20% aqueous TFA at 80 °C for 10 min. PTH amino acids were identified by HPLC according to Bhown et al. (1978). Aliquots of each step also were counted for radioactivity.

Results

Reconstitution of cAMP-Dependent Enzyme. Both R^{II} and the chymotryptic proteolytic fragment of R^{II} were combined with a 5% molar excess of C subunit and dialyzed under conditions that typically lead to the formation of holoenzyme. After removal of excess C subunit by ion-exchange chromatography, the formation of holoenzyme and Ch-R^{II}–C was monitored by measuring the dependency of kinase activity on added cAMP. In both cases, enzymatic activity was negligible in the absence of cAMP. Polyacrylamide gel electrophoresis also confirmed that both the native R^{II} and the proteolytic fragment of R^{II} were present in amounts that were equivalent on a molar basis to the amount of C subunit (data not shown). Both lines of evidence established that this proteolytic fragment of R^{II} is capable of reassociating with the C subunit with a high affinity to form a stable complex that is inactive in the absence of cAMP.

Cyclic Nucleotide Binding. Cyclic nucleotide binding was measured by equilibrium dialysis, by ammonium sulfate precipitation followed by filtration, and by two different

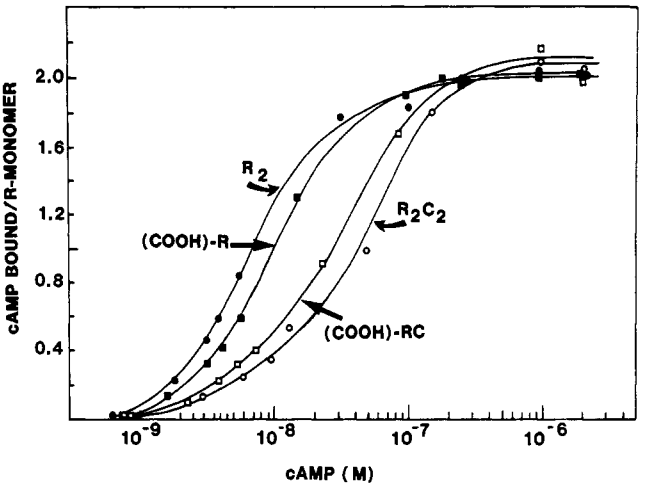

FIGURE 1: Binding of cAMP to R^{II}_2, Ch-R^{II}, holoenzyme II, and the reconstituted complex between Ch-R^{II} and the C subunit. R^{II} dimer (40 nM), holoenzyme II (45 nM), Ch-R^{II} (90 nM), or Ch-R^{II}–C (95 nM) was incubated with various concentrations of [^{3}H]cAMP (0–2.0 μM). Binding of cAMP to the different proteins was measured by equilibrium dialysis. R_2C_2 = holoenzyme (○), R_2 = R^{II} dimer (●), (COOH)-RC = complex between Ch-R^{II} and the C subunit (□), and (COOH)-R = Ch-R^{II} (■).

Millipore filtration procedures. Method A is carried out in low salt, whereas with method B high salt concentrations are used and histone is included (Bubis & Taylor, 1985). Binding constants for [^{3}H]cAMP were characterized for the R^{II} dimer, for Ch-R^{II}, for the type II holoenzyme, and, finally, for the reconstituted complex between Ch-R^{II} and the C subunit. Figure 1 illustrates the results that were obtained by equilibrium dialysis, and equivalent results were seen with ammonium sulfate precipitation or Millipore filtration method B. Millipore filtration method A showed 1 mol of cAMP bound per mole of R^{II} monomer or Ch-R^{II}, whereas the other methods showed a stoichiometry of 2 mol of cAMP bound per mole of R^{II} monomer or Ch-R^{II}. The apparent K_d's for the native R subunit and for the proteolytic fragment Ch-R^{II} did not differ substantially; however, for both proteins, the apparent K_d(cAMP) was dependent on the aggregation state. Complex formation with C subunit in both cases resulted in an increase in apparent K_d(cAMP). In the case of the native protein, the K_d(cAMP) shifted from 7 nM for R_2 to 40 nM for R_2C_2. The proteolytic fragment of R^{II} showed a K_d(cAMP) of 10 nM, which increased to 30 nM when the protein was complexed with the C subunit. Autophosphorylation of R^{II} or Ch-R^{II} by the C subunit had little effect on the K_d(cAMP). K_d(cAMP) was 8 and 11 nM for the phosphorylated R^{II} and Ch-R^{II}, respectively.

Binding of the photoaffinity analogue 8-N_3cAMP to both holoenzyme II and the Ch-R^{II}–C complex also was characterized. As shown previously by Kerlavage and Taylor (1982), a maximum of 1 mol of 8-N_3cAMP bound per mole of R^{II} monomer was obtained with Millipore filtration method B. However, as indicated in Figure 2, when equilibrium dialysis was used to measure binding, a stoichiometry of 2 mol of 8-N_3cAMP/mol of R^{II} monomer was obtained.

Covalent Modification of Proteolytic Fragments of R^{II} by 8-N_3cAMP. In addition to the chymotryptic proteolytic fragment described above, a similar proteolytic fragment that was formed by endogenous proteolysis was isolated. This fragment begins at Cys-97, which was confirmed by gas-phase sequencing. Photolabeling of both of these proteins was compared to the covalent modification that had been characterized previously for R^{II}. The various proteins (1–5 mg in 1–2 mL) were incubated with 8-N_3[^{3}H]cAMP and subse-

Table I: Sequence Analysis of the New Tryptic Peptide Modified in the Chymotryptic Fragment of Porcine R^{II} by 8-N_3cAMP

	192								200										210		213
Peptide[a]	Ser	Val	Gly	Gln	Tyr	Asp	Asn	His	Gly	Ser	Phe	Gly	Glu	Leu	Ala	Leu	Met	Tyr	Asn	Thr	Pro	Arg
Gas Phase Sequencing																						
Modified Peptide	→	→	→	→		→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Unmodified Peptide	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Manual Sequencing																						
Radioactivity[b] (10% cpm/step)	18	22	44	25	324	277	216	96	50	72	48											

[a] The sequence indicated is based on the homologous peptide in bovine R^{II}. [b] Only the first 11 residues were manually sequenced.

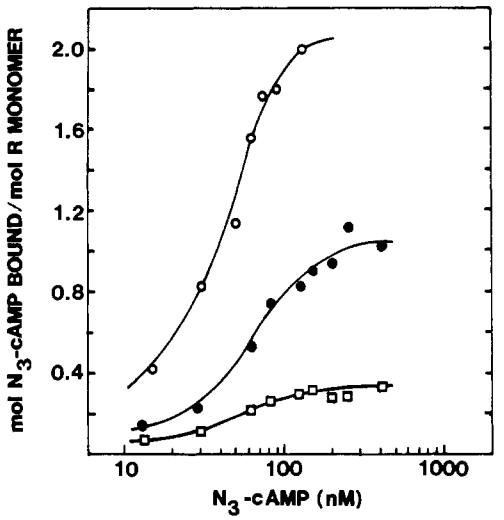

FIGURE 2: Binding of 8-N_3cAMP to type II holoenzyme. Holoenzyme type II (10 nM) was incubated with various concentrations of 8-N_3[^{3}H]cAMP (0–0.4 μM). 8-N_3cAMP was measured by equilibrium dialysis (○), Millipore filtration assay A (□), and Millipore filtration assay B (●).

quently irradiated, as described under Experimental Procedures. After digestion with TPCK-treated trypsin, the resultant peptides were resolved by HPLC with a 0.1% TFA to CH_3CN gradient. The resultant profiles are summarized in Figure 3. As described previously, the native R^{II} dimer or the reconstituted holoenzyme was covalently modified with a stoichiometry that exceeds 0.5 mol/mol at a single site (Figure 3, top). In contrast, two peptides were photolabeled in the endogenous proteolytic fragment of R^{II} (Figure 3, middle). The identical two peaks were observed in the fragment generated by chymotrypsin (data not shown).

The peptides modified by 8-N_3[^{3}H]cAMP were purified further by HPLC with a 0.01 N ammonium phosphate (pH 6.8) to CH_3CN gradient (Figure 3, inset). The peptide corresponding to the first peak of radioactivity (Figure 3, top panel) was sequenced by solid-phase methods, by gas-phase sequencing, and by manual sequencing. The summarized sequence found by all the methods was Asn-Ile-Ser-His-X-Glu-Glu-Gln-Leu-Val-Lys, and this peptide is identical with the peptide that is modified stoichiometrically in the native R^{II}. The unidentified residue at step 5 corresponds to a Tyr in the unmodified peptide. Counting aliquots of each step of the manual or solid support sequencing showed that the radioactivity was associated with step 5 in peptide A (Tyr-381) (data not shown). Due presumably to the negative charge of 8-N_3[^{3}H]cAMP, the radiolabeled residue remains on the filter in the gas-phase sequencer, and no radioactivity or amino acid peak was detected in any step. The peptide isolated from the second peak of radioactivity (Figure 3, middle panel) was sequenced by gas-phase methods. The identical profile was observed following photolabeling of 5 mg of the chymotryptic

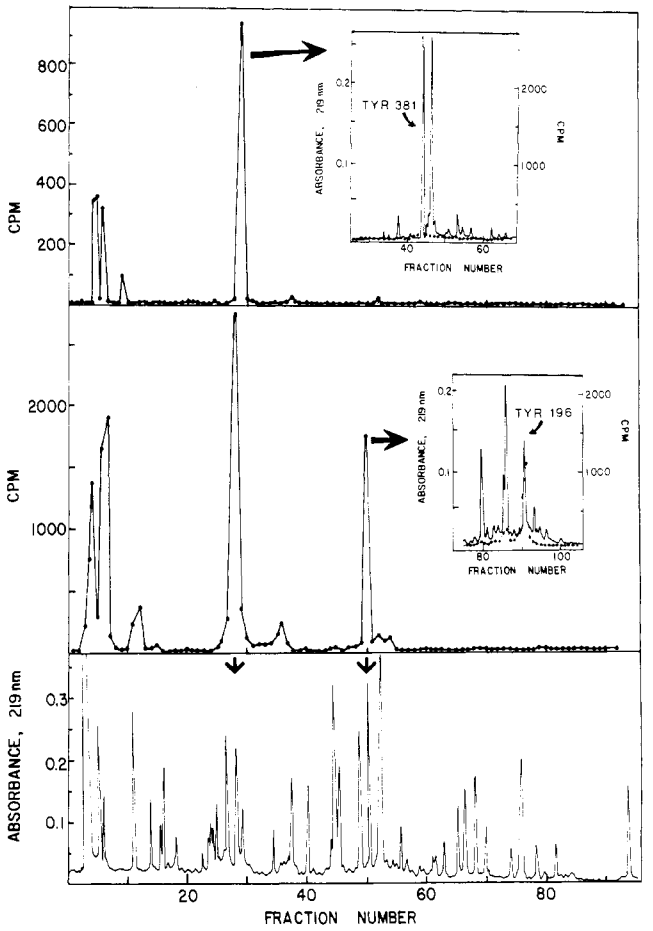

FIGURE 3: High-performance liquid chromatography of the 8-N_3-[^{3}H]cAMP-modified holoenzyme and of the 8-N_3cAMP-labeled endogenous proteolytic fragment of R^{II}. Holoenzyme II (8 nmol) or the endogenous proteolytic fragment of R^{II} (41 nmol) was incubated with 120 nmol of 8-N_3[^{3}H]cAMP and then irradiated as described under Experimental Procedures. After digestion with TPCK-trypsin, the peptides were separated by HPLC with a Vydac C_{18} column and a 120-min linear gradient of 0.1% TFA (pH 2.15) to CH_3CN (0–60%). Radioactivity for the holoenzyme is shown in the top panel. Radioactivity and absorbance at 219 nm for the fragment of R^{II} generated by endogenous proteolysis are shown in the middle and lower panels, respectively. A similar profile was observed for the chymotryptic fragment of R^{II}. Also shown are the rechromatographs (insets of top and middle panels) of the radioactive peaks on a second Vydac C_{18} column with a 120-min linear gradient of 0.01 N ammonium phosphate (pH 6.9) to CH_3CN (0–30%).

proteolytic fragment. The sequence of that peptide was carried out with both gas-phase and manual methods so that radioactivity could be recovered (Table I). The only blank step in the sequence was residue 5, which is a Tyr in the unmodified peptide. Counting aliquots of each step of the manual sequencing showed that the radioactivity was associated with step 5. As with the first peptide, the labeled residue at step 5 stayed bound to the filter of the gas-phase sequencer, and

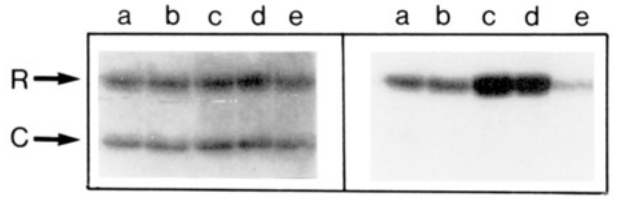

FIGURE 4: SDS–polyacrylamide gel electrophoresis of holoenzyme modified with trace amounts of 8-N_3[^{32}P]cAMP in the presence of different analogues of cAMP. Holoenzyme (1.2 μM) was incubated with 12.5 μM various cAMP derivatives and 29.5 nM 8-N_3[^{32}P]cAMP as described Experimental Procedures. Proteins were then separated by SDS–polyacrylamide gel electrophoresis on a 10% gel. (Left panel) Protein staining with Coomassie blue; (right panel) autoradiogram. R = regulatory subunit; C = catalytic subunit. (a) Control (no cAMP analogue added); (b) cAMP); (c) N^6-monobutyryl-cAMP; (d) N^6-[[(6-aminohexyl)carbamoyl]methyl]-cAMP; (e) 8-(methylamino)-cAMP.

no radioactivity or amino acid peak was detected. On the basis of this sequence, the modified residue was identified as Tyr-196. The stoichiometry for modification of Ch-R^{II} was 0.30 and 0.55 mol/mol of monomeric subunit for Tyr-196 and Tyr-381, respectively.

Correlation of Site-Selective Occupancy of cAMP-Binding Sites with Photolabeling. In order to correlate photolabeling of Tyr-196 and Tyr-381 with occupancy of the two cAMP-binding sites in the linear sequence, [^{3}H]cAMP or 8-N_3-[^{32}P]cAMP binding to both holoenzyme II and the Ch-R^{II} complex was carried out in the presence of a 0–50-fold molar excess of derivatives of cAMP that show a strong preference for one of the two cAMP-binding sites.

Synergistic Effect of cAMP Analogues on Photoaffinity Labeling. When either holoenzyme II or Ch-R^{II} was incubated with saturating amounts of nonradioactive cAMP derivatives and a trace amount of 8-N_3[^{32}P]cAMP, a clear synergistic effect was observed. Both SDS–polyacrylamide gel electrophoresis (Figure 4) and two different filtration procedures (data not shown) showed that photolabeling by 8-N_3cAMP was increased in the presence of N^6-substituted analogues of cAMP, whereas when an 8-substituted analogue such as 8-(methylamino)-cAMP was used, a decrease in 8-N_3cAMP photolabeling was observed.

The specificity of the effect of the different cAMP derivatives on the covalent modification by 8-N_3cAMP was assessed by digesting the modified proteins with trypsin and then resolving the resulting peptides by HPLC (Figure 5) or, alternatively, by peptide gels (data not shown). For holoenzyme II, where 8-N_3cAMP characteristically only labels one peptide, the synergistic increase in labeling was due exclusively to an increase in the covalent modification of Tyr-381 when photolabeling was carried out in the presence of N^6-monobutyryl-cAMP. A similar effect was observed with 8-N_3cAMP when the photolabeling of holoenzyme II was carried out in the presence of N^6-[[(6-aminohexyl)carbamoyl]methyl]-cAMP (data not shown). On the other hand, the labeling of Tyr-381 was nearly abolished in the presence of 8-(methylamino)-cAMP.

In the case of the chymotryptic proteolytic fragment where two peptides were photolabeled with 8-N_3[^{3}H]cAMP, synergistic labeling of protein was also observed when photolabeling was carried out in the presence of N^6-monobutyryl-cAMP. As seen in Figure 6, HPLC separation of the tryptic peptides of 8-N_3cAMP-modified Ch-R^{II} showed that this synergism was due entirely to the enhanced photolabeling of Tyr-381, similar to what was observed for the holoenzyme. In contrast, ra-

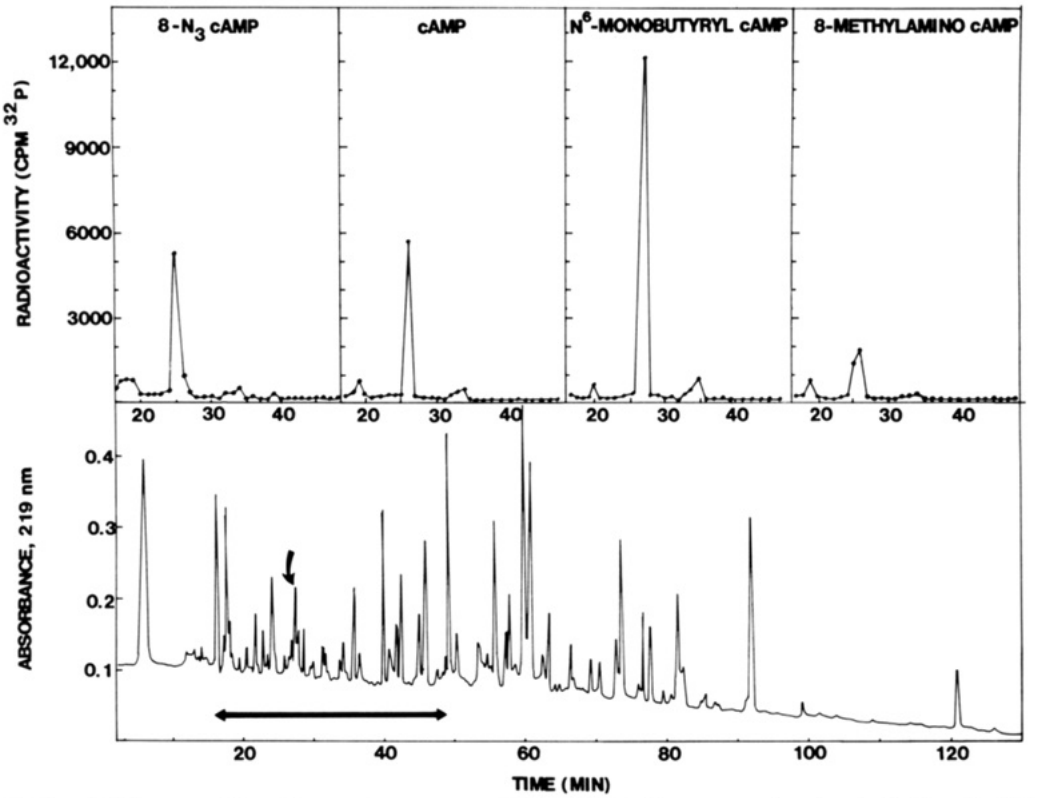

FIGURE 5: Photolabeling of holoenzyme II by subsaturating amounts of 8-N_3cAMP in the presence of various derivatives of cAMP. Holoenzyme (2.9 nmol) was incubated with 0.074 nmol of 8-N_3[^{32}P]cAMP and 34 nmol of nonradioactive cAMP analogues. Samples were incubated, irradiated, and separated by HPLC after treatment with TPCK-trypsin as described under Experimental Procedures. The tryptic peptides were eluted with a 5-min linear gradient from 0 to 10% CH_3CN followed by a 120-min linear gradient from 10 to 50% CH_3CN. (Top) Radioactivity; (bottom) absorbance at 219 nm. Underlined are the fractions for which radioactivity profiles were plotted on the top panel for each cAMP analogue used. Also shown with an arrow is the position of the peak region where the 8-N_3cAMP-modified peptide runs under these HPLC conditions.

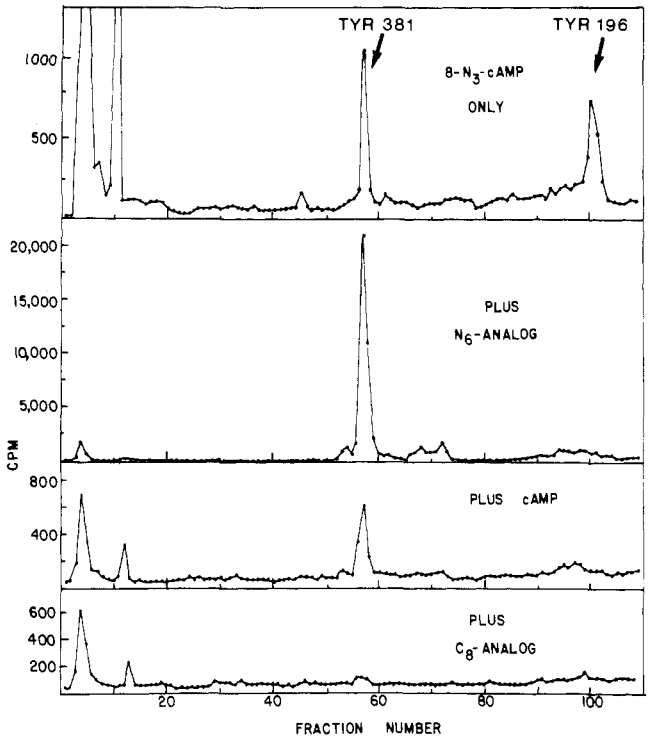

FIGURE 6: Photolabeling of CH-R^{II} by trace amounts of 8-N_3-[^{32}P]cAMP in the presence of various derivatives of cAMP. Ch-R^{II} (6.5 nmol) was incubated with 0.09 nmol of 8-N_3[^{32}P]cAMP and 34 nmol of nonradioactive analogues of cAMP. Samples were incubated and were photolyzed as described under Experimental Procedures. After an overnight digestion with TPCK-trypsin, the peptides were eluted by HPLC with a 120-min linear gradient of 0.1% TFA to CH_3CN (0–30%) followed by a 60-min linear gradient from 30 to 60% CH_3CN. Shown are the radioactivity profiles obtained with the different cyclic nucleotide used. N_6-analog = N^6-monobutyryl-cAMP; C_8-analog = 8-(methylamino)-cAMP.

diolabeling of the second peptide containing Tyr-196 disappeared completely in the presence of N^6-monobutyryl-cAMP (Figure 6). 8-(Methylamino)-cAMP abolished labeling of both peptides, whereas cAMP abolished labeling of Tyr-196 and reduced the labeling of Tyr-381. Similar results were observed by peptide gels (data not shown).

The covalent modification in the presence of saturating amounts of 8-N_3[^{32}P]cAMP showed a stoichiometry of 0.6 mol/mol of R monomer into Tyr-381 of the native protein (Figure 5, left panel on top) and 0.5 mol/mol and 0.4 mol/mol into Tyr-381 and Tyr-196, respectively, of the proteolytic fragment (Figure 6, top).

Discussion

The fact that each protomer of the R subunit binds two molecules of cAMP was initially missed by several investigators because the conventional filter disk assay artifactually and selectively excludes one class of binding site. We also reported that only 1 mol of 8-N_3cAMP was bound per R monomer in contrast to cAMP (Kerlavage & Taylor, 1982). These results were based on a modified version of the filter disk assay (method B), which is capable of detecting 2 mol of cAMP/R monomer. No binding of 8-N_3cAMP to holoenzyme was detected with the conventional membrane filtration procedure (method A). A parallel measurement of the activation of the holoenzyme suggested at that time that occupancy of only one of the two cyclic nucleotide binding sites in the R^{II} monomer was sufficient to cause complete dissociation of the holoenzyme. Ogreid and Doskeland (1982) subsequently demonstrated that 8-N_3cAMP actually does interact with both types of cyclic nucleotide binding sites of R^{II}, but the rate of

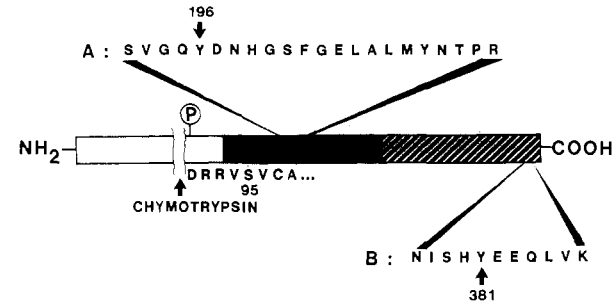

FIGURE 7: Localization of radioactive peptides in the primary structure of R^{II}. Two peptides were photolabeled by 8-N_3cAMP in the chymotrypic fragment of R^{II}. Both peptides were characterized by manual, solid-phase, and/or gas-phase sequencing as described under Results. Peptide A is located in cAMP-binding domain A, and peptide B is located in cAMP-domain B. Arrows show the amino acid residues modified by 8-N_3cAMP tyrosine-196 and tyrosine-381. Also shown is the NH_2-terminal region sequenced by gas-phase sequencing for the chymotryptic fragment of R^{II}.

exchange of 8-N_3[^{3}H]cAMP with cAMP was 20000 times faster from site A than from site B. This rapid dissociation of 8-N_3cAMP from stie A of R^{II} most likely explains our earlier binding results and prevented detection of binding to the second site by filter disk assay B. If 8-N_3cAMP binding to R^{II} is measured by equilibrium dialysis, 2 mol of 8-N_3cAMP is bound per mole of R^{II} monomer, which is consistent with the results of Ogreid and Doskeland (1982) and demonstrates that 8-N_3cAMP interacts with site A as well as site B in the process of activation of the holoenzyme.

The R^{II} subunit contains a "hinge" region that is very susceptible to proteolysis by endogenous or exogenous proteases. Limited proteolysis of R^{II} with chymotrypsin generates a 37-kDa fragment that retains the two tandem cAMP-binding sites but that has lost the region of interaction between subunits in the dimer. Chymotrypsin cleaves at Phe-90, which just preceeds the site of autophosphorylation (Figure 7). This fragment, Ch-R^{II}, is capable of forming a dimeric complex with the C subunit, and this complex is dependent on the addition of cAMP for activation. Covalent modification of this proteolytic fragment with 8-N_3cAMP differed from covalent modification of the R^{II} dimer (Kerlavage & Taylor, 1980) or of the reconstituted type II holoenzyme (Kerlavage & Taylor, 1982). In addition to the covalent modification of Tyr-381, which also is seen in R^{II}, a second residue was modified in the proteolytic fragment. This second site of covalent modification was identified as Tyr-196. The endogenous proteolytic fragment of R^{II} is cleaved before Cys-97. This fragment lacks the two arginine residues that precede the autophosphorylation site in R^{II} and, in consequence, does not form a stable complex with the C subunit (Weldon & Taylor, 1983); however, it showed the same pattern of photolabeling that was seen with the chymotryptic fragment.

An initial comparison of the amino acid sequence of R^{II} with CAP showed clearly that the two cAMP binding domains of R^{II} were homologous to the cAMP-binding domain of CAP (Weber et al., 1982). Further model building of each of the cAMP-binding domains of R^{II} has been carried out by actually building the R^{II} sequences into the coordinates of the CAP crystal structure. Since this model was shown to be consistent with the previously observed photolabeling of R^I and R^{II}, it was used to interpret the photolabeling results described here for the proteolytic fragments of R^{II} (Weber et al., 1986). According to the proposed model for cAMP-binding domain B, Tyr-381 in R^{II} would be located on the C helix, which interacts with the adenine ring of cAMP in CAP, and therefore, one would predict that this residue would be a candidate

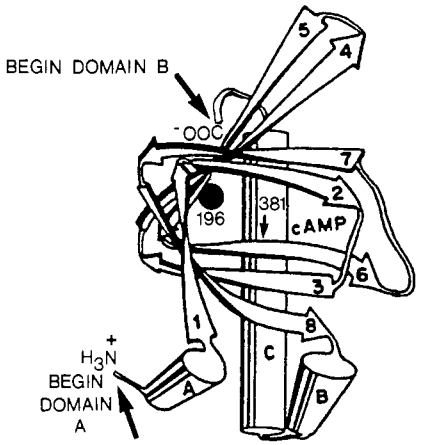

FIGURE 8: Model of cAMP-binding domain A of R^{II} showing the new modification site when Ch-R^{II} is photolabeled with 8-N_3cAMP. View of the cAMP binding domain of CAP with the amino acid sequence of cAMP-binding domain A of R^{II} superimposed on it. Shown with an arrow is where Tyr-381 will be located if cAMP-binding domain B of R^{II} is superimposed onto the cAMP-binding domain of CAP in a similar manner. This model predicts that the polypeptide chain of R^{II} folds in a manner analogous to CAP in the region containing the cAMP-binding domains.

for covalent modification by 8-N_3cAMP in both the native R^{II} and the 37-kDa fragment of R^{II}. The modification of Tyr-196 in Ch-R^{II} is most likely due to 8-N_3cAMP bound to cAMP-binding domain A on the basis of its localization in the primary structure of R^{II} (Figure 7). The environment surrounding the adenine ring in site A clearly has been slightly altered in the proteolytic fragment of R^{II} since this Tyr is not photolabeled in the native R^{II}. It is possible that in the native R^{II} Tyr-196 forms a hydrogen bond with the NH_2-terminal region of R^{II}, but when this region is removed by proteolysis, Tyr-196 rotates toward the bound cAMP, and in this conformation, Tyr-196 is close to the adenine ring. This would explain why Tyr-196 is not photolabeled in the native protein. This would also suggest that Tyr-196 may be a contact point between the N-terminal portion of R^{II} and the cAMP-binding domains, and this region may contribute to the intersubunit interactions that Ogreid et al. (1985) described in the R^{I} dimer.

Photolabeling of holoenzyme type I with 8-N_3cAMP also leads to the modification of two residues, Trp-260 and Tyr-371. This photoaffinity labeling of R^{I} has been correlated with occupancy of cAMP binding sites by carrying out the photolabeling in the presence of various analogues of cAMP that bind preferentially to one site (Bubis & Taylor, 1987). These studies established that modification of Trp-260, although it is the beginning of domain B, was due to 8-N_3cAMP bound to domain A, whereas modification of Tyr-371 results from 8-N_3cAMP bound to domain B. A similar correlation for R^{II} and Ch-R^{II} has been demonstrated here by photolabeling holoenzyme II or the proteolytic fragments of R^{II} with trace amounts of 8-N_3cAMP in the presence of a variety of analogues that show a strong preference for one of the cAMP-binding sites. As shown in Figure 4, the HPLC separation of labeled tryptic peptides of the holoenzyme showed that modification of Tyr-381 was increased synergistically when N^6 derivatives of cAMP were used. Only the presence of C-8 derivatives of cAMP such as 8-(methylamino)-cAMP diminished the labeling of Tyr-381. For the proteolytic fragments where both Tyr-196 and Tyr-381 were photolabeled by 8-N_3cAMP, the peptide containing Tyr-381 behaved similarly as in holoenzyme II, but the peptide containing Tyr-196 disappeared completely when N^6 substituents of cAMP were used (Figure 6). Modification of Tyr-196 thus can be correlated directly with occupancy of site A, which is the site that prefers N^6 derivatives of cAMP. Conversely, occupancy of site B, which prefers C-8-substituted analogues of cAMP, correlates with the covalent modification of Tyr-381.

Figure 8 depicts the models that have been proposed for each of the two cAMP-binding domains of R^{II} with domain A as an example. Photolabeling of R^{I} provides a preliminary basis for orienting these two domains since Trp-260 must be in close contact to the C-8 position of the adenine ring of 8-N_3cAMP that is bound to domain A. The predicted location of Tyr-196 is also indicated on this model. On the basis of this model, the position of Tyr-196 actually could be very close to Trp-260 in R^{I} in the tertiary structure even though these two residues lie far apart in the linear sequence. The fact that the two cAMP-binding domains are tandem gene-duplicated domains mandates that the two domains are in close contact, and this also would be consistent with the positive cooperativity that is seen for cAMP binding. Photoaffinity labeling appears to be a very precise mechanism for mapping specific regions of each cAMP binding site. In some cases, photoaffinity labeling also is capable of detecting subtle changes in the immediate environment of the cAMP-binding sites such as those described here for some of the proteolytic fragments of R^{II}.

Registry No. 8-N_3cAMP, 31966-52-6; Tyr, 60-18-4; protein kinase, 9026-43-1.

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