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Covalent Modification of Both cAMP Binding Sites in cAMP-Dependent Protein Kinase I by 8-Azidoadenosine 3′,5′-Monophosphate†

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ABSTRACT: Reconstituted porcine cAMP-dependent protein kinase type I was labeled with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) to study cyclic nucleotide binding and to identify amino acid residues that are either in or in close proximity to the cAMP binding sites. The photoaffinity analogue 8-N_3cAMP behaved as cAMP itself with respect to cyclic nucleotide binding. For both cAMP and 8-N_3cAMP, (1) 2 mol of nucleotide was bound per mole of type I regulatory subunit monomer (R^I), (2) the apparent K_d's observed were approximately 10–17 nM on the basis of either Millipore filtration assays, equilibrium dialysis, or ammonium sulfate precipitation, (3) Scatchard plots showed positive cooperativity, and (4) the Hill coefficients were approximately 1.5–1.6. After photolysis and addition of an excess of cAMP, approximately 1 mol of 8-N_3cAMP/mol of R^I monomer was covalently incorporated. Tryptic digestion of the labeled protein revealed that two unique tryptic peptides were modified. Proline-271 and tyrosine-371 were identified as the two residues that were covalently modified by 8-N_3cAMP in R^I. These results contrast with the type II regulatory subunit (R^{II}) where 8-N_3cAMP modified covalently a single tyrosine residue [Kerlavage, A. R., & Taylor, S. S. (1980) *J. Biol. Chem. 255*, 8483–8488]. R^I contains two adjacent regions of sequence homology in the COOH-terminal fragment that binds two molecules of cAMP. On the basis of the homology between the cAMP binding domain of the *Escherichia coli* catabolite gene activator protein (CAP) and the two cAMP-binding domains of R^I, a model has been proposed that is consistent with the covalent modifications described above. The model suggests that the modification of proline-271 is due to 8-N_3cAMP bound to cAMP binding domain A and modification of tyrosine-371 results from 8-N_3cAMP bound to cAMP binding domain B.

The major receptor protein for cAMP, and as such the primary mediator of cAMP action in eukaryotic cells, is cAMP-dependent protein kinase (EC 2.7.1.37). The native holoenzyme exists as an inactive tetramer consisting of two regulatory (R)[1] and two catalytic (C) subunits. On binding cAMP, the holoenzyme is dissociated into two active catalytic subunits carrying the ATP:protein phosphotransferase activity and a dimeric regulatory subunit that binds cAMP, according to the following scheme:

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

Several lines of evidence support the idea that dissociation involves the formation of a ternary complex involving R, C, and cAMP (Builder et al., 1980; Chau et al., 1980; Armstrong & Kaiser, 1978). Fluorescence data with etheno-cAMP further suggested that dissociation and thus activation do not occur until all four cAMP binding sites were occupied (Smith et al., 1981).

There are two major classes of cAMP-dependent protein kinase that are designated as type I and type II on the basis of elution from DEAE-cellulose (Corbin et al., 1975; Corbin & Keely, 1977). The two isozymes have virtually identical catalytic subunits but dissimilar regulatory subunits (Hofmann et al., 1975; Zoller et al., 1979). In both types of regulatory subunits, however, limited proteolysis has demonstrated a similar type of domain structure (Corbin et al., 1978; Potter & Taylor, 1979; Takio et al., 1982). The NH_2-terminal domain accounts for approximately 25% of the polypeptide chain and appears to be involved primarily in maintaining the dimeric structure of the native protein. The COOH-terminal domain on the other hand retains the cAMP binding sites although it is monomeric. Initial studies indicated that the R subunit bound only one molecule of cAMP per monomer; however,

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[1] Abbreviations: 8-N_3cAMP, 8-azidoadenosine 3′,5′-monophosphate; R subunit, regulatory subunit of the cAMP-dependent protein kinase; C subunit, catalytic subunit of the cAMP-dependent protein kinase; CAP, *Escherichia coli* catabolite gene activator protein; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; K_d, apparent equilibrium dissociation constant; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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later studies confirmed the initial results of Corbin et al. (1978), Weber et al. (1979), and Døskeland (1978) that indicated that each protomer had two cAMP binding sites. Subsequent work has established that the ability of cAMP analogues to dissociate cAMP from these two sites differs with N-6-substituted analogues showing a preference for one site and C-8-substituted analogues for the other site (Corbin et al., 1982; Døskeland et al., 1983).

The presence of two unique cAMP binding sites is consistent with the amino acid sequence of bovine R^{II}, which clearly shows an in tandem gene duplication in the COOH-terminal end of the molecule (Takio et al., 1982). Correlation of these gene-duplicated regions with the cAMP binding sites has been most firmly established by comparison with another homologous cAMP binding protein, the *Escherichia coli* catabolite gene activator protein (CAP) (Weber et al., 1982). The crystal structure of CAP reveals that it has a DNA binding domain and a cAMP binding domain (McKay et al., 1982). By comparing the amino acid sequences of CAP and R^{II}, Weber et al. (1982) established that the cAMP binding domains in CAP and R^{II} are homologous and that the in tandem gene duplicated region of R^{II} corresponds to two contiguous cAMP binding domains. Titani et al. (1984) have recently reported the amino acid sequence of bovine R^{I} and have shown that it also contains two homologous cAMP binding regions.

Although affinity labeling has been used to identify several specific amino acid residues in the C subunit that are associated with either the ATP (Zoller & Taylor, 1979) or the peptide binding sites (Bramson et al., 1982), only one affinity analogue has been used with the R subunit. 8-N_3cAMP is a highly specific photoaffinity probe for the R subunit of cAMP-dependent protein kinase and can be used to label the R subunit selectively in crude extracts (Haley, 1975; Pomerantz et al., 1975). 8-N_3cAMP has been shown to covalently incorporate into a single tyrosine residue in the type II R subunit, and this residue is located at the COOH-terminal end of the molecule (Kerlavage & Taylor, 1980; Kerlavage & Taylor, 1982).

In this study the type I regulatory subunit from porcine skeletal muscle was modified with 8-N_3cAMP. The binding of 8-N_3cAMP was characterized by a variety of methods and was compared with cAMP binding. Two sites of covalent modification were identified. These sites have been correlated with the two cAMP binding sites and with CAP.

Experimental Procedures

Materials. Reagents were obtained from the following sources: [2,8-^{3}H]cAMP (27 Ci/mmol), ICN; 8-N_3[2-^{3}H]-cAMP (16 Ci/mmol), New England Nuclear; CM-Sephadex, Pharmacia Fine Chemicals; Cytoscint, West Chem; L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, Worthington; polyamide sheets and trifluoroacetic acid (sequanal grade), Pierce; acetonitrile (HPLC grade), Fisher Scientific; cAMP, 8-N_3cAMP, ATP, bovine serum albumin, histone IIA, and Kemptide, Sigma. 8-N_3[^{32}P]cAMP (20.1 Ci/mmol) was kindly provided by Dr. Boyd Haley, Division of Biochemistry, University of Wyoming.

Proteins. R^{I} subunit was purified from porcine skeletal muscle as described previously (Zick & Taylor, 1982). C subunit was prepared from porcine heart according to Nelson & Taylor (1981). Holoenzyme was reconstituted by dialysis of R^{I} with a slight excess (5–10%) of C subunit for 5 days against 40 mM potassium phosphate (pH 6.1), 10% glycerol, 5 mM 2-mercaptoethanol, 10^{-4} M ATP, and 5×10^{-4} M $MgCl_2$. Excess C subunit was removed by addition of CM-Sephadex (2 mL of resin/mg of excess C subunit). Excess ATP and $MgCl_2$ were removed by dialysis against 40 mM potassium phosphate buffer (pH 6.8) containing 2 mM EDTA, 10% glycerol, and 5 mM 2-mercaptoethanol. 2-Mercaptoethanol was removed prior to 8-N_3cAMP binding by dialysis against the same buffer lacking 2-mercaptoethanol. 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.

Measurement of Cyclic Nucleotide Binding. Cyclic nucleotide binding was measured by Millipore filtration, equilibrium dialysis, and ammonium sulfate precipitation. In all cases, the initial binding was carried out in buffer I (50 mM potassium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, 1 mg/mL BSA, pH 6.8). When 8-N_3cAMP binding was measured, 2-mercaptoethanol was omitted from all buffers. The binding reaction mixture contained 20 nM holoenzyme and various concentrations of [^{3}H]cAMP (0.0–2.0 μM) or 8-N_3[^{3}H]cAMP (0.0–0.4 μM). After an overnight incubation (16 h) at 4 °C, two different Millipore filtration methods were used (Kerlavage & Taylor, 1982). In method A, which is a modification of the Gilman assay (Gilman, 1970), a 20-μL aliquot of the reaction mixture was transferred to a filter reservoir (0.45-μm Millipore HA filter) containing 5 mL of ice-cold buffer A (25 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4). The filters were washed under vacuum with 25 mL of additional buffer A, placed in scintillation vials, dried with a lamp, and counted in 6 mL of Cytoscint. In method B, which is based on the assay of Sugden & Corbin (1976), 150 μL of buffer II (50 mM potassium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, 2 M NaCl, and 0.5 mg/mL histone type IIA, pH 6.8) was added to 50 μL of the initial binding reaction mixture. The mixture was incubated for 1–2 h on ice, and an 80-μL aliquot was transferred to a filter reservoir containing 5 mL of ice-cold buffer B (10 mM potassium phosphate, 1 mM EDTA, pH 6.8). The filters were washed with wash buffer B and counted as in method A. An alternative method for measuring cyclic nucleotide binding was based on the procedure described by Døskeland & Ueland (1975). In this case, 450 μL of buffer C [3.8 M $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 6.8] was added to 50 μL of the initial binding reaction mixture indicated above. The mixture was incubated for 1–2 h on ice, and a 200-μL aliquot was transferred to a filter reservoir containing 5 mL of ice-cold buffer C. The ammonium sulfate precipitate was collected, washed with buffer C, and counted as in method A.

Cyclic nucleotide binding was also measured by equilibrium dialysis. Equilibrium dialyses were carried out in a 250-μL eight-cell chamber apparatus obtained from Hoefer Scientific. The binding reaction mixture (200 μL) containing protein and cyclic nucleotide was placed in one side of the dialysis membrane, and 200 μL of buffer I was placed on the opposite side. After an overnight incubation (16 h) at 4 °C, a 20-μL aliquot was transferred into scintillation vials and counted in 6 mL of Cytoscint.

All the cyclic nucleotide binding determinations were done in duplicate. The counts from Millipore filtration assays A and B were corrected for 25% quenching due to the filter membrane. The counts from the ammonium sulfate assay were corrected for 50% quenching due to the filter membrane and the amount of ammonium sulfate present on it.

Photoaffinity Labeling and Covalent Incorporation of 8-N_3cAMP. Holoenzyme type I was incubated with 8-N_3-[^{3}H]cAMP under the conditions described for Millipore filtration or equilibrium dialysis. The samples were irradiated

at 4 °C with a UVS-11 lamp (254 nm) for 10 min at a distance of approximately 8 cm with occasional mixing.

The covalent incorporation of 8-N_3cAMP after photolysis was measured in the equilibrium dialysis chamber at room temperature by dialyzing for 16 h against 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, 1 mg/mL BSA, and 30 μM cAMP. Aliquots were transferred into scintillation vials and counted. After being chased with nonradioactive cAMP, aliquots also were taken for Millipore filtration assays.

Isolation of Covalently Modified Peptides from $R^I_2C_2$. Holoenzyme type I (23 nmol) was incubated overnight (16 h) at 4 °C in 50 mM potassium phosphate (pH 6.8) containing 1 mM EDTA and a 2-fold molar excess of 8-N_3[^{3}H]cAMP (0.016 Ci/mmol) over cAMP binding sites and photolyzed as described above. After photolysis, the dissociated C subunit was removed by stirring the reaction mixture with CM-Sephadex at a pH of 6.1. 8-N_3cAMP-labeled R^I was carboxymethylated (Crestfield et al., 1963), dialyzed exhaustively against 50 mM NH_4HCO_3 (pH 8.4), and incubated with trypsin at 37 °C overnight with 1:50 (w/w) TPCK-trypsin to protein. The reaction mixture was lyophilized, redissolved, and resolved by high-performance liquid chromatography (HPLC).

HPLC was carried out on an Altex 3200 system with a Waters C_{18} μBondapak column (0.39 × 30 cm). The buffers employed were (a) 0.1% TFA (pH 2.11) and (b) 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% at a flow rate of 1 mL/min. Absorbance was monitored at 219 nm on a Hitachi spectrophotometer equipped with a flow-through cell.

Amino Acid Analyses. Analyses were performed on a Beckman Model 118C automated amino acid analyzer. Samples were hydrolyzed in vacuo at 110 °C in 6 M HCl for 24 h.

Sequencing. Manual dansyl-Edman degradation was carried out by two-dimensional thin-layer chromatography on polyamide sheets (8 × 8 cm), according to the method of Hartley (1970). NH_2-terminal residues were identified by the same method.

Solid-phase sequencing was carried out on an automatic Sequenator modeled after the instrument described by Doolittle et al. (1977) with the method of Laursen et al. (1975). Peptides were coupled to phenylene diisothiocyanate activated β-aminopropyl glass or aminoaryl glass (200–400 mesh). Phenylthiohydantoin amino acids were identified by HPLC carried out according to Bhown et al. (1978) on a Waters C_{18} μBondapak column.

Protein Kinase Assay. Protein kinase was assayed spectrophotometrically according to the procedure of Cook et al. (1982), with Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out on 1.5-mm, 12.5% slab gels in the presence of SDS according to the method of Laemmli (1970). Nondenaturing polyacrylamide gel electrophoresis was carried out on 1.5-mm, 7.5% polyacrylamide slab running gels with 5% polyacrylamide stacking gels, both prepared in 40 mM potassium phosphate buffer, pH 7.0.

Results

Binding of cyclic nucleotides to cAMP-dependent protein kinase I was characterized with reconstituted holoenzyme that was prepared from porcine R^I and C subunits. Formation of holoenzyme was carried out in the presence of MgATP and a slight excess of C subunit as described under Experimental

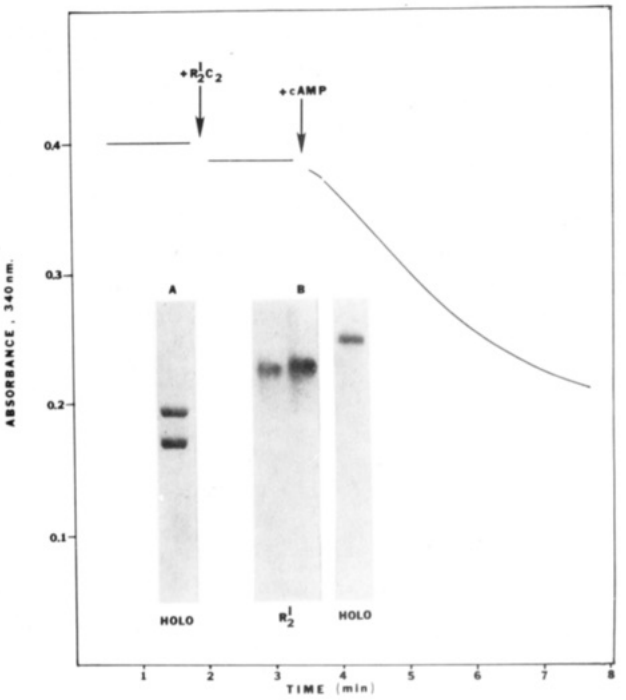

FIGURE 1: Integrity of holoenzyme type I. The reconstitution of holoenzyme was assessed enzymatically by determining the dependence of catalytic activity on added cAMP with the coupled spectrophotometric assay of Cook et al. (1982). The inset shows (A) a SDS–polyacrylamide gel electrophoresis of the holoenzyme (HOLO) and (B) a polyacrylamide gel electrophoresis under nondenaturing conditions of the holoenzyme (HOLO) and the free dimeric regulatory subunitf (R^I_2).

Procedures. After removal of the excess C subunit by ion-exchange chromatography, the formation of holoenzyme was checked by three criteria. (1) The dependency of enzymatic activity on added cAMP was established by a coupled spectrophotometric assay. (2) The relative proportions of R and C subunits were monitored visually by protein staining of SDS–polyacrylamide gels. (3) The integrity of the holoenzyme was checked by polyacrylamide gel electrophoresis run under nondenaturing conditions. As seen in Figure 1, enzymatic activity was negligible in the absence of cAMP, and R and C subunits following electrophoresis under denaturing conditions had similar intensities on the basis of Coomassie blue staining (Figure 1, inset A). Electrophoresis of holoenzyme under nondenaturing conditions showed a single band that migrated in a position that was distinct from the free dimeric R subunit (R^I_2) (Figure 1, inset B). Under these conditions free C subunit did not migrate into the gel.

Binding of cAMP to holoenzyme was measured by several techniques. Two different Millipore filtration procedures were used. Method A is a standard low-salt method that resembles the original procedure described by Gilman (1970). The alternative method, method B, included high salt and histone in the buffer and is similar to the assay described by Sugden & Corbin (1976). As was seen with the type II holoenzyme, the standard Millipore assay, method A, indicated that 1 mol of cAMP was bound per mole of R^I monomer whereas with method B 2 mol of cAMP/mol of R^I monomer was bound (Figure 2, top). Equilibrium dialysis (Figure 2, center) confirmed that 2 mol of cAMP was bound per mole of R^I monomer. Finally, a third procedure was used that was originally described by Døskeland & Ueland (1975). In this case, the holoenzyme was precipitated with ammonium sulfate and then subjected to Millipore filtration. Under these conditions both cAMP binding sites were measured as well (Figure 2, bottom).

Table I: Cyclic Nucleotide Binding to Holoenzyme Type I

assay procedure	mol of cAMP bound/ mol of R^I monomer	K_d (cAMP) (nM)[a]	mol of 8-N_3cAMP bound/ mol of R^I monomer	K_d (8-N_3cAMP) (nM)[a]
method A	0.8	10	0.9	13
method B	1.9	13	1.7	17
equilibrium dialysis	2.0	11	2.1	14
ammonium sulfate method	1.9	11	2.2	13

[a] Apparent K_d's were approximated from half-maximal binding from graphs in Figure 2.

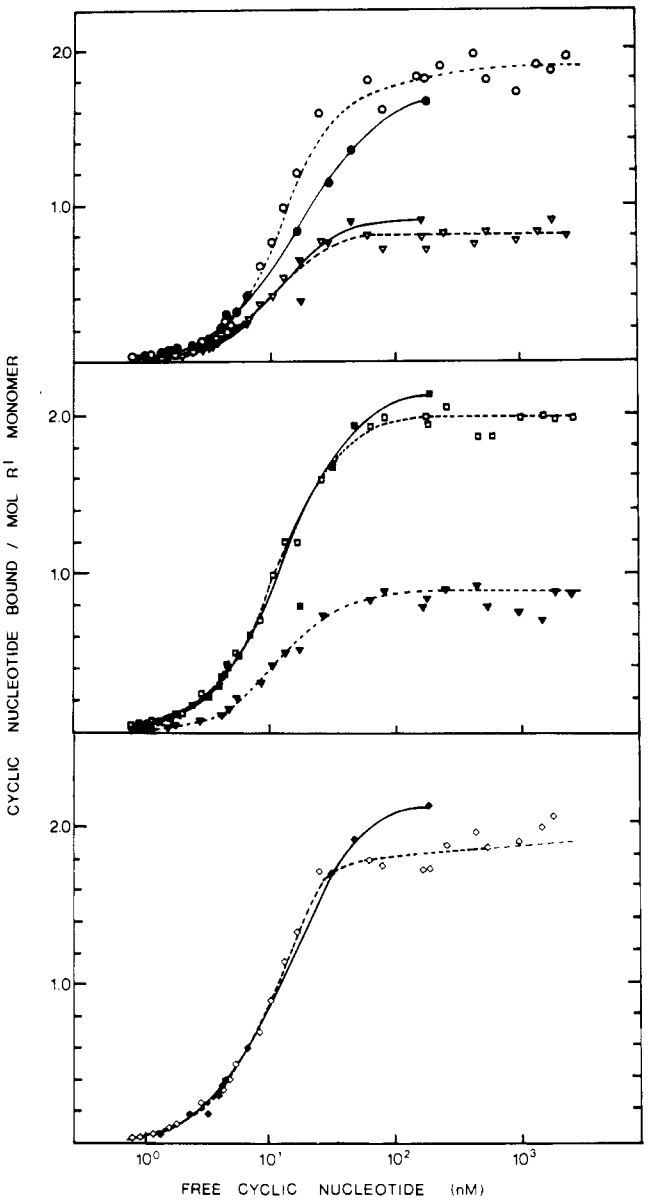

FIGURE 2: Cyclic nucleotide binding to holoenzyme type I. Holoenzyme type I (20 nM) was incubated with various concentrations of [^{3}H]cAMP (0.0–2.0 μM) or 8-N_3[^{3}H]cAMP (0.0–0.4 μM) as described under Experimental Procedures. Dashed curves and solid curves show cAMP and 8-N_3cAMP binding, respectively. (Top) Millipore filtration assay A (▽, ▼) and Millipore filtration assay B (○, ●); (center) equilibrium dialysis (□, ■) and Millipore filtration assay A after equilibrium dialysis (▼); (bottom) ammonium sulfate precipitation followed by Millipore filtration assay (◇, ◆).

The apparent K_d's for cAMP binding were approximately 10–13 nM either with Millipore assays, equilibrium dialysis, or ammonium sulfate precipitation (Table I). The Scatchard plot showed positive cooperativity for the cAMP binding sites, and a Hill coefficient of 1.53 was determined.

Further verification that all residual cAMP was removed from the holoenzyme was obtained by measuring cAMP binding at 4 and 30 °C. At 4 °C, residual bound cAMP does not exchange with added [^{3}H]cAMP. If cAMP binding to the free R subunit is measured at 4 °C, it has been observed that very little [^{3}H]cAMP is bound unless there is an opportunity for nonradioactive bound cAMP to exchange with the radioactive cAMP in the assay (Hoyer et al., 1980). At 30 °C this exchange can occur. The holoenzyme that was used for the studies described here likewise showed no difference in cAMP binding at 4 and 30 °C, and this procedure also was used as a routine verification that the holoenzyme was free of bound nucleotides.

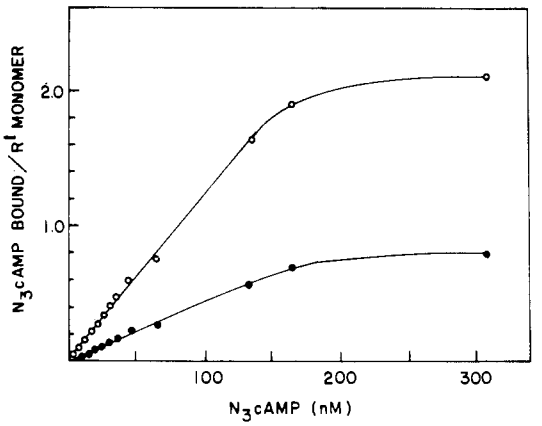

FIGURE 3: Determination of covalent incorporation of 8-N_3cAMP to holoenzyme type I. Holoenzyme type I (20 nM) was incubated with various concentrations of 8-N_3[^{3}H]cAMP as in Figure 2. Binding of 8-N_3cAMP was determined by equilibrium dialysis (○). Samples were irradiated at 4 °C with UV light (254 nm) for 10 min. Covalent incorporation of 8-N_3cAMP into the protein was determined by equilibrium dialysis against an excess of cAMP (●).

Having established the cAMP binding properties of the porcine type I holoenzyme, a parallel set of experiments was carried out with the cAMP analogue, 8-N_3cAMP, in order to establish that 8-N_3cAMP was binding to R^I in a manner that was analogous to cAMP. Equilibrium dialysis, ammonium sulfate precipitation, and Millipore filtration with method B all indicated that 2 mol of 8-N_3cAMP was bound per mole of R^I monomer (Figure 2). The apparent K_d's for 8-N_3cAMP, which are summarized in Table I, were also found to be analogous to the K_d's for cAMP. A Scatchard plot for 8-N_3cAMP also showed positive cooperativity and was nearly identical with the comparable plot for cAMP. A Hill coefficient of 1.58 was determined.

After characterizing the binding properties of 8-N_3cAMP to the type I holoenzyme in the absence of photolysis, the extent of covalent incorporation following photolysis was evaluated. 8-N_3cAMP was bound to holoenzyme overnight as described and then photolyzed. Covalent incorporation was monitored by carrying out dialysis at room temperature in the presence of a 100-fold excess of cAMP. Under these conditions, approximately 1 mol of 8-N_3cAMP/mol of R^I monomer was incorporated (Figure 3). In order to demonstrate that the binding of 8-N_3cAMP was specific for the cAMP binding sites of R^I, samples of holoenzyme I were incubated with a fixed concentration of 8-N_3[^{32}P]cAMP and increasing concentrations of nonradioactive cAMP. The samples were

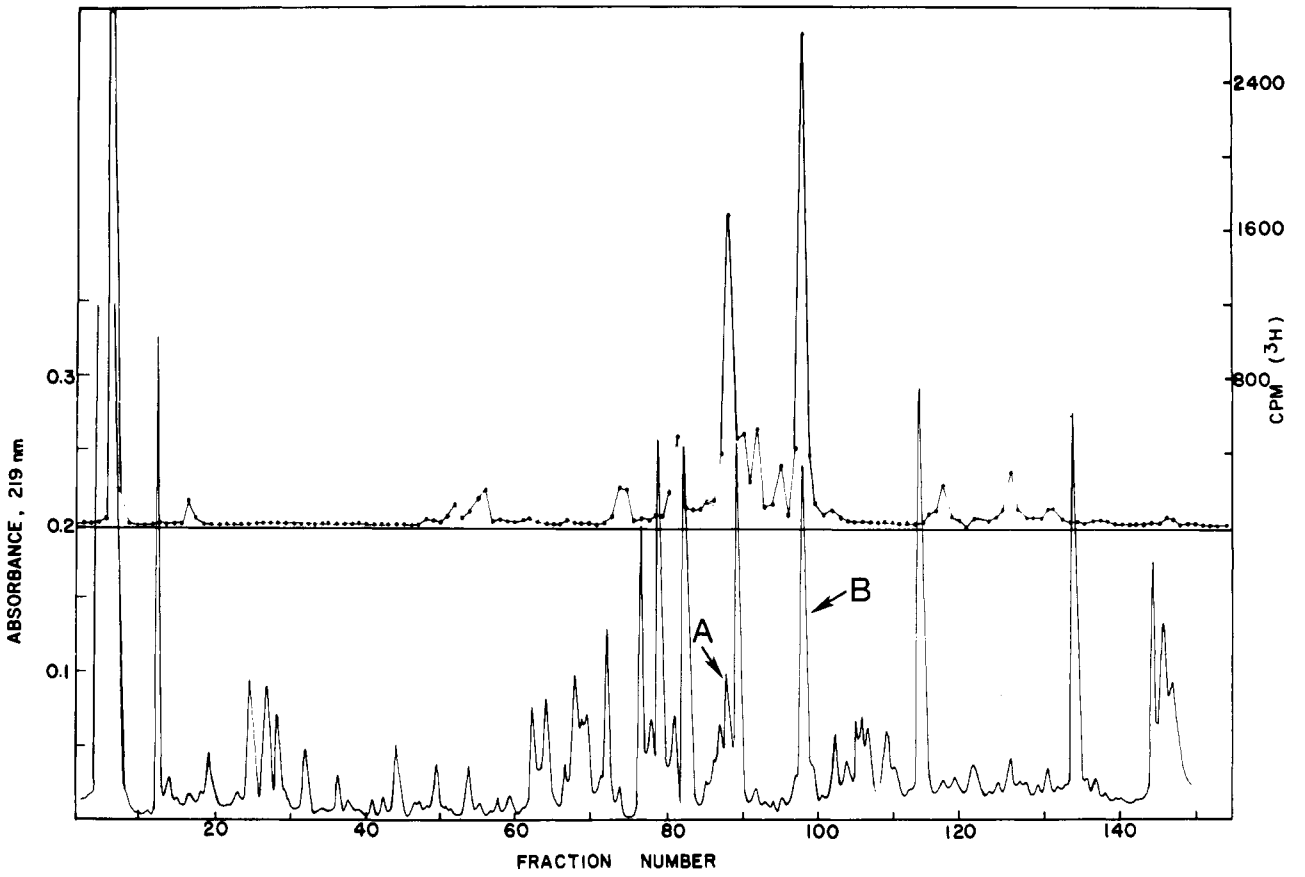

FIGURE 4: High-performance liquid chromatography separation of trypsin digest of R^I from 8-N_3cAMP-labeled $R^I_2C_2$. The buffers employed were (a) 0.1% TFA (pH 2.11) and (b) CH_3CN. The tryptic peptides were eluted with a 120-min linear gradient from 0% to 30% b and a 60-min linear gradient from 30% to 60% b. (Top) Radioactivity (cpm of [^{3}H]); (bottom) absorbance at 219 nm.

photolyzed and separated by SDS–polyacrylamide gel electrophoresis. The radioactivity incorporated into R^I was determined by autoradiography. 8-N_3cAMP modification of R^I was shown to be completely blocked by the addition of an excess of cAMP (data not shown).

Holoenzyme type I labeled with 8-N_3[^{3}H]cAMP on a preparative scale bound 2.1 mol of 8-N_3cAMP/mol of R^I monomer, but only 1.2 mol of 8-N_3cAMP/mol of R^I monomer remained covalently bound. Subsequent incubation of the holoenzyme with [^{3}H]cAMP after photolysis indicated that the protein was still capable of binding an additional 1.0 mol of cAMP after 1.2 mol of 8-N_3cAMP had been covalently incorporated.

The specificity of the covalent modification was assessed by digesting the covalently modified protein with trypsin and then resolving the resulting peptides by HPLC. The results seen in Figure 4 showed that two peptides were specifically photolabeled. When the peptides were immobilized to glass beads and sequenced, counting aliquots of each step showed that the radioactivity was associated with step 5 in peptide A and with step 9 in peptide B (Figure 5). The first four residues of peptide A were identified by manual dansyl-Edman degradation. Peptide B was sequenced in its entirety by solid-phase procedures (Table II). On the basis of the sequence, amino acid composition, and homology with the bovine R^I sequence (Titani et al., 1984), the modified residue in peptide A was identified as tyrosine-371 (Table II). The sequence of peptide B identified the modified residue as proline-271.

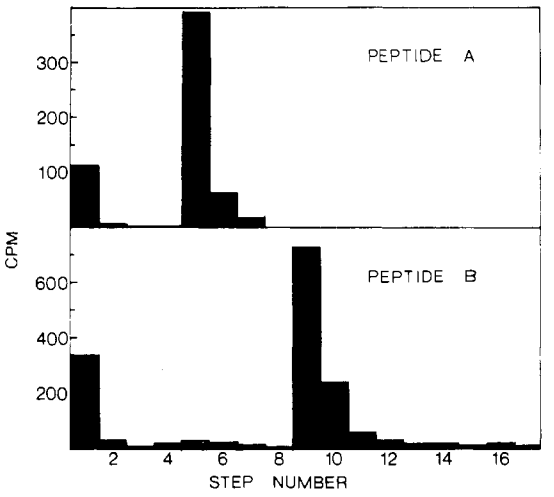

FIGURE 5: Determination of amino acid residues modified by 8-N_3cAMP. Peptides A and B were sequenced by solid-phase sequencing as described under Experimental Procedures: (top) peptide A; (bottom) peptide B.

DISCUSSION

Binding of 8-N_3cAMP to the type I holoenzyme differed in a number of significant ways from binding to the type II enzyme that was characterized previously (Kerlavage & Taylor, 1982). In the case of protein kinase II, it was not possible to demonstrate binding to two sites by Millipore filtration even with high salt and histone. In contrast, binding of 8-N_3cAMP to protein kinase I was indistinguishable from the binding of cAMP. Millipore filtration, equilibrium dialysis, and ammonium sulfate precipitation indicated that two cAMP sites per R^I monomer were capable of binding 8-N_3cAMP with a high affinity (K_d = 13–17 nM) that was comparable to the K_d for cAMP (=10–13 nM).

Covalent modification of R^I with 8-N_3cAMP also differed from R^{II} where a single tyrosine residue was shown to be

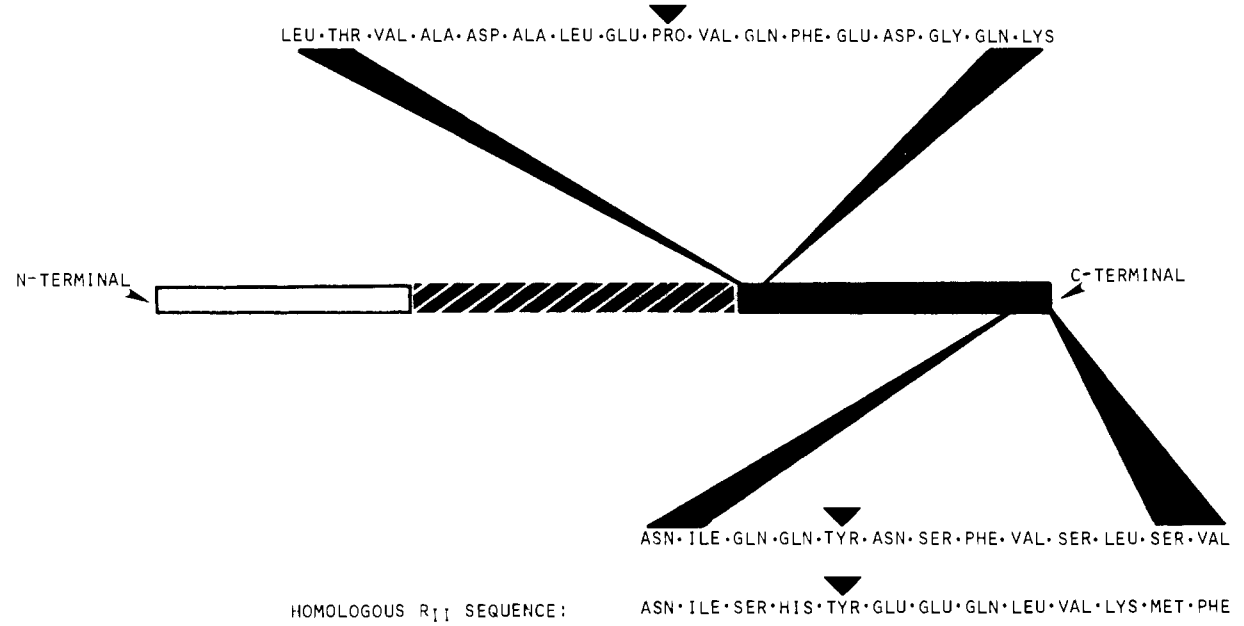

FIGURE 6: Localization of radioactive peptides in primary structure of R^I. Peptides A and B were characterized by amino acid composition and sequencing. Both are located in the cAMP binding domain B of R^I. Arrows show the amino acid residues modified by 8-N_3cAMP: proline-271 and tyrosine-371. Also shown is the homologous tyrosine-381 that becomes modified by 8-N_3cAMP in R^{II}.

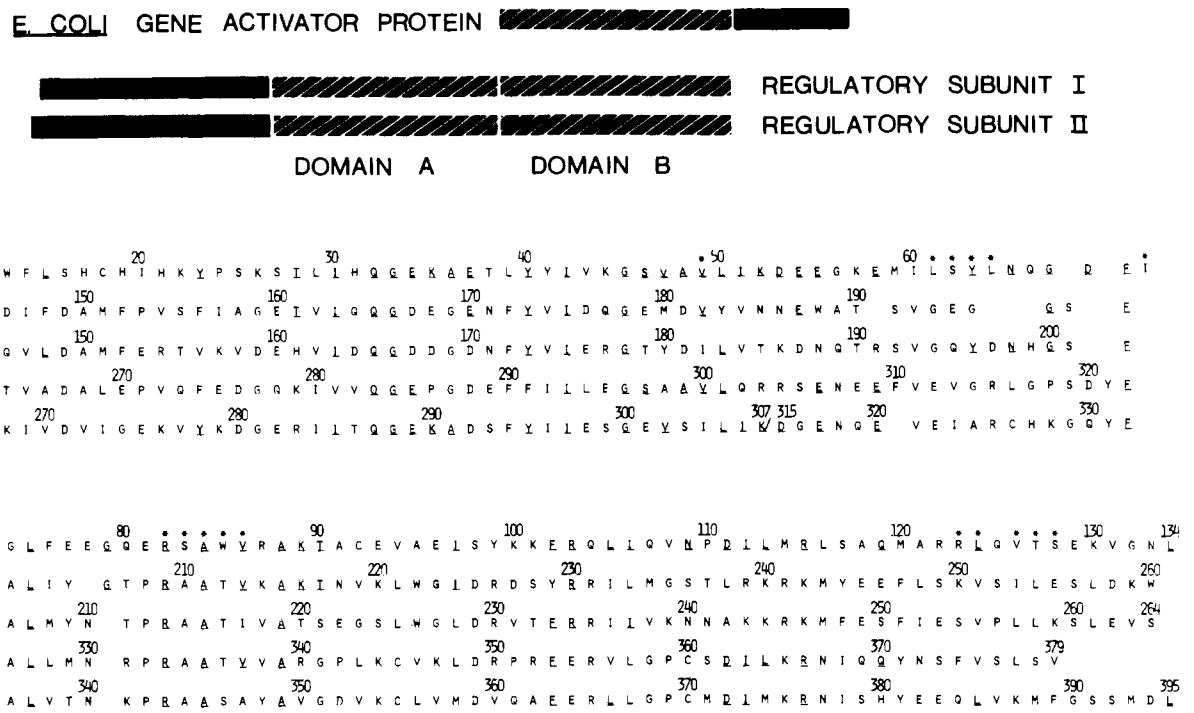

FIGURE 7: Amino acid sequence homology between the cAMP binding domain of CAP and cAMP binding domains A and B of R^I and R^{II}. Underlining indicates amino acids that are identical between the CAP sequence and the other sequences. (*) Amino acids that are close to cAMP in CAP (Weber et al., 1982).

covalently modified. Although R^I was covalently modified with a stoichiometry that approximated 1 mol/mol of R^I, the HPLC separation of labeled tryptic peptides revealed that two unique peptides were modified. These labeled residues correspond to proline-271 and tyrosine-371 on the basis of the amino acid sequence of R^I presented by Titani et al. (1984). The tyrosine residue is homologous to tyrosine-381, which is labeled with 8-N_3cAMP in R^{II} (Figure 6).

When the amino acid sequence of R^{II} was compared with CAP, the *E. coli* cAMP binding protein, it was clear that the two cAMP binding domains (A and B) of R^{II} were homologous to the cAMP binding domain of CAP (Weber et al., 1982). A similar comparison can be made for R^I (Figure 7). With this homology in mind, the localization of the covalent modifications observed was examined with respect to the known structure of CAP. The results of such a comparison are indicated diagramatically in Figure 8. This figure depicts the basic structure of the cAMP binding domain of CAP. If the amino acid sequences of the cAMP binding domains of R are superimposed directly on that crystal structure of CAP, one can obtain a theoretical localization for the various amino acid residues, and this has been done for domain B in Figure 8. If the polypeptide chain in R is folding in the same manner as in CAP as the sequence homology would suggest and if cAMP in general is binding similarly in both molecules, then the affinity labeling results should be consistent in indicating that

Table II: Amino Acid Sequence of the Two Tryptic Peptides of the Regulatory Subunit Type I Modified by 8-N_3cAMP[a]

Peptide A:	Asn-Ile-Gln-Gln-Tyr-Asn-Ser-Phe-Val-Ser-Leu-Ser-Val
Dansyl-Edman:	→ → → →
Peptide B:	Leu-Thr-Val-Ala-Asp-Ala-Leu-Glu-Pro-Val-Gln-Phe-Glu-Asp-Gly-Gln-Lys
Dansyl-Edman:	→
Solid Phase Sequencing:	→ → → → → → → → → → → → → → → →

[a] Only the first four residues of peptide A were sequenced. The remaining sequence indicated is based on the homologous peptide in bovine R^I and is consistent with the composition of peptide A.

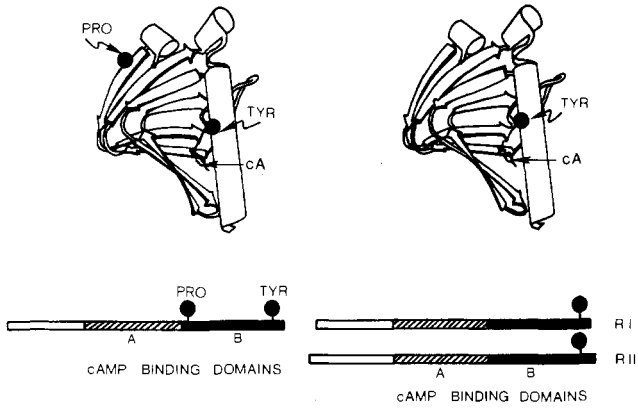

FIGURE 8: Model of cAMP binding domain B of R^I showing proline and tyrosine modification sites. View of the cAMP binding domain of CAP (McKay et al., 1982) with the sequence of R^I superimposed on it. This model assumes that the polypeptide chain of R^I folds in a manner analogous to the cAMP binding domain of CAP. Bound cAMP is also indicated with the adenine ring interacting primarily with the long helix. (Left) Although the proline and tyrosine residues both lie within the linear sequence contained in cAMP binding domain B, they are far removed in the tertiary structure. Localization of the tyrosine and proline residues in the linear sequence are indicated below. (Right) The circle indicates the homologous tyrosine residue that is modified by 8-N_3cAMP in both R^I and R^{II}. Localization of the tyrosine residues in the linear sequence is also indicated.

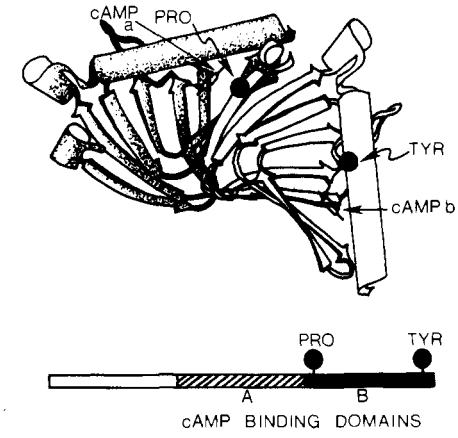

FIGURE 9: Proposed structural model for the COOH-terminal region of R^I containing the two adjacent and homologous cAMP binding domains. Arrows show the location of the modified proline-271 and tyrosine-371, and the location of these residues is correlated to cAMP bound to both cAMP binding sites.

the photoactivatable group is in close proximity to the modified residue. In fact, a close examination of the 8-N_3cAMP modification sites is probably one of the best ways short of solving the crystal structure of R to test whether the sequence homologies can be extended to structural homologies and common cAMP binding sites. When the above comparisons are made with CAP and the cAMP binding domains B of both R^I and R^{II}, the tyrosine residues that are modified (Tyr-371 in R^I and Tyr-381 in R^{II}) would be located in the major C helix of CAP (Figure 8, right). It is this C helix in CAP that provides the major sites of interaction of the protein with the adenine ring of cAMP where the photoactivatable azido group in 8-N_3cAMP is located. Furthermore, the side chain of Tyr-371 in the C helix would be oriented such that it faces in the direction of the adenine ring.[2] This provides convincing biochemical evidence for both R^I and R^{II} that modification of the tyrosine residue is derived from 8-N_3cAMP bound to domain B and that in a more general sense cAMP is binding to R in a manner that is analogous to CAP even though cAMP does not bind with a high affinity to CAP. It should be emphasized strongly that the analogy is general at this point, indicating only that when cAMP is bound to site B in R, the adenine ring of the cyclic nucleotide interacts with a region that is analogous to the C helix in CAP. Obviously, a detailed comparison of the immediate environment of the cAMP binding sites in R^I, R^{II}, and CAP will require detailed and precise model building and must take into account the extensive analogue studies that have been carried out with each protein.

Covalent modification of the proline residue was also compared in a similar manner. Since Pro-271 actually falls within the cAMP binding domain B, it was first analyzed to see if Pro-271 and Tyr-371 were actually in close proximity to each other in the tertiary structure of the protein despite their distance in the primary structure. If the homology with CAP is valid, the proline residue is 20 Å or more removed from the tyrosine,[2] thus making it highly unlikely that both residues would be modified by the same 8-N_3cAMP molecule bound to domain B (Figure 8, left). On the other hand, the sequence predicts that R^I contains two in-tandem domains for binding cAMP. If these two domains are aligned as shown in Figure 9, it is clearly possible that proline, although it is a component of domain B, could be modified by 8-N_3cAMP bound to domain A. In this case, the adenine ring of the cyclic nucleotide bound to domain A would come into very close proximity to the first β-sheet of domain B where the proline would be located. The labeling of R^I with 8-N_3cAMP therefore not only provides information about cAMP binding to domains A and B but also potentially provides information as to how the two domains might be oriented with respect to one another in the native protein. Once again, detailed model building will be required to more rigorously compare the cAMP binding sites in both molecules and to look more closely at possible orientations of domains A and B.

[2] I. Weber, personal communication.

A final point can be made in that in spite of 8-N_3cAMP binding to both sites and presumably labeling both sites, a stoichiometry for covalent incorporation of greater than 1 mol of 8-N_3cAMP/mol of R^I monomer was rarely achieved. These results might be fortuitious. On the other hand, they might also suggest that covalent modification at both sites simultaneously in the same molecule may not be possible. The results show clearly that cAMP can bind to the remaining sites following covalent modification; however, covalent modification may lead to conformational changes or may fix a given domain in a static configuration that would preclude 8-N_3cAMP binding in the remaining site in a manner that would lead to covalent modification.

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Registry No. 8-N_3cAMP, 31966-52-6; cAMP, 60-92-4; protein kinase, 9026-43-1; L-proline, 147-85-3; L-tyrosine, 60-18-4.

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