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Correlation of Photolabeling with Occupancy of cAMP Binding Sites in the Regulatory Subunit of cAMP-Dependent Protein Kinase I†

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ABSTRACT: Each regulatory subunit of the cAMP-dependent protein kinase contains two in-tandem cAMP binding sites. Photolabeling of holoenzyme I with 8-azidoadenosine 3′,5′-monophosphate (8-N_3-cAMP) leads to the covalent modification of two residues, Trp-260 and Tyr-371. In order to correlate photolabeling of these two residues with occupancy of each specific cAMP binding site, photolabeling was carried out in the presence of various analogues of cAMP that bind preferentially to one site. Photolabeling of holoenzyme I after dissociation of 60% of 8-N_3-[^{3}H]cAMP with an excess of N^6-monobutyryl-cAMP nearly abolished the incorporation of 8-N_3-cAMP into Trp-260, whereas the modification of Tyr-371 was reduced by 49%. When 8-N_3-[^{32}P]cAMP was bound under equilibrium conditions in the presence of various cAMP analogues, N^6-monobutyryl-cAMP also selectively abolished incorporation of radioactivity into Trp-260, whereas 8-(methylamino)-cAMP preferentially reduced the covalent modification of Tyr-371. Photolabeling with trace amounts of 8-N_3-[^{32}P]cAMP in the presence of saturating amounts of N^6-monobutyryl-cAMP led to the covalent modification of only Tyr-371. In addition, photolabeling of Tyr-371 was enhanced synergistically in the presence of N^6-monobutyryl-cAMP. MgATP reduced the covalent modification of both Trp-260 and Tyr-371 but showed no selectivity for either site. These studies support a model that correlates photolabeling of Trp-260 with occupancy of cAMP binding site A and photolabeling of Tyr-371 with occupancy of cAMP binding site B. Thus, Trp-260, although it lies at the boundary between domain A and domain B, must be in close contact with the cyclic nucleotide that is bound to domain A. The results also establish unambiguously that N^6-substituted analogues of cAMP which are selective for the fast dissociation site preferentially bind to the first cAMP binding site in the linear sequence (site A), whereas C-8-substituted analogues which are selective for the slow dissociation site preferentially bind to the second site (site B). These two sites are correlated with other features that are known to distinguish the two cAMP binding sites.

Two general classes of cAMP-dependent protein kinases have been characterized, type I and type II, on the basis of elution from (diethylaminoethyl)cellulose (Corbin et al., 1975). Both isoforms exist as inactive tetramers containing two regulatory subunits (R)[1] and two catalytic subunits (C). The primary differences in the holoenzymes can be attributed to differences in the regulatory subunits (Hofmann et al., 1975; Zoller et al., 1979), and there are at least three unique genes that code for distinct regulatory subunits (Lee et al., 1983; Weldon et al., 1985; Stein & Rubin, 1985; Jahnsen et al., 1986). Despite

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[1] Abbreviations: 8-N_3-cAMP, 8-azidoadenosine 3′,5′-monophosphate; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; CAP, *Escherichia coli* catabolite gene activator protein; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Trp, tryptophan; Tyr, tyrosine; K_d, apparent equilibrium dissociation constant; K_i, apparent equilibrium inhibition constant; M_r, molecular weight; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

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this heterogeneity, each R-subunit retains certain common features. Each regulatory subunit is a dimeric protein, and each protomer has a well-defined domain structure that is conserved. In general, the amino-terminal region of the polypeptide chain is the point of contact between subunits in the dimer and also contains an essential site for interaction with the C-subunit (Potter & Taylor, 1979; Reimann, 1986; Weldon & Taylor, 1985). In contrast, the cAMP binding sites are associated with the carboxy-terminal region (Corbin et al., 1978; Potter & Taylor, 1979; Takio et al., 1980, 1982).

Each R-subunit monomer has two cAMP binding sites (Corbin et al., 1978; Døskeland, 1978), and positive cooperativity is observed between the two sites (Hoppe et al., 1978; Døskeland & Øgreid, 1984). The two cAMP binding sites differ significantly in their dissociation rates for [^{3}H]cAMP and in their relative affinities for analogues of cAMP (Rannels & Corbin, 1980; Øgreid & Døskeland, 1981). Only the slow dissociation site is measured by the conventional filter disk assay (Øgreid & Døskeland, 1980), and derivatives of cAMP containing C-8 substituents preferentially displace cAMP from this site (Rannels & Corbin, 1980; Corbin & Rannels, 1981). In contrast, the rapidly exchanging site generally interacts preferentially with N^6-substituted derivatives of cAMP (Corbin et al., 1982; Døskeland et al., 1983). The utilization of these site-directed analogues has demonstrated a clear synergistic relationship between the two sites (Corbin et al., 1982; Robinson-Steiner & Corbin, 1983).

The presence of two cAMP binding sites is consistent with the amino acid sequences of R^I and R^{II}, which both show two in-tandem regions of internal homology in the carboxy-terminal region (Takio et al., 1984; Titani et al., 1984). The most compelling argument for assigning these gene duplicated segments to the two cAMP binding sites is provided by a comparison of the R-subunit with the *Escherichia coli* catabolite gene activator protein (CAP), which is the major cAMP binding protein in prokaryotes. This molecule also has a distinct domain structure with the carboxy-terminal region corresponding to the DNA-binding domain. The amino-terminal cAMP-binding domain is approximately the same size as the gene-duplicated segments in R, and the homologies in amino acid sequence in each of these cAMP-binding domains are extensive (Weber et al., 1982, 1987).

Although the kinetic and equilibrium studies with analogues of cAMP clearly distinguish two classes of cAMP binding sites, they cannot be used to actually discriminate, for example, whether the fast dissociation site corresponds to the first or second cAMP-binding domain in the protein. Photolabeling with 8-N_3-cAMP is the only approach that so far has identified specific amino acid residues that are in close proximity to the cAMP binding sites. In the case of R^I, photolabeling with 8-N_3-cAMP led to the covalent modification of two residues (Bubis & Taylor, 1985). However, since these studies were carried out under saturating conditions, and since 8-N_3-cAMP binds to both sites and does not show a strong preference for either site (Døskeland & Øgreid, 1982), it was not possible to correlate these covalent modifications with occupancy of a specific site. On the basis of the homology of the R-subunits with the crystal structure of CAP, a model has been predicted that correlates photolabeling of specific residues with occupancy of each cAMP binding site (Weber et al., 1987). In order to confirm this model and to unambiguously correlate the covalent modifications with 8-N_3-cAMP bound to each domain, the type I holoenzyme was photolabeled in the presence of various analogues of cAMP that show a strong preference for one site. This approach has allowed us to correlate photoaffinity labeling with analogue specificity and to correlate the two functionally distinct sites with their actual localization in the polypeptide chain.

Experimental Procedures

Materials. Reagents were obtained from the following sources: [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; [2-^{3}H]ATP (25.5 Ci/mmol), Amersham; cAMP, 8-N_3-cAMP, ATP, 8-(methylamino)-cAMP, N^6-monobutyryl-cAMP, bovine serum albumin (BSA), histone IIA, and CM-Sepharose CL-6B, Sigma; Cytoscint and Betaphase, Westchem; L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, Cooper Biomedical or United States Biochemical Corp.; trifluoroacetic acid (TFA, sequanal grade), Pierce; acetonitrile (HPLC grade), Fisher Scientific; filters, type HA (0.45 μm), Millipore Corp.; glass fiber paper GF/C, Whatman.

Proteins. R^I-subunit was purified from porcine skeletal muscle (Zick & Taylor, 1982) and C-subunit from porcine heart (Nelson & Taylor, 1981). Holoenzyme was reconstituted by dialysis of R^I with a 5% excess of C-subunit against 25 mM potassium phosphate (pH 6.5), 5% glycerol, 5 mM β-mercaptoethanol, 5×10^{-4} M $MgCl_2$, and 10^{-4} M ATP. After adjusting the pH to 6.1, excess C-subunit was removed with 2 mL of CM-Sepharose CL-6B/mg of excess C-subunit. Excess ATP and $MgCl_2$ were removed by dialysis against 25 mM potassium phosphate (pH 6.5), 5% glycerol, 2 mM EDTA, and 5 mM β-mercaptoethanol. β-Mercaptoethanol was removed by dialysis just prior to 8-N_3-cAMP binding. All dialyses were carried out at 4 °C. Protein concentration was determined by the method of Bradford (1976). In calculating molarity, M_r values of 48 000 and 38 000 were used for R^I and C, respectively.

Measurements of Cyclic Nucleotide Binding. Cyclic nucleotide binding was measured by two filter disk assays. Method A was carried out in the presence of low salt (buffer I, 50 mM potassium phosphate, 2 mM EDTA, 5 mM β-mercaptoethanol, 1 mg/mL BSA, pH 6.8) whereas method B was carried out in the same buffer containing 2 M NaCl and 0.5 mg/mL histone IIA instead of BSA (buffer II). When 8-N_3-cAMP binding was measured, β-mercaptoethanol was omitted from all buffers. Equilibrium dialysis and ammonium sulfate precipitation followed by filtration also were used to measure cyclic nucleotide binding as described previously (Bubis & Taylor, 1985). The binding mixture contained 20 nM holoenzyme and various concentrations of [^{3}H]cAMP or 8-N_3-[^{3}H]cAMP up to 1.5 μM. Cyclic nucleotide binding was also carried out in buffer A [25 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 10 mM magnesium acetate, 1 mg/mL BSA, pH 6.5] in the absence or presence of 5 mM ATP.

Measurements of ATP Binding to Holoenzyme. Holoenzyme (30 nM) was incubated for 16 h at 4 °C with various concentrations of [^{3}H]ATP (0.0–1.0 μM) in buffer A or in buffer B (25 mM MES, 10 mM magnesium acetate, 2 M NaCl, 0.5 mg/mL histone IIA, pH 6.5). ATP binding was measured by equilibrium dialysis, Millipore filtration, and ammonium sulfate precipitation.

Competition for Cyclic Nucleotide Binding by cAMP Analogues. Competition experiments were carried out under equilibrium conditions in buffer I. Reaction mixtures (0.4 mL) contained 2.0 nM holoenzyme (8 nM cAMP binding sites), 30 nM [^{3}H]cAMP or 8-N_3-[^{3}H]cAMP and a 0–50-fold excess of analogues of cAMP over radioactive nucleotide. The reaction was initiated by the addition of holoenzyme. The

binding of [^{3}H]cAMP or 8-N_3-[^{3}H]cAMP was assayed after incubating overnight at 4 °C by using the two filter disk assays (A and B), equilibrium dialysis, and ammonium sulfate precipitation as described above.

Displacement of Bound Cyclic Nucleotide by cAMP Analogues. Holoenzyme (20 nM) was incubated for 16 h at 4 °C with 160 nM ^{3}H-labeled cyclic nucleotide in buffer I. Dissociation of bound cyclic nucleotide as a function of time was initiated by the addition of a 15-fold excess of nonradioactive cyclic nucleotide. After thorough mixing, aliquots were withdrawn at designated time intervals and assayed by ammonium sulfate precipitation. Similar measurements were carried out by using the filter disk assay B, following incubation in buffer II.

Photoaffinity Labeling in the Presence of cAMP Analogues. Photolabeling in the presence of analogues was carried out under three different conditions. In the first case, holoenzyme (3.7 nmol) was incubated with 37 nmol of 8-N_3-[^{3}H]cAMP for 16 h at 4 °C, which was sufficient to fully saturate both cyclic nucleotide binding sites. cAMP analogues (580 nmol) were added subsequently and incubated at 4 °C for 13 h. In the second case, holoenzyme (1.75 nmol) was incubated for 16 h at 4 °C with 18 nmol of 8-N_3-[^{32}P]cAMP and sufficient cAMP analogue to saturate approximately 50–60% of the cAMP binding sites with the radioactive photoaffinity analogue. In the final case, holoenzyme (1.9 nmol) was incubated overnight for 16 h, at 4 °C, with 0.07 nmol of 8-N_3-[^{32}P]-cAMP and 20 nmol of nonradioactive analogue of cAMP, in 25 mM potassium phosphate (pH 6.5) containing 2 mM EDTA. Samples were irradiated at 4 °C with a UVS-11 lamp (254 nm) for 10 min at a distance of approximately 5 cm with occasional mixing. The covalent incorporation of 8-N_3-cAMP after photolysis was followed by, (1) polyacrylamide gel electrophoreses, (2) TCA precipitation (10%) followed by filtration through GF/C glass fiber filters, (3) equilibrium dialysis or filter disk assays after chasing with 30 mM cold cAMP at room temperature for 16 h to remove noncovalently incorporated analogue, and (4) peptide mapping of the modified protein after treatment with TPCK-treated trypsin, using high-performance liquid chromatography (HPLC).

High-Performance Liquid Chromatography (HPLC). HPLC was carried out on an Altex 3200 system with a Vydac C_{18} column (0.46 × 25 cm). The buffers employed were (a) 0.1% TFA (pH 2.15) and (b) CH_3CN. Absorbance was monitored at 219 nm on a SF 769 Kratos spectrophotometer equipped with a flow-through cell. When necessary, peptides were rechromatographed on a second Vydac C_{18} column with a gradient of 10 mM sodium phosphate (pH 6.8) to CH_3CN prior to sequencing. In some cases, the peptides were resolved initially with the sodium phosphate to CH_3CN gradient and then rechromatographed with the TFA to CH_3CN gradient. This latter procedure gave better yields when the peptides were subsequently sequenced. Details for each run are given in the figure and table legends.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out on 12.5% slab gels (1.5 mm) in the presence of sodium dodecyl sulfate (SDS) according to the method of Laemmli (1970).

Protein Kinase Assay. Protein kinase was assayed spectrophotometrically (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 a 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 for 1 h.

Table I: Site Selectivity of cAMP Analogues Used[a]

analogue	K_i(cAMP)/K_i(analogue) site A	K_i(cAMP)/K_i(analogue) site B	specificity A/B	specificity B/A
cAMP	1.0	1.0	1.0	1.0
8-N_3-cAMP	0.55	2.2	0.25	4.0
8-(methylamino)-cAMP	0.21	3.5	0.06	17.0
N^6-monobutyryl-cAMP	3.6	0.093	38	0.026

[a] Døskeland et al. (1983).

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.

Results

In order to correlate photolabeling of R^1 with occupancy of specific cAMP binding sites, it was necessary to establish conditions that would distinguish the two sites. Conditions therefore were established that would maximize differential occupancy of both sites.

Binding of cAMP and 8-N_3-cAMP. Several methods were used to measure directly the binding of both [^{3}H]cAMP and 8-N_3-[^{3}H]cAMP. With one exception these methods gave equivalent results. The observed K_d (cyclic nucleotide) was 10–20 nM with each method using both nucleotides. Equilibrium dialysis, ammonium sulfate precipitation, and the filter disk assay B all showed 1.9–2.0 mol of cyclic nucleotide bound per mole of R^1-monomer and could thus be used interchangeably to measure total cyclic nucleotide binding. The exception for quantitating cyclic nucleotide binding was the filter disk assay A, which consistently showed only 1 mol of cyclic nucleotide binding per mole of R^1-monomer. This filter disk assay A thus has the potential to discriminate between the sites when used in conjunction with one of the other methods.

Selectivity of Cyclic Nucleotide Binding. In order to establish the selectivity of binding to each of the cAMP binding sites, a series of cAMP analogues were used. Two general classes of cAMP analogues were selected—those that contained a substituent at the C-8 position of the adenine ring and those that were substituted at the N^6 position. Within each category, analogues were specifically chosen because of their preference for selectively binding to one of the cAMP binding sites in the R-subunit. Some representative analogues used are summarized in Table I together with estimates of their site selectivity.

Equilibrium studies were used to establish conditions that would maximally distinguish selective occupancy of one cAMP binding site. Holoenzyme was incubated with a 3.75-fold molar excess of [^{3}H]cAMP over cAMP binding sites, which is sufficient to fully saturate both sites, and with increasing concentrations of unlabeled cAMP analogues. An indication of selectivity of binding was established by measuring cAMP binding with several methods. As indicated in Figure 1, a general gauge of selectivity could be established, in some cases, by comparing the filter disk assay A, which is capable of detecting only one class of binding sites, with equilibrium dialysis, ammonium sulfate precipitation, or the filter disk assay B. Figure 1A shows the inhibition of [^{3}H]cAMP binding by 8-N_3-cAMP measured by the filter disk assay A and by equilibrium dialysis. As shown by both methods, 8-N_3-cAMP behaved similar to cAMP itself in competing for the two cAMP binding sites. On the other hand, in the case of N^6-monobutyryl-cAMP, equilibrium dialysis indicated that 50%

Table II: Sequence Analysis of the Two Tryptic Peptides of R-Subunit Type I Modified by 8-N_3-cAMP

	367												379
peptide 1[b]	Asn	Ile	Gln	Gln	Tyr	Asn	Ser	Phe	Val	Ser	Leu	Ser	Val
gas-phase sequencing													
modified peptide	→	→	→	→		→	→	→	→	→	→	→	→
unmodified peptide	→	→	→	→	→	→	→	→	→	→	→	→	→
radioactivity[a] (solid-phase sequencing) (cpm/step)	120	20	10	10	380	80	30	15					
	251											262	
peptide 2[b]	Val	Ser	Ile	Leu	Glu	Ser	Leu	Asp	Lys	Trp	Glu	Arg	
gas-phase sequencing													
modified peptide	→	→	→	→	→	→	→	→	→		→	→	
unmodified peptide	→	→	→	→	→	→	→	→	→	→	→	→	
radioactivity[a] (solid-phase sequencing) (cpm/step)	350	40	15	30	45	35	28	15	730	250	60	30	
linkage to solid-phase support	*								*				

[a] Bubis & Taylor (1985). [b] Residues are numbered according to the published sequence of Titani et al. (1984).

of the bound cyclic nucleotide was displaced when the N^6-substituted analogue and [^{3}H]cAMP were present in equivalent amounts, whereas the binding measured by method A indicated that less than 5% of the bound nucleotide was displaced (Figure 1B). In the presence of a C-8 analogue, such as 8-(methylamino)-cAMP, [^{3}H]cAMP is displaced more rapidly than with cAMP, but the two assays do not distinguish as clearly whether the rapid dissociation is site selective (Figure 1C). These results established a distinct selective behavior for these analogues and suggested that the two sites might be best differentiated in the presence of N^6-substituted analogues of cAMP. Binding alone, however, cannot be correlated direclty with the occupancy of specific sites on the polypeptide chain.

Correlation of Cyclic Nucleotide Binding with Occupancy of Specific Sites. The analogue selectivity provides a mechanism for directly correlating photoaffinity labeling with occupancy of a specific cAMP binding site. In addition, coupling of photolabeling with selective occupancy of the cAMP binding sites enables one to establish directly which site in the linear sequence is preferred by which analogues. When both cAMP binding sites of R^I are saturated with 8-N_3-[^{3}H]cAMP, two amino acid residues are covalently modified following photolysis. The specificity of this photolabeling is seen in Figure 2, which represents an HPLC elution profile of tryptic peptides derived from R^I photolabeled in the presence of saturating amounts of 8-N_3-[^{3}H]cAMP. Each peak of radioactivity was rechromatographed on a second HPLC column as shown in the insets. The first region (fractions 68–70) corresponds to residues 367–379, and the photolabeled residue was identified as Tyr–371 (Bubis & Taylor, 1985). The second peak (fraction 76) corresponds to residues 251–262, and the site of modification is Trp-260. This second site of covalent modification was previously identified as Pro-271; however, the combination of the two different HPLC gradient systems used here, together with the utilization of a better resolving C_{18} column, indicated that the previous assignment was incorrect. In the earlier study the photolabeled peptide was, in fact, masked by the peptide containing Pro-271, and the HPLC system that was used at that time did not resolve these two peptides. In the experiment described here more protein was used, which enhanced the yields. In addition, the modified HPLC protocol used here clearly resolves the two peaks and shows unambiguously that the site of covalent modification corresponds to Trp-260. The assignments for covalent modification were confirmed by sequence analysis (Figure 3 and Table II). For both peptides, no identifiable PTH-amino acid was seen at the site of covalent modification as would be anticipated. These assignments also were consistent with the solid-phase sequencing described earlier (Bubis & Taylor, 1985). In the case of peptide 1, radioactivity eluted at step

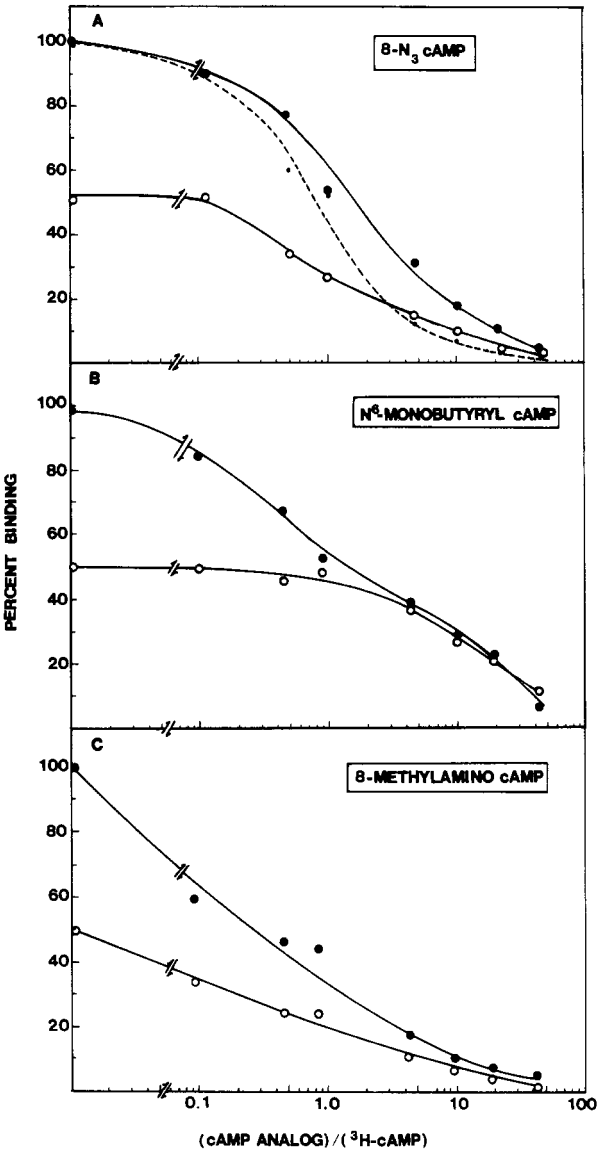


FIGURE 1: Inhibition of cAMP binding by cAMP analogues. Holoenzyme (2 nM) was incubated with 30 nM [^{3}H]cAMP and a 0–50-fold excess of derivatives of cAMP over radioactive cyclic nucleotide. The binding of [^{3}H]cAMP was assayed by using equilibrium dialysis (●) and the filter disk assay A (○). (A) [^{3}H]cAMP vs. 8-N_3-cAMP. The dashed line shows [^{3}H]cAMP vs. cAMP measured by equilibrium dialysis (•). (B) [^{3}H]cAMP vs. N^6-monobutyryl-cAMP. (C) [^{3}H]cAMP vs. 8-(methylamino)-cAMP.

5, which corresponds to Tyr-371. In the case of peptide 2, the radioactivity eluted at step 9. This is because the peptide was linked to the solid support by the ϵ-NH_2 group of the Lys (Table II), so that all of the remaining peptide eluted once

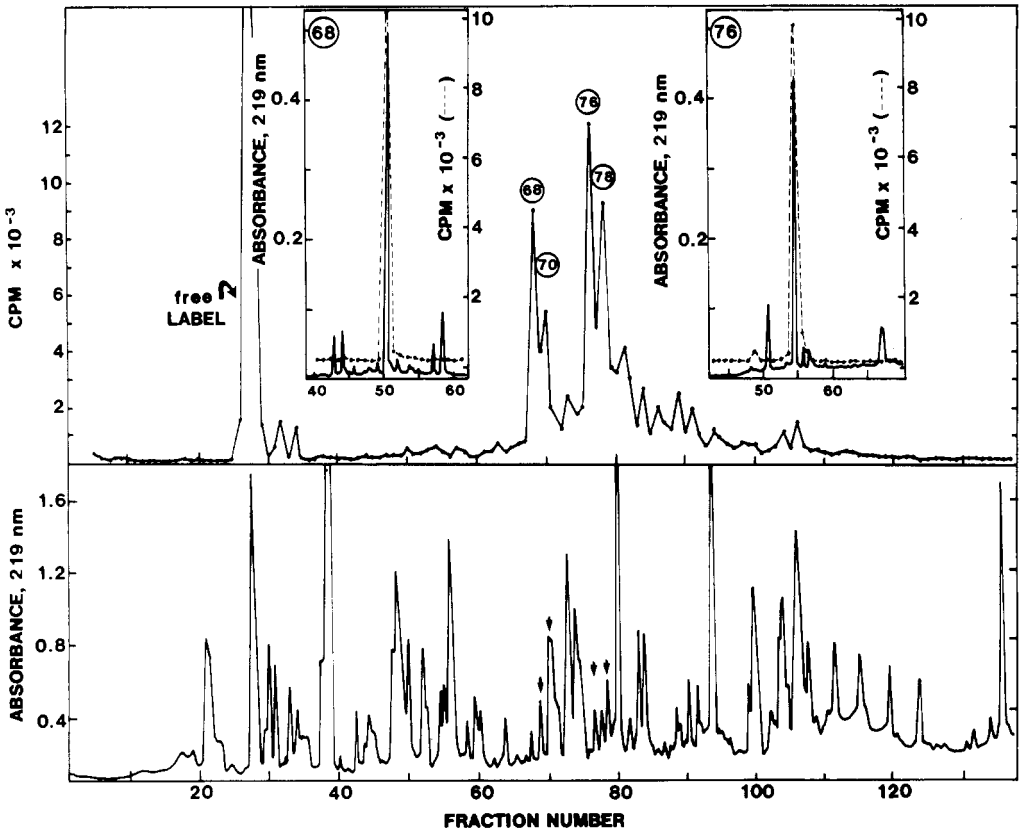


FIGURE 2: High-performance liquid chromatography separation of 8-N_3-cAMP-modified R^I. Regulatory subunit (30 nmol) was incubated with 300 nmol of 8-N_3-[^{3}H]cAMP for 30 min at 30 °C followed by an incubation for 4 h at 4 °C. The sample was then irradiated on ice for 10 min as described under Experimental Procedures. After an incubation with trypsin at 37 °C overnight, R^I peptides were separated by HPLC with a Vydac C_{18} column and a gradient of 10 mM sodium phosphate (pH 6.8) to CH_3CN. The tryptic peptides were eluted with a 120-min linear gradient from 0% to 30% CH_3CN followed by a 60-min linear gradient from 30% to 60% CH_3CN. (Top) Radioactivity (cpm of ^{3}H); (bottom) absorbance at 219 nm. Also shown are the rechromatographs (insets, top panel) of the radioactive peaks on a second Vydac C_{18} column with a gradient of 0.1% TFA (pH 2.15) to CH_3CN. The peptides were eluted with a 80-min linear gradient from 0% to 40% CH_3CN.

the Lys–Trp bond was cleaved. Of the three remaining residues, Trp, Glu, and Arg, only the Trp was absent in the sequence. In some cases, incomplete proteolysis accounts for the appearance of a doublet; in other cases, several peaks such as fractions 68, 70, and 78 gave the same sequence. The sodium phosphate to CH_3CN gradient, in particular, will resolve the cyclic phosphate derivative from the hydrolyzed monophosphate. The assignments of all of the peaks in Figure 2 were confirmed by rechromatographing each radiolabeled peak as described under Experimental Procedures and sequencing the labeled peptides. In each case, the radiolabeled residue gave no recognizable PTH-amino acid in contrast to corresponding unlabeled peptides where the Tyr and Trp residues were clearly identified.

Total binding of 8-N_3-[^{3}H]cAMP also was correlated with total covalent incorporation, which was measured by TCA precipitation of the photolabeled protein. In Figure 2, where both sites were fully saturated (2 mol of 8-N_3-cAMP bound/mol of R^I), covalent incorporation was 1.2 mol of 8-N_3-[^{3}H]cAMP/mol of R^I-monomer, which corresponds to 60%. Quantitation of covalent incorporation into each site was established by determining the radioactivity incorporated into each peak following elution from HPLC. In Figure 2, the incorporation of radioactivity into Tyr-371 and Trp-260 was approximately 64% (0.64 mol/mol of R^I) and 40% (0.40 mol/mol of R^I), respectively. Recovery following HPLC was close to 90%. This approximate level of covalent incorporation was seen consistently although the relative incorporation into each site fluctuated slightly in any given experiment.

In order to correlate this photolabeling with occupancy of a specific site in the polypeptide chain, selective occupancy

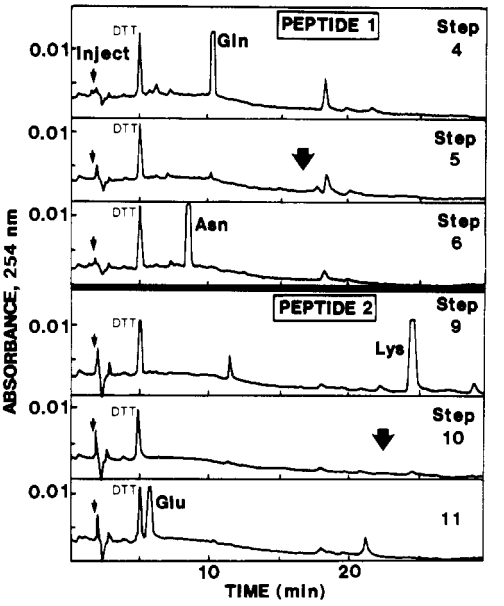


FIGURE 3: Identification of PTH-amino acids flanking the covalent modification site of peptides 1 and 2. The two peptides indicated in Table II were sequenced by the gas-phase method, and PTH-amino acids were identified following HPLC chromatography as described under Experimental Procedures. The elution positions of Tyr and Trp are indicated by arrows. Tyr is clearly identified as step 5 of the unmodified peptide 1 and Trp as step 10 of the unmodified peptide 2. The elution position of dithiothreitol (DTT) is also indicated.

of the two cyclic nucleotide binding sites was established by measuring the dissociation of 8-N_3[^{3}H]cAMP in the presence of cyclic nucleotide analogues that are capable of discrimi-

Table III: 8-N_3-cAMP Modification of Holoenzyme after Dissociation with Various Analogues of cAMP[a]

cyclic nucleotide added	calcd total binding[b] (mol/mol of R-monomer)	calcd covalent incorpn[c] (mol/mol of R-monomer)	incorpn[d] (mol/mol of R-monomer)	
			Tyr-371	Trp-360
none (control)	2.00	1.20 (1.20)	0.60	0.50
cAMP	0.28	nd (0.07)	0.00	0.06
N^6-monobutyryl-cAMP	0.94	nd (0.37)	0.31	0.03
8-(methylamino)-cAMP	0.32	nd (0.13)	0.09	0.03

[a] Holoenzyme (3.7 nmol) was incubated with 37 nmol of 8-N_3-[^{3}H]cAMP for 16 h at 4 °C. Analogues (580 nmol) were added and incubated at 4 °C for 13 h. Samples were irradiated on ice for 10 min. Free C-subunit was removed with CM-Sepharose, and R^I peptides were separated by HPLC after treatment with TPCK–trypsin. The buffers employed were (a) 0.1% TFA (pH 2.15) and (b) CH_3CN. The tryptic peptides were eluted with 120-min linear gradient from 0% to 30% CH_3CN followed by a 60-min linear gradient from 30% to 60% CH_3CN at a flow rate of 1 mL/min. [b] Total 8-N_3-cAMP binding was assayed by ammonium sulfate precipitation. [c] Covalent incorporation was assayed by TCA precipitation and by equilibrium dialysis after chasing with 30 mM nonradioactive cAMP for 16 h at room temperature. In parentheses is also shown the corrected total covalent incorporation, assuming 90% recovery of peptide from each HPLC run. nd, not determined. [d] Incorporation into Tyr-371 and Trp-260 was measured by calculating the radioactivity recovered from each peak from the respective HPLC runs.

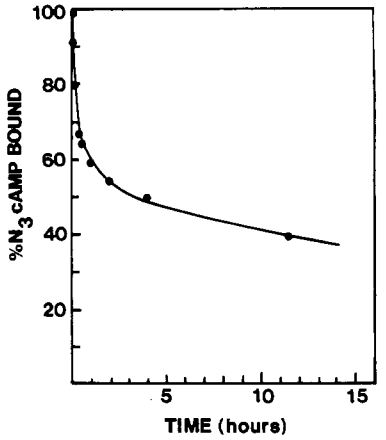


FIGURE 4: Displacement of 8-N_3-cAMP by N^6-monobutyryl-cAMP. Holoenzyme (20 nM) was incubated with 160 nM 8-N_3-[^{3}H]cAMP for 16 h at 4 °C. Dissociation of bound 8-N_3-cAMP was initiated by the addition of a 15-fold excess of nonradioactive N^6-monobutyryl-cAMP. Aliquots were withdrawn at the designated time intervals and assayed by ammonium sulfate precipitation.

nating between the two sites. Under these conditions, both cAMP binding sites were first saturated with radiolabeled nucleotide. Dissociation in the presence of unlabeled cAMP analogues was then measured as a function of time. Binding was measured by ammonium sulfate precipitation or filter disk assay B, which both measure total cAMP binding. Figure 4 shows an example of the displacement of 8-N_3-[^{3}H]cAMP by N^6-monobutyryl-cAMP. On the basis of these dissociation curves, photolabeling was repeated when approximately 40–60% of the 8-N_3-[^{3}H]cAMP had been displaced with N^6-monobutyryl-cAMP. As seen in Table III when photolabeling was carried out after 53% of the 8-N_3-[^{3}H]cAMP had been displaced with N^6-monobutyryl-cAMP, the incorporation of radioactivity into Trp-260 was reduced by greater than 90% while the incorporation of radioactivity into Tyr-371 was reduced by less than 50%. When 84% of 8-N_3-[^{3}H]cAMP was displaced by 8-(methylamino)-cAMP, the incorporation of radioactivity was reduced equivalently into both labeled peptides (Table III). In this particular experiment the incorporation of radioactivity into Tyr-371 and Trp-260 was approximately 60% and 50%, respectively, when both binding sites where saturated with 8-N_3-[^{3}H]cAMP and no analogue was added.

A second approach also was used to correlate photolabeling with occupancy of a specific cAMP binding site. Under these conditions, the binding studies described earlier for [^{3}H]cAMP were repeated with 8-N_3-[^{3}H]cAMP. The results were essentially identical with those shown in Figure 1 (data not shown). On the basis of these curves, sufficient analogue was used in conjunction with 8-N_3-[^{32}P]cAMP to saturate approximately 50–60% of the sites with the radioactive photoaffinity analogue under equilibrium conditions. The sample was then irradiated as described under Experimental Procedures, dialyzed, and digested with TPCK–trypsin. In this experiment, the incorporation into Trp-260 was slightly greater than into Tyr-371 when all sites were saturated with 8-N_3-[^{32}P]cAMP (Figure 5A) and 60% of the total 8-N_3-cAMP that was bound was photoincorporated. When photolabeling was carried out in the presence of cAMP, incorporation into both residues was reduced equivalently (Figure 5B). Photolabeling in the presence of N^6-monobutyryl-cAMP nearly abolished the covalent modification of Trp-260 (Figure 5C). In contrast, when 8-N_3-[^{32}P]cAMP was bound in the presence of 8-(methylamino)-cAMP, the incorporation of radioactivity into Trp-260 was actually increased, whereas the modification of Tyr-371 was reduced by approximately 40% (Figure 5D). Covalent modification of Tyr-371 also was more efficient when the other site was occupied by the nonphotoreactive N^6-substituted analogue (Figure 5C).

Correlation of Synergism with Occupancy of Each Site. It is well-established that the two cAMP binding sites interact synergistically (Robinson-Steiner & Corbin, 1983), and this synergism coupled with analogue selectivity can be used to distinguish type I and II holoenzymes (Øgreid et al., 1985). Photoaffinity labeling under conditions that synergistically direct a cAMP analogue to one cAMP binding site provided a mechanism for directly correlating synergism with occupancy of specific sites in the polypeptide chain. The general synergistic relationship can be seen in Figure 6. As indicated, when photolabeling was carried out in the presence of N^6-monobutyryl-cAMP, the total covalent incorporation of radioactivity into the protein was increased. To determine whether this enhanced incorporation could be accounted for by the preferential directing of 8-N_3-[^{32}P]cAMP to one site, the photolabeled proteins shown in figure 6 were digested with trypsin and the resulting peptides resolved by HPLC. As seen in Table IV, when enough N^6-monobutyryl-cAMP was added to fully saturate both cAMP binding sites, radioactivity into Tyr-371 was markedly enhanced whereas photolabeling of Trp-260 was nearly abolished. Total incorporation of radioactivity into both sites was reduced in the presence of either cAMP or 8-(methylamino)-cAMP.

Effect of MgATP on cAMP Binding and on Photolabeling by 8-N_3-cAMP. The type I cAMP-dependent protein kinase has a high-affinity binding site for MgATP, and the affinity of the holoenzyme for cAMP is reduced in the presence of MgATP (Hofmann et al., 1975; Hoppe et al., 1978). In the case of the porcine enzyme, the K_d (cAMP) in the absence of MgATP (20 nM) was increased 20–50-fold in the presence

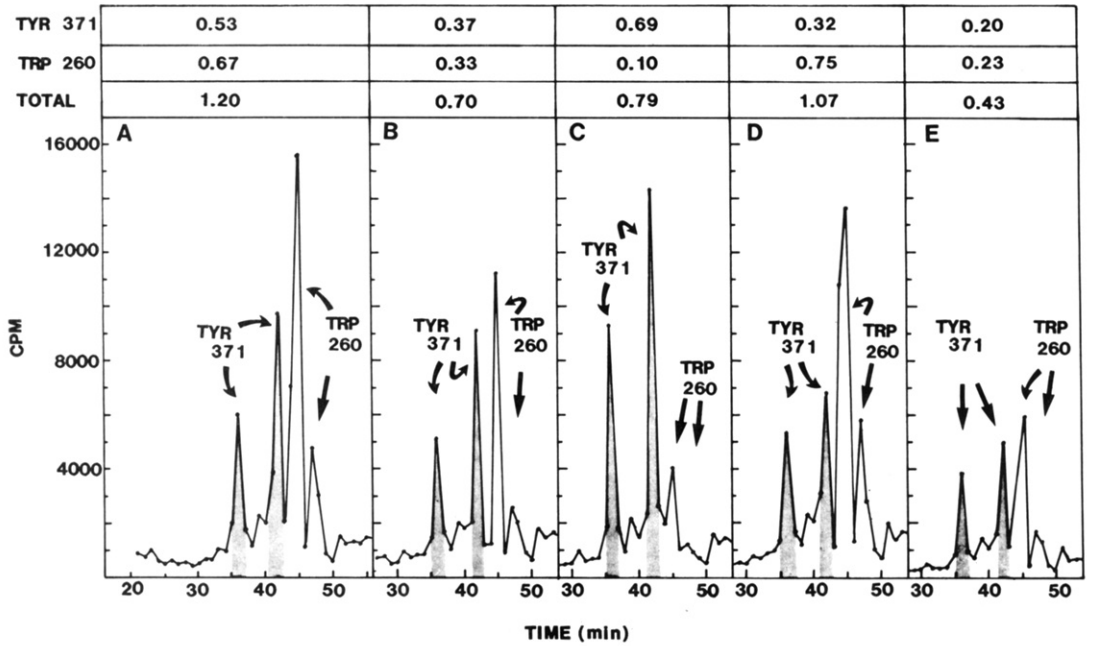


FIGURE 5: Effect of different cyclic nucleotides or MgATP on 8-N_3-cAMP labeling of holoenzyme. Holoenzyme (1.75 nmol) was incubated with 18 nmol of 8-N_3-[^{32}P]cAMP in the absence of nucleotides (A) or in the presence of 27 nmol of cAMP (B), 27 nmol of N^6-monobutyryl cAMP (C), 6 nmol of 8-(methylamino)-cAMP (D), or 17 mM magnesium acetate and 5 mM ATP (E). The concentrations of cyclic nucleotide analogues used were enough to saturate approximately 50–60% of the cAMP binding sites with 8-N_3-[^{32}P]cAMP. The concentration of MgATP used was sufficient to block 60% of the cyclic nucleotide binding. Samples were irradiated on ice for 10 min, and holoenzyme peptides were separated by HPLC with a Vydac C_{18} column and a gradient of 0.1% TFA (pH 2.15) to CH_3CN, after an overnight treatment with TPCK–trypsin. The peptides were eluted with a 10-min linear gradient from 0% to 15% CH_3CN, a 60-min linear gradient from 15% to 30% CH_3CN, and a 30-min linear gradient from 30% to 100%. Shown are the radioactivity profiles (cpm of ^{32}P). The shaded area corresponds to radioactivity associated with Tyr-371. The stoichiometries of incorporation on a mole per mole basis are summarized above. Total incorporation is based on moles of 8-N_3-cAMP per mole of R^I-monomer.

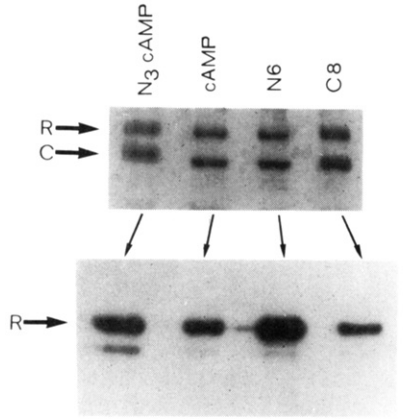


FIGURE 6: SDS–polyacrylamide gel electrophoresis of holoenzyme modified with trace amounts of 8-N_3-[^{32}P]cAMP in the presence of different analogues of cAMP. Holoenzyme (10 μg) was modified as described in Table IV. (Top) Protein staining with Coomassie blue; (bottom) autoradiogram. N6 = N^6-monobutyryl-cAMP, C8 = 8-(methylamino)-cAMP.

Table IV: Binding and Photolabeling of Holoenzyme by Trace Amounts of 8-N_3-cAMP in the Presence of Different cAMP Derivatives[a]

cyclic nucleotide added	calcd total binding (cpm)	radioactivity incorpd (cpm)			calcd covalent incorpn (cpm)
		Tyr-371	Trp-260	total	
none (control)	62 700	18 300	13 000	32 100	36 900
cAMP	54 900	12 000	3 500	15 500	18 700
N^6-monobutyryl-cAMP	143 900	64 000	300	64 300	67 000
8-(methylamino)-cAMP	57 000	9 800	4 300	14 100	15 400

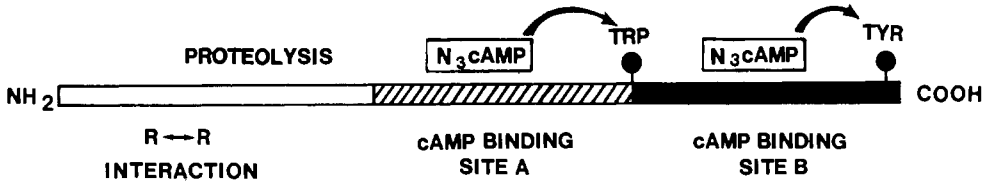
[a] Holoenzyme (1.9 nmol) was incubated with 20 nmol of various cAMP derivatives and 0.07 nmol of 8-N_3-[^{32}P]cAMP as described under Experimental Procedures. Samples were photolyzed on ice for 10 min, and peptides were separated by HPLC after proteolysis with trypsin. Tryptic peptides were separated by using a gradient of 0.1% TFA (pH 2.15) to CH_3CN with a 10-min linear gradient from 0% to 15% CH_3CN, a 60-min linear gradient from 15% to 30% CH_3CN, and a 30-min linear gradient from 30% to 100% CH_3CN. Total 8-N_3-cAMP binding and covalent incorporation were assayed by ammonium sulfate and TCA precipitations, respectively. The radioactivity incorporated into Trp-260 and Tyr-371 were measured by calculating the radioactivity recovered for each peak from the respective HPLC runs.

of MgATP. The apparent K_d in the presence of MgATP also varied depending on the assay used. A K_d of 400 nM was observed when the ammonium sulfate method was used, whereas a value of 1000 nM was measured when the Millipore filtration method A was used. These values were obtained with 20 nM holoenzyme in buffer A and 5 mM MgATP. The high-affinity binding of MgATP also could be demonstrated by equilibrium dialysis or by the filter disk assay in buffer A, which both gave an apparent K_d for ATP binding of 15–20 nM. However, in buffer B no high-affinity binding of MgATP was observed, indicating most likely that the holoenzyme was dissociating under these conditions.

In order to determine if MgATP was selectively effecting occupancy of one of the cAMP binding sites, 8-N_3-[^{32}P]cAMP was bound in the presence of a concentration of 17 mM magnesium acetate and 5 mM ATP, which was sufficient to dissociate 60% of the cyclic nucleotide. Photolabeling under these conditions showed reduced labeling of both Tyr-371 and Trp-260; however, there was no indication of a preferential effect on either site (Figure 5E).

Discussion

Although early reports indicated that one molecule of cAMP was bound per each monomeric regulatory subunit, subsequent evidence has clearly established that each protomer of the regulatory subunit of cAMP-dependent protein kinase has two



Designation in Literature	A, I, 2	B, II, 1
Dissociation of cAMP	fast	slow
Analog Preference	N^6-substituted	C_8-substituted
Standard Millipore Assay	no	yes
Order of Binding	second	first

FIGURE 7: Model correlating photolabeling with occupancy of specific cAMP binding sites. Photolabeling of Trp-260 has been correlated with occupancy of site A and photolabeling of Tyr-371 with occupancy of site B. Localization of both amino acid residues and the two cAMP binding sites in the linear sequence of R^I are indicated. Also shown is the amino-terminal domain which contains the "hinge" region that is susceptible to proteolysis and the interaction site between R-subunits in the dimer. The table summarizes some of the properties associated with the two different in-tandem cAMP binding sites in the holoenzyme.

high-affinity binding sites for cAMP (Corbin et al., 1978). Part of the original confusion arose from the assay techniques that were utilized to measure bound cAMP. The intitial reports relied on a conventional Millipore filtration technique that only detects one class of sites, whereas when this assay was modified to include high salt and histone, both classes of sites were measured. The reason for this discrepancy is still not apparent; however, using equilibrium dialysis and including a piece of Millipore filter in the dialysis chamber containing the R-subunit showed that all of the R-subunit was absorbed to the filter and, furthermore, that binding to the Millipore filter in low salt led to the selective displacement of 1 mol of cAMP per R-monomer (Builder et al., 1980). Ammonium sulfate precipitation (Døskeland, 1978) and equilibrium dialysis also measure both cAMP binding sites. Equilibrium dialysis is probably the most reliable and consistent method for determining direct binding.

cAMP binds in a manner that shows positive cooperativity, and Hill coefficients in the range of 1.4–1.6 have been calculated (Hoppe et al., 1978; Bubis & Taylor, 1985). Several laboratories have demonstrated that cAMP dissociates from the two sites at different rates (Døskeland, 1978; Rannels & Corbin, 1980). These two sites can thus be distinguished on the basis of relative fast and slow dissociation rates. It has been further shown that various analogues of cAMP showed a preference for dissociating bound cAMP from the two sites (Rannels & Corbin, 1980; Corbin & Rannels, 1981). As a general rule, N^6-substituted analogues of cAMP will preferentially dissociate bound cAMP from the fast dissociation site whereas C-8-substituted analogues show preference for the slow dissociation site.

This study has tried to definitively correlate the two functionally distinct cAMP binding sites with their position in the polypeptide chain. The sites that are photolabeled with 8-N_3-cAMP also have been correlated with each cAMP binding site in the linear sequence. Although it was established earlier that a single tyrosine is covalently modified in R^{II} by 8-N_3-cAMP (Kerlavage & Taylor, 1980), it has not been rigorously shown that this site is the one that prefers C-8-substituted analogues of cAMP since the labeling was done under saturating conditions and since 8-N_3-cAMP interacts with both types of cyclic nucleotide binding sites (Øgreid & Døskeland, 1982) and does not show a strong selective preference for one site. The possibility thus remained that this site was simply readily photolabeled regardless of its analogue preference. Stoichiometric labeling with photoaffinity analogues is rare and suggests that the tyrosine residue is not only ideally positioned for photolabeling but also must be well shielded from the solvent. The fact that R^I is covalently modified at two sites (Bubis & Taylor, 1985) provided a good model system for correlating photolabeling with the two sites in the linear sequence, and the known selectivity of the two sites enabled us to functionally distinguish them.

Comparison of the R^I sequence with the crystal structure of CAP suggested that Trp-260 and Tyr-371 are most likely well separated in the tertiary structure, thus making it unlikely that 8-N_3-cAMP bound to one site was responsible for both covalent modifications (Bubis & Taylor, 1985; Weber et al., 1987). This led to the hypothesis that labeling of Tyr-371 is due to 8-N_3-cAMP bound to domain B and that Trp-260 is modified by 8-N_3-cAMP bound to domain A. This hypothesis has been confirmed here chemically by carrying out photolabeling of R^I in the presence of a variety of analogues that show a strong preference for one of the cAMP binding sites. As summarized in Figure 7, photolabeling has established that N^6-substituted analogues preferentially displace cAMP from the first cAMP binding site whereas C-8-substituted analogues select for the second cAMP binding site. These photolabeling results can be correlated directly with the previous binding studies as well. For example, 8-N_3-[^{3}H]cAMP was selectively displaced from the fast dissociation site by N^6-monobutyryl-cAMP, based on the binding assays. Photolabeling under these conditions established that the covalent modification of Trp-260 was lost preferentially as a consequence of selectively displacing the 8-N_3-[^{3}H]cAMP from this fast dissociation site. Modification of Trp-260 can therefore be correlated directly

with occupancy of the fast dissociation site. Conversely, occupancy of the slow dissociation site correlates with the covalent modification of Tyr-371. N^6-Substituted analogues of cAMP also synergistically enhance binding to the second cAMP binding site. Finally, these results can be correlated with the kinetic studies of Øgreid and Døskeland (1981) which established that binding of cAMP to the slow dissociation site (site B) precedes and allosterically enhances cAMP binding to the fast dissociation site (site A) in the holoenzyme.

The evidence presented here also identifies at least one contact point between the two cAMP-binding domains in the tertiary structure. Tyr-371 is located at the end of the second cAMP binding site (domain B), whereas Trp-260 terminates domain A and lies right at the start of domain B. This study confirms the hypothesis that Trp-260 is modified by cyclic nucleotide that is actually bound to domain A. This Trp must thus lie in close proximity to the C-8 position of the adenine ring of the cyclic nucleotide bound to domain A. This finding is consistent with the model of the cAMP-binding domains based on the crystal structure of CAP (Weber et al., 1987).

Throughout the course of characterizing these cAMP binding sites several nomenclatures have been utilized. These are summarized in Figure 7 together with some of the properties that distinguish the two sites. We have chosen to designate the two sites as A and B for several reasons. First, this represents the clear orientation of the sites in the polypeptide chain extending from the amino- to the carboxy-terminal end of the protein. Second, this nomenclature seems to represent the best clarification of sites when reference is made to the various isoenzyme forms that have consistently been classified as I and II. The numerical designations of 1 and 2 based on location in the polypeptide chain from the amino to the carboxy terminus are opposite to the numerical designations that have previously been used in the literature (Corbin et al., 1978). Thus, the A and B nomenclature seems most appropriate.

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