Proc. Nat. Acad. Sci. USA
Vol. 70, No. 9, pp. 2529–2533, September 1973

Cyclic Adenosine Monophosphate Receptor: Loss of cAMP-Dependent DNA Binding Activity after Proteolysis in the Presence of Cyclic Adenosine Monophosphate

(binding assay/DNA-cellulose chromatography/conformation)

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Communicated by Severo Ochoa, May 14, 1973

ABSTRACT **The cAMP receptor requires cAMP for DNA binding at pH 8.0 but shows cAMP-independent DNA binding at pH 6.0. Incubation of the cAMP receptor with proteolytic enzymes in the presence of cAMP results in loss of DNA-binding ability at pH 8, while it is still able to bind cAMP and DNA at pH 6. Incubation with proteolytic enzyme in the absence of cAMP does not affect the DNA-binding properties of the cAMP receptor. After proteolysis in the presence of cAMP, analysis by sodium dodecyl sulfate–acrylamide-gel electrophoresis shows that the 22,500-dalton subunit characteristic of the untreated protein has been completely replaced by a 12,500-dalton fragment.**

The cyclic AMP (cAMP) receptor is a DNA-binding protein in which the affinity for DNA is dependent on cAMP (1–3). cAMP receptor acts as an auxiliary factor for the RNA polymerase holoenzyme for catabolite-repressible operons where initiation of transcription requires the coincident binding of both cAMP receptor and RNA polymerase to the promoter (4, 5). cAMP receptor is considered an allosteric protein in which cAMP binding elicits a conformational change in cAMP receptor necessary for DNA binding (6). We will present evidence showing that limited proteolytic digestion of cAMP receptor in the presence (but not in the absence) of cAMP results in a modified form of cAMP receptor in which the characteristic 22,500-dalton subunit of native cAMP receptor has been replaced by a 12,500 (±500)-dalton fragment. The modified cAMP receptor, although able to bind cAMP and to form complexes with poly(dA-dT)·poly(dA-dT)[d(A-T)] at pH 6.0 (cAMP-independent binding), is unable to carry out the cAMP-dependent binding of d(A-T) at pH 8.0 characteristic of native cAMP receptor.

MATERIALS AND METHODS

Materials. Phenylmethanesulfonylfluoride, dithiothreitol, trypsin, chymotrypsin, subtilisin, cAMP, cGMP, 5′-AMP, ADP, ATP, dATP, TTP, dCTP, and Bis–Tris–propane buffer were products of Sigma. Nitrocellulose filters (0.45-μm pore size, 25-mm diameter) were obtained from Matheson–Higgins. Tritiated cAMP, TTP, and dCTP were obtained from New England Nuclear Corp. dITP was prepared by nitrous acid deamination of dATP by the method of Inman and Baldwin (7). Labeled and unlabeled d(A-T) and poly(dI-dC)·poly(dI-dC)[d(I-C)]were prepared with *Escherichia coli* DNA polymerase (8) with a specific activity of 1000.

The cAMP receptor used in these studies was prepared from *E. coli* B by a modified procedure to be presented elsewhere, and was judged to be at least 95% pure by Na dodecyl sulfate–gel electrophoresis. This material was equally as active in promoting *gal* transcription *in vitro* as cAMP receptor prepared by the method of Anderson *et al.* (1).

[³H]cAMP Binding. The assay was similar to that of Anderson *et al.* (1) with the ammonium sulfate precipitate collected on a Whatman GFC glass-fiber filter rather than by centrifugation.

[³H]d(A-T) or [³H]d(I-C) Binding. To lower the blank adsorption of the labeled polymers, the nitrocellulose filters were soaked in 0.1 M KOH for 30 min at room temperature (25°) (9) and then placed in 20 mM Tris·HCl (pH 7.8)–50 mM NaCl before use. After the binding assay the filters were dried and counted in liquifluor toluene in a Beckman LS 230 scintillation counter. All incubations were done in polystyrene tubes.

DNA–cellulose was prepared by the method of Alberts *et al.* (10), with heat-denatured calf-thymus DNA.

RESULTS

Binding of cAMP receptor to DNA containing catabolite repressible operons requires cAMP (2, 3); this is also true for formation of cAMP receptor–d(A-T) and cAMP receptor–d(I-C) complexes (Fig. 1). The data show that the cAMP concentration required for half-maximum binding varies with the polydeoxynucleotide assayed. Half-maximum binding to cAMP receptor occurred at a concentration of 7×10^{-7} M cAMP for [³H]d(I-C) and 1×10^{-5} M cAMP for [³H]d(A-T). Other experiments have indicated that cAMP receptor + d(I-C) has a higher affinity for cAMP than either cAMP receptor alone or cAMP receptor + d(A-T); this finding is consonant with the data showing that a low concentration of cAMP is required for d(I-C) binding. The apparent high affinity of cAMP receptor + cAMP for d(I-C) relative to d(A-T) is in contrast to the much higher affinity of the *lac* repressor for d(A-T) relative to d(I-C) (11). These differences may reflect some properties of the promoter and operator sequence or secondary structure that would be recognized by cAMP receptor and *lac* repressor.

The pH response for the formation of d(A-T)– or d(I-C)–cAMP receptor complex is interesting (Fig. 2), with the optimum pH for deoxypolymer binding at pH 8.0. As the pH of the binding assay was raised to pH 10, complete loss of complex formation resulted. RNA polymerase–[³H]d(A-T) complexes are retained on nitrocellulose filters at pH 10, indicating that the inability of cAMP receptor to bind to de-

Abbreviations: d(A-T), poly(dA-dT)·poly(dA-dT); d(I-C), poly(dI-dC)·poly(dI-dC).

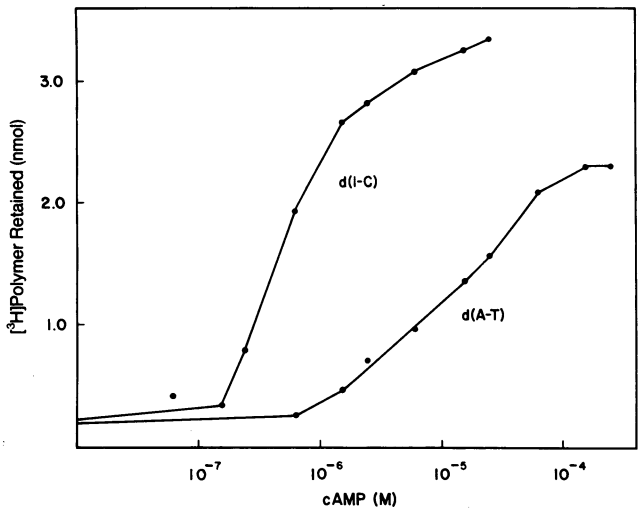

FIG. 1. Binding of cAMP receptor to d(A-T) or d(I-C) as a function of cAMP concentration. The incubation contained (final volume, 0.25 ml): 40 mM Bis–Tris–propane buffer (pH 8.0), 2.5 μg of cAMP receptor, 4.5 nmol of [³H]d(A-T) (1944 cpm/nmol) or 4.5 nmol of [³H]d(I-C) (1494 cpm/nmol), and cAMP as indicated. The mixtures were incubated for 5 min at 37°. After addition of 0.75 ml of 50 mM NaCl, they were filtered onto nitrocellulose membranes.

oxypolymers is not a property of the filters at alkaline pH. As the pH was lowered to 6.0, there was an increase in the cAMP-independent binding of cAMP receptor to both (dA-T) and d(I-C) to values approaching that obtained for the cAMP + cAMP receptor assays at this pH. The data suggest that at pH 8, cAMP is required to induce a conformational change

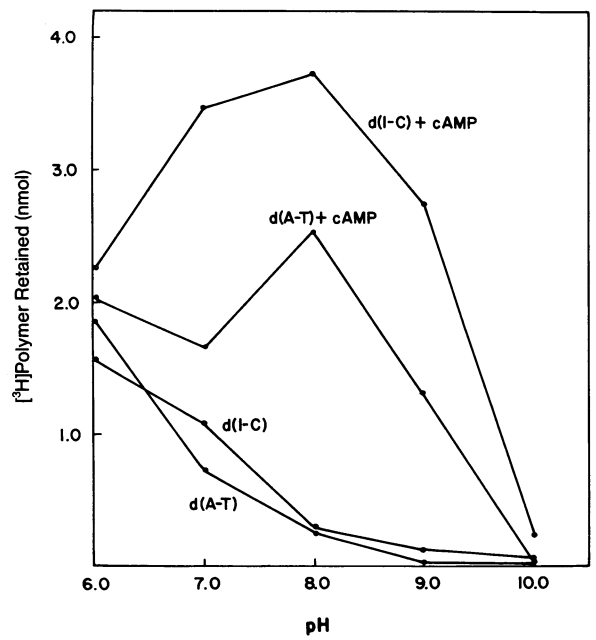

FIG. 2. Deoxypolymer binding by cAMP receptor as a function of pH and cAMP. The incubations contained (final volume, 0.25 ml): 40 mM Bis–Tris–propane buffer at the pH indicated, 2.5 μg of cAMP receptor, 4.5 nmol of [³H]d(A-T) or [³H]d(I-C), and, where indicated, 4×10^{-4} M cAMP. The mixtures were incubated for 5 min at 37°. After addition of 0.75 ml of 50 mM NaCl, they were filtered onto nitrocellulose membranes.

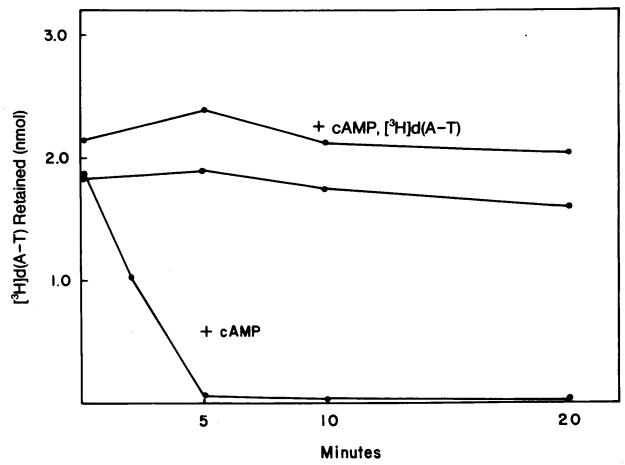

FIG. 3. Time-course for inactivation of cAMP receptor incubated with cAMP + subtilisin. The incubations contained (final volume, 0.25 ml): 40 mM Bis–Tris–propane buffer (pH 8.0), 0.4 mM dithiothreitol, 2 μg of cAMP receptor, and 0.1 μg of subtilisin; where indicated 10 nmol of cAMP and 4.5 nmol of [³H]-d(A-T) were added before incubation at 37° for the periods indicated. In the assay for binding, each tube received 1 nmol of phenylmethanesulfonylfluoride, 100 nmol of cAMP, and to the sets which lacked it, 4.5 nmol of [³H]d(A-T). The mixtures were incubated for 5 min at 37°. After addition of 0.75 ml of 50 mM NaCl, they were filtered onto nitrocellulose membranes.

in cAMP receptor required for DNA binding; this conformation could occur spontaneously at pH 6 in the absence of cAMP.

The data presented previously, and thus far in this paper, suggest that the response of cAMP receptor to cAMP probably involves a conformational alteration resulting in an increase in the affinity of cAMP receptor for DNA. As an experimentally simple approach to this problem we have looked

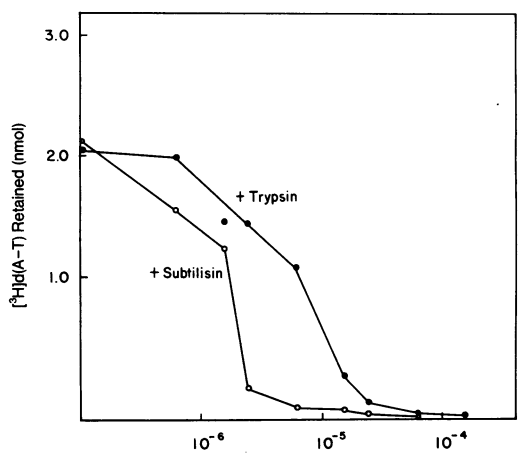

FIG. 4. Dependence of cAMP concentration for inactivation of cAMP receptor by subtilisin and trypsin. The incubations contained (final volume, 0.25 ml): 40 mM Bis–Tris–propane buffer (pH 8.0), 2.5 μg of cAMP receptor, and 0.5 μg of either subtilisin or trypsin. After addition of cAMP at the concentrations indicated, the mixtures were incubated for 10 min at 37°, after which the following were added: 1 nmol of phenylmethanesulfonylfluoride, 4.5 nmol of [³H]d(A-T), and 100 nmol of cAMP. After 5 min at 37°, 0.75 ml of 50 mM NaCl were added. The mixture was then filtered onto nitrocellulose membranes.

TABLE 1. *Effect of proteolytic enzymes on cAMP receptor*

Additions to incubation	[^{3}H]d(A-T) retained (nmol)
None	2.94
cAMP	2.88
Chymotrypsin (0.5 μg)	3.05
Chymotrypsin (0.5 μg) + cAMP	0.03
Subtilisin (0.5 μg)	2.55
Subtilisin (0.5 μg) + cAMP	0.02
Trypsin (0.5 μg)	2.83
Trypsin (0.5 μg) + cAMP	0.41

The reactions contained (final volume, 0.25 ml): 40 mM Bis-Tris-propane buffer (pH 8.0), 5 μg of cAMP receptor and, where indicated, 0.4 mM cAMP and the proteases. After the mixtures were incubated for 10 min at 37°, the following were added: 4 μM phenylmethanesulfonylfluoride, 4.5 nmol of [^{3}H]d(A-T), and 0.4 mM cAMP where omitted in the first incubation. After 5 min at 37°, 0.75 ml of 50 mM NaCl was added and the mixture was filtered onto a nitrocellulose membrane.

at the effect of proteolytic enzymes (12) on the structural and functional properties of cAMP receptor. If cAMP alters the conformation of cAMP receptor, than one may assume that this alteration would be reflected in a change in the availability of susceptible peptide bonds rendering cAMP receptor either more or less stable relative to cAMP receptor incubated with the proteolytic enzymes in the absence of cAMP. With three proteases of markedly differing peptide-bond specificity, the results were identical with regard to d(A-T) binding (Table 1). cAMP receptor incubated with trypsin, chymotrypsin, or subtilisin retained its [^{3}H]d(A-T)-binding capacity when subsequently assayed in the presence of cAMP; in contrast, incubation of cAMP receptor with the proteolytic enzymes in the presence of cAMP resulted in loss of the ability to bind d(A-T).

Incubation of 2 μg of cAMP receptor with 0.1 μg of subtilisin in the presence of 40 μM cAMP resulted in a complete loss of d(A-T) binding within 10 min at 37° (Fig. 3). In the absence of cAMP no effect of subtilisin on d(A-T) binding was evident, even after 20 min when the mixture was subsequently assayed after addition of cAMP (0.4 mM). cAMP receptor bound to [^{3}H]d(A-T) in the presence of 40 μM cAMP was also resistant to subtilisin, indicating that the susceptible area in cAMP receptor was protected on binding to DNA. Similar results were obtained with trypsin in place of subtilisin.

The cAMP requirement for the trypsin-induced loss of polymer binding by cAMP receptor is shown in Table 2. Incubation of 5 μg of cAMP receptor with 1 μg of trypsin for 10 min at 37° led to a 10% loss of [^{3}H]d(A-T) binding. Incubation in the presence of cAMP (0.4 mM) resulted in complete loss of d(A-T) binding by the trypsinized cAMP receptor. The specific requirement for cAMP for the trypsin effect is seen in the inability of cGMP, 5′-AMP, or ATP to lower cAMP receptor binding of d(A-T). The lack of effect of cGMP on the trypsin degradation of cAMP receptor is interesting, since cGMP competes with cAMP for binding to cAMP receptor (1, 6, 13).

Since the proteolytic loss of deoxypolymer binding by cAMP receptor is cAMP dependent, it was possible to determine the cAMP concentration required for this response. The samples were treated with protease for 10 min at 37°. As an assay for residual [^{3}H]d(A-T) binding, an additional 100 nmol of cAMP was added after protease treatment. The cAMP concentration required for loss of half the d(A-T) binding capacity is about 8 μM cAMP for trypsin and 3 μM cAMP for subtilisin (Fig. 4). These values are within the range of cAMP concentrations required for half-maximum binding of cAMP receptor to d(A-T) and d(I-C) shown in Fig. 1. The different cAMP concentrations required for inactivation of cAMP receptor by trypsin and subtilisin may reflect the specificity of trypsin for hydrolysis of lysine- and arginine-containing peptide links, while subtilisin is a relatively nonspecific proteolytic enzyme.

Preliminary experiments showed that although cAMP-dependent d(A-T) binding at pH 8 was lost after protease digestion, the modified cAMP receptor was still able to bind d(A-T) at pH 6; as shown in Fig. 2 formation of a cAMP receptor–d(A-T) complex at pH 6 does not require cAMP. Using this property we could prepare subtilisin-modified cAMP receptor by chromatography on DNA–cellulose (Fig. 5). 10 mg of cAMP receptor were incubated with 0.3 mg of subtilisin and 50 μM cAMP until 95% of the cAMP-dependent d(A-T)-binding activity was lost. After the pH of the mixture was adjusted to 6.5, the cAMP receptor was adsorbed onto a DNA–cellulose column and eluted with a linear salt gradient in Buffer A. A major protein absorbance peak comprising 78% of the input cAMP receptor emerged at 0.2 M NaCl in Buffer A. The fractions were assayed for d(A-T) binding at pH 6.0 (cAMP-independent), cAMP-dependent d(I-C)-binding at pH 8.0, and also for cAMP binding where indicated in Fig. 5. As shown, two of the characteristic activities of cAMP receptor were coincident with the 280-nm absorbance profile (tubes 12–16; the UV-absorbing peak at the beginning of the elution is mostly due to cAMP and subtilisin); the subtilisin-modified cAMP receptor was still able to bind [^{3}H]cAMP and [^{3}H]d(A-T) at pH 6.0, but showed no cAMP-dependent [^{3}H]d(I-C) [or [^{3}H]d(A-T)] binding at pH 8.0. The high affinity of the subtilisin-modified cAMP receptor for DNA–cellulose as well as for d(A-T) at the low pH demonstrates that a DNA-binding site of cAMP receptor was not

TABLE 2. *cAMP requirement for trypsin-induced loss of d(A-T) binding by cAMP receptor*

Additions to incubation	[^{3}H]d(A-T) retained (nmol)
None	3.09
cAMP	2.62
cAMP + trypsin (1 μg)	0.01
cGMP + trypsin (1 μg)	2.60
5′-AMP + trypsin (1 μg)	2.87
ATP + trypsin (1 μg)	2.66
Trypsin (1 μg)	2.81

The reactions contained (final volume, 0.25 ml): 40 mM Bis-Tris-propane buffer (pH 8.0), 5 μg of cAMP receptor, and where indicated, the nucleotides were added to 0.4 mM. After 10 min at 37°, the following were added: 4 μM phenylmethanesulfonylfluoride, 4.5 nmol of [^{3}H]d(A-T), and 0.4 mM cAMP where omitted in the first incubation. After 5 min at 37°, 0.75 ml of 50 mM NaCl was added, and the mixture was filtered onto a nitrocellulose membrane.

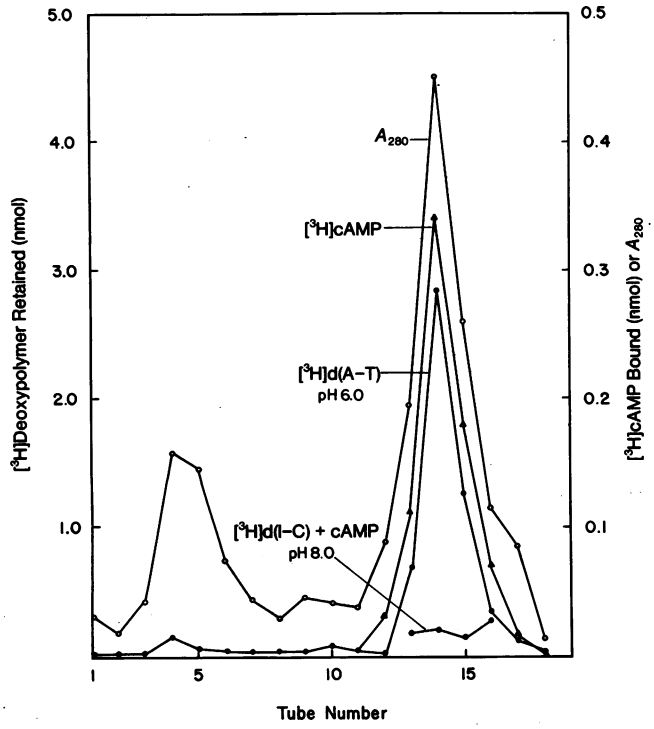

FIG. 5. DNA–cellulose chromatography of cAMP receptor modified by subtilisin + cAMP. To 10 mg of cAMP receptor in 10 ml of 10 mM potassium phosphate (pH 7.0), 0.1 M KCl, 0.1 mM dithiothreitol, and 1 mM EDTA, were added 50 μl of 10 mM cAMP and 0.5 ml of subtilisin (0.2 mg/ml). After 5 min at 37°, an additional 1 ml of subtilisin (0.2 mg/ml) was added, and the incubation was continued for another 15 min at 37°. Then 100 nmol of phenylmethanesulfonylfluoride were added, and the tube was placed in ice. Before addition of subtilisin, 5 μl of the cAMP receptor solution bound 3.24 nmol of [^{3}H]d(A-T) in the cAMP-dependent assay (Fig. 1, pH 8.0); after digestion with subtilisin, 5 μl of the mixture bound only 0.13 nmol of [^{3}H]d(A-T). The modified cAMP receptor was adjusted to pH 6.5 and run onto a 1.9 × 8-cm DNA–cellulose column equilibrated with 20 mM potassium phosphate (pH 6.5)–0.1 mM dithiothreitol–1 mM EDTA (Buffer A). After the column was washed with about 50 ml of Buffer A, the cAMP receptor was eluted with a linear gradient (total volume, 120 ml) of 0.02–1.0 M NaCl in Buffer A. Fractions were assayed with 10-μl aliquots for [^{3}H]d(A-T) binding at pH 6.0 (no cAMP) and for cAMP-dependent binding of [^{3}H]d(I-C) at pH 8.0 by the assay conditions shown in the legend to Fig. 2. [^{3}H]cAMP binding was assayed with 50 μl of the indicated fractions.

destroyed after subtilisin digestion. The number of pmol of [^{3}H]cAMP bound per mg of modified cAMP receptor was comparable to that shown by unmodified cAMP receptor after DNA–cellulose chromatography, under conditions identical to those shown in the legend to Fig. 5 with omission of subtilisin. Since cAMP receptor contains two identical subunits, it presumably has two cAMP-binding sites (1, 6); only one of them may be functional (low cAMP binding constant) in the native cAMP receptor. Although modified cAMP receptor bound cAMP, it is not certain whether binding occurred to the same site as in the native cAMP receptor or whether the modification has altered the properties of the cAMP-binding sites. DNA binding at pH 6 (cAMP-independent) may be due to a DNA-binding site that ordinarily does not function at pH 8 or may merely be a consequence

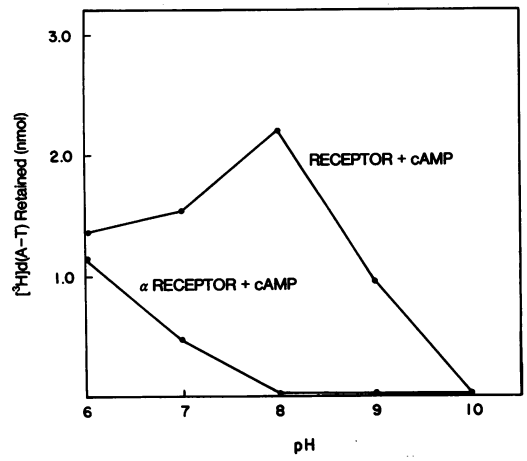

FIG. 6. [^{3}H]d(A-T) binding of subtilisin-modified cAMP receptor as a function of pH. Binding assays were done with 3.4 μg of modified cAMP receptor (α) (from tube 14 of Fig. 5) and 2.5 μg of native cAMP receptor by the procedure indicated in the legend to Fig. 2.

of an increase in the net positive charge of cAMP receptor (or α cAMP receptor) as the pH is lowered from 8 to 6. Since cAMP receptor has an isoelectric point of 9.12 (1), the protein would be positively charged at pH 8.0, where DNA binding is cAMP dependent.

Comparison of the pH profiles for d(A-T) binding, from the peak fractions of the subtilisin-modified cAMP receptor and native cAMP receptor obtained after DNA–cellulose chromatography, illustrates the profound effect of proteolytic modification on the cAMP-dependent response (Fig. 6). While native cAMP receptor showed its characteristic pH profile with a maximum [^{3}H]d(A-T) binding at pH 8 in the presence of cAMP (see also Fig. 2), the subtilisin-modified cAMP receptor (α) showed a pH profile essentially identical to that obtained for native cAMP receptor when the binding assays were done in the absence of cAMP.

Samples of the peak fractions from the DNA–cellulose column on which the subtilisin-digested material was chromatographed were compared electrophoretically with unmodified cAMP receptor to determine the nature of the subtilisin modification. The cAMP receptor was first dissociated into its subunit form by Na dodecyl sulfate, and the denatured proteins were resolved by Na dodecyl sulfate–acrylamide-gel electrophoresis (Fig. 7). Native cAMP receptor consists of two identical subunits of 22,500 daltons (1, 2), and this was found for the unmodified cAMP receptor (Fig. 7, *top*). After subtilisin modification in the presence of cAMP, the modified cAMP receptor showed a complete loss of the 22,500-dalton subunit, which was replaced by a modified subunit species termed α of about 12,500 (±500) daltons (Fig. 7, *bottom*). A protein of similar size was formed after incubation of cAMP receptor with trypsin or chymotrypsin in the presence of cAMP. Incubation of cAMP receptor with trypsin in the absence of cAMP has no apparent effect on the 22,500-dalton subunits.

DISCUSSION

The results presented are consonant with a proposal that cAMP induces a conformational change in cAMP receptor that is necessary for DNA binding at pH 8. The data also show that the binding by cAMP receptor to d(A-T) or d(I-C)

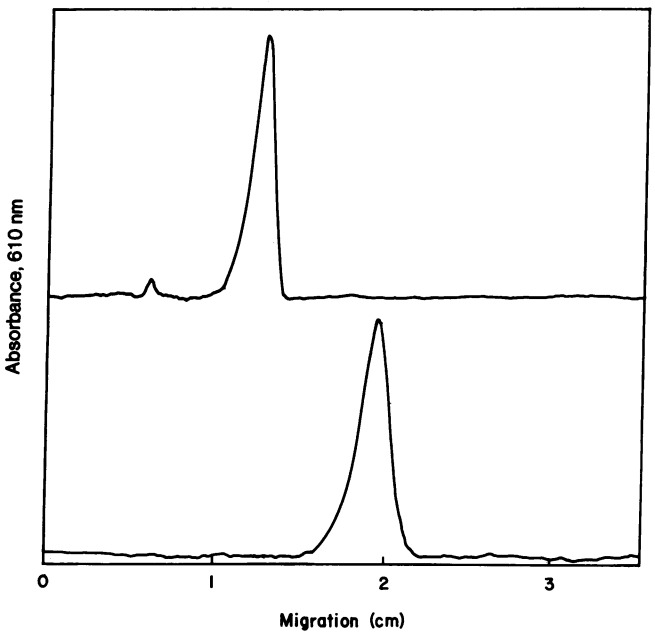

FIG. 7. Na dodecyl sulfate–gel electrophoresis of cAMP receptor and subtilisin-modified CRP. 5 μg of native cAMP receptor (*top*) and 6.8 μg of subtilisin-modified cAMP receptor (α) (*bottom*) (from tube 14 of Fig. 6) were resolved on 10% acrylamide gels containing 0.1% Na dodecyl sulfate by the method of Shapiro *et al.* (14), stained with coomassie blue (15), and scanned at 610 nm in a Beckman ACTA III equipped with a gel-scanning device.

can occur at pH 6 in the absence of added cAMP, suggesting that the proposed conformational change may occur spontaneously at the lower pH. The ability of cAMP, but not cGMP, to support formation of cAMP receptor–d(I-C) as well as cAMP receptor–d(A-T) complexes would tend to rule out a direct Watson–Crick type of hydrogen-bonding role of the adenine moiety of cAMP with DNA thymine and is in keeping with the allosteric properties of cAMP receptor.

Allosteric proteins are assumed to undergo conformational changes in tertiary structure in response to the effector molecule. That such changes do occur is shown by the increased lability of cAMP receptor induced by cAMP in the presence of proteolytic enzymes. The effect of the proteases on cAMP receptor is especially interesting since the cAMP requirement for loss of d(A-T) binding at pH 8.0 and subunit cleavage is striking. Incubation of cAMP receptor with trypsin, chymotrypsin, or subtilisin all produce a similar response; in the absence of cAMP the cAMP-dependent d(A-T)-binding by cAMP receptor at pH 8 is retained, while protease digestion in the presence of cAMP leads to complete loss of this property. In the presence of cAMP, all three proteases produce the α fragment of about 12,500 daltons, indicating that the attack had occurred in a similar region of the native cAMP receptor protomer close to the middle of the 22,500-dalton subunit. Our data demonstrate that cAMP- and DNA-binding sites (although not necessarily identical to the sites that function in unmodified cAMP receptor) of α cAMP receptor (subtilisin + cAMP modified cAMP receptor) were still present and functional under appropriate assay conditions; the critical difference between native and modified cAMP receptor is the inability of the modified cAMP receptor to respond to cAMP at pH 8 with the characteristic large increase in affinity for DNA evinced by native cAMP receptor.

Platt *et al.* (16) have shown that *lac* repressor treated with trypsin or chymotrypsin results in inactivation of operator binding without affecting inducer binding. After incubation of native repressor with the proteases, a resistant core of about 28,000 daltons was found after Na dodecyl sulfate–acrylamide-gel electrophoresis; the molecular weight of the untreated *lac* repressor is 38,000 daltons. No protective effect was noted when trypsin digestion of the *lac* repressor was done in the presence of an inducer, isopropylthiogalactoside. In contrast to the results presented on the effect of trypsin on cAMP receptor, in which subunit cleavage with loss of d(A-T) binding at pH 8 requires cAMP, the *lac* repressor shows a loss of DNA binding independent of the presence of the effector molecule. In part, these differences in trypsin susceptibility reflect the biological properties of the protein, cAMP receptor will not bind DNA in the absence of cAMP, and one may assume that the DNA-binding region may not be exposed to attack. The repressor binds to the operator only in the absence of the inducer, and the DNA-binding region would ordinarily be exposed to attack.

We acknowledge the expert technical assistance of Mr. Earl Fronk and Mrs. Rose Koo. This work was supported in part by grants from NIH (GM-18673) and a City University of New York Faculty Research Award (01475) to J.S.K.

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