Characterization of the binding of cAMP and cGMP to the CRP*598 mutant of the E. coli cAMP receptor protein

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ABSTRACT

Wild type cAMP receptor protein (CRP) activates in vitro lac transcription only in the presence of cAMP. In contrast the mutant CRP*598 (Arg-142 to His, Ala-144 to Thr) can activate lac transcription in the absence of cyclic nucleotide or at concentrations of cAMP below that required by CRP. To further characterize the properties of CRP*598, the binding of cAMP and cGMP to CRP and CRP*598 has been determined. The intrinsic binding constant (K) values obtained for cAMP binding are: CRP, \(1.9 \times 10^4\) M\(^{-1}\); CRP*598, \(3.8 \times 10^5\) M\(^{-1}\). The K values obtained for cGMP binding are: CRP, \(2.9 \times 10^4\) M\(^{-1}\); CRP*598, \(2.7 \times 10^4\) M\(^{-1}\). The results indicate that the affinity of CRP and CRP*598 for cGMP is relatively unchanged while the affinity of CRP*598 for cAMP is approximately twenty times greater than that shown by CRP. Binding of cAMP by CRP and cGMP by CRP or CRP*598 exhibits slight negative cooperativity. The major difference seen is that CRP*598 binds cAMP with strong positive cooperativity. The importance of the unsubstituted N\(^8\) position of the adenine moiety is also shown by the similar affinity of both forms of CRP for N\(^6\)-butyryl cAMP. The cAMP binding properties evinced by CRP*598 suggest that its intrinsically altered conformation may be related to that assumed by CRP in a CRP-DNA or a cAMP-CRP-DNA complex.

INTRODUCTION

The cAMP receptor protein (CRP) is composed of two identical 23,619 Da subunits (1-3). The CRP monomer has a two-domain structure in which the large N-terminal domain is responsible for cAMP binding and subunit-subunit interaction; the smaller C-terminal domain is involved in DNA binding (4-6). Binding of cAMP elicits a conformational change which minimally involves an alteration in the relative orientation of the large and small domains (7-11). The CRP*598 mutations (Arg-142 to His, Ala-144 to Thr) have been mapped (12) within the D\(\alpha\) helix close to the hinge connecting the two domains of the CRP subunits (5,6). CRP* mutants are able to support in vitro transcription from the lac P\(^+\) promoter in the presence of low concentrations of cAMP, high concentrations of cGMP or in the absence of added cyclic nucleotide (12-17). Such mutants in the absence of cAMP evoke a conformational state related to that shown by cAMP-CRP (16,17). DNase I footprinting experiments indicated that cAMP-CRP*598 binds to its site on the lac promoter while unliganded CRP*598 and cGMP-CRP*598 form a stable complex with the lac promoter only in the presence of RNA polymerase showing cooperative binding between two heterologous proteins (17). Straney et al. (18) have presented evidence showing that RNA polymerase stabilizes binding of CRP in the lac P\(^+\) open complex. This cooperative binding is consistent with the involvement of contact between CRP and RNA polymerase in transcriptional activation. In this study we have compared the cyclic nucleotide binding properties of CRP*598 and CRP. The results indicate that the affinity of mutant CRP*598 and wild type CRP for cGMP is similar. In contrast the affinity of CRP*598 for cAMP is greater than that shown by CRP. The most striking difference is the strong positive cooperativity shown for cAMP binding by CRP*598.

MATERIALS AND METHODS

Materials
Reagents were obtained as follows: lysozyme, casein, cAMP and other cyclic nucleotides, Sigma Chemical Co.; ZetaChrom SP-100 capsule, CUNO, Inc.; Sephacryl S-200 and S-Sepharose Fast Flow, Pharmacia; \(^{[3]}H\)cAMP and \(^{[3]}H\)cGMP, ICN; polymin P, Gallard Schlessinger; Ecolume, Isolab.

Proteins
CRP was purified from E. coli strain pp47 containing the recombinant plasmid pHA7 (2) donated by H. Aiba (University of Tsukuba, Ibaraki, Japan). CRP*598 was purified from E. coli strain G817 containing the recombinant plasmid pZ598 (12). CRP and CRP*598 were purified by the method of Eilen et al. (7) with the following modifications. After lysis, polymin P addition and centrifugation, the supernatant is adjusted to pH 6.5 with 1 M acetic acid and loaded onto a ZetaChrom SP-100 capsule (equilibrated with 50 mM sodium phosphate (pH 6.5), 0.1 M dithiothreitol, 0.1 mM PMSF (phenylmethanesulfonyl fluoride))

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and 5% glycerol). After washing the capsule with the equilibration buffer, CRP or CRP*598 is eluted with 300 mL of 50 mM sodium phosphate (pH 7.5), 0.5 M NaCl, 0.1 mM dithiothreitol, 0.1 mM PMSF, 5% glycerol. Fractions of 10 mL are collected and assayed for [3H]cAMP binding. The most active fractions are pooled and precipitated by addition of ammonium sulfate to 60% saturation at pH 6.8. After 30 minutes (or overnight) the protein is collected by centrifuging at 12,000 rpm for 20 minutes and the precipitate is dissolved in 5 mL of 20 mM sodium phosphate (pH 6.8), 0.1 mM dithiothreitol and 5% glycerol. Chromatography on Sephacryl S-200 is carried out as given in Ellen et al. (7). Fractions containing CRP are pooled and precipitated with ammonium sulfate (60% saturation at a pH of 6.8-7.0). After 30 minutes (or overnight) the protein is collected by centrifuging at 12,000 rpm for 20 minutes. The precipitate is dissolved in 100 mL of buffer A: 20 mM sodium phosphate (pH 6.8), 0.1 mM dithiothreitol, 1 mM EDTA and 5% glycerol and loaded onto a column of S-Sepharose Fast Flow (bed volume = 60 mL) equilibrated with Buffer A. After washing the column with 100 mL of Buffer A, a linear gradient is run with 400 mL Buffer A and 400 mL Buffer A + 0.5 M NaCl. Before pooling, the purity of the fractions containing CRP or CRP* is assessed by SDS-polyacrylamide gel electrophoresis. The concentration of CRP and CRP* was determined using the following extinction coefficient: ε_{280nm} = 9.2 (19).

RNA polymerase was isolated from E. coli K12 by the method of Burgess and Jendrisak (20). RNA polymerase holoenzyme concentration was determined using the following extinction coefficient: ε_{280nm} = 6.7 (21).

**Abortive Initiation Assay**

A modification of the abortive initiation assay of Malan et al. (22) was used to determine the effect of CRP* on CRP-dependent transcription from the lac promoter. The 203-base pair fragment containing the lac P' promoter was prepared as indicated in Li and Krakow (23). The reaction mixture contained (final volume 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, the indicated concentration of cAMP or cGMP, 2 mM lac P' DNA fragment and 40 mM RNA polymerase holoenzyme to which was added 20 nM CRP or CRP*. After incubation at 37°C for 10 minutes, 0.5 mM ApA and 50 nM [3H]UTP (9200 cpm/pmol) were added. The reaction was allowed to proceed for 15 minutes at 37°C and was terminated by addition of 10 μL 0.5 M EDTA. The radioactive products were resolved by paper chromatography in WASP solvent (24). After cutting the chromatography strip into 1 cm segments the amount of ApApUpU synthesized was determined by counting appropriate segments in Ecolume.

**Assays for Cyclic Nucleotide Binding**

Binding of cAMP or cGMP was measured by equilibrium dialysis and ammonium sulfate precipitation methods.

**(A) Equilibrium Dialysis-** The experiments were performed essentially as described in Takahashi et al. (19) using a Hoefer EMD101 apparatus. The binding assays were performed in a mixture containing: 40 mM Tris-HCl (pH 8.0), 0.4 M KCl, 1 mM dithiothreitol and 1 mM EDTA and 4 to 6 μM CRP or CRP*598. The concentration of [3H]cAMP (125 cpm/pmol) was varied from 2×10^{-6} M to 4×10^{-4} M for CRP and from 2.5×10^{-7} M to 5×10^{-5} M for CRP*598. The concentration of [3H]cGMP (200 cpm/pmol) was varied from 4×10^{-6} M to 4×10^{-4} M for CRP and CRP*598. The dialysis membranes (Spectro/Por 2) were boiled for 5 min in 5% (w/v) sodium bicarbonate containing 50 mM EDTA and then rinsed with deionized water. [3H]cAMP solution (0.2 mL) was introduced into one half-cell and CRP or CRP* solution (0.2 mL) into the other cell. Dialysis was allowed to occur at 4°C for 12 h. Two samples (20 μL) from each half-cell were added to scintillation vials containing 5 mL of Ecolume and counted.

**(B) Ammonium Sulfate Precipitation-** The binding assays were performed in a reaction mixture containing (final volume 100 μL): 40 mM Tris-HCl (pH 8.0), 0.4 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 μg casein and 2 μM CRP or CRP*598. The concentration of [3H]cAMP (283 cpm/pmol) was varied from 5×10^{-7} M to 1×10^{-4} M for CRP and from 1×10^{-7} M to 2.5×10^{-5} M for CRP*598. The concentration of [3H]cGMP was varied from 1×10^{-6} M to 1.5×10^{-4} M for CRP and CRP*598. After 30 min at 0°C, 0.6 mL of a solution of saturated ammonium sulfate (pH 8.0) was added and after 30 min at 0°C the samples were centrifuged at 10,000 rpm for 5 min. The supernatant was removed by aspiration and the pellets were dissolved in 500 μL of water. Radioactivity was determined by counting in 5 mL of Ecolume. Blanks lacking CRP or CRP* were run at all cyclic nucleotide concentrations and the values were subtracted from the test samples.

**Competitive Binding Assay**

The incubation mixtures contained (final volume 100 μL): 40 mM Tris-HCl (pH 8.0), 400 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 50 μg casein, 2 μM CRP or CRP*598, 2 μM [3H]cGMP (284 cpm/pmol) and the indicated analog over an appropriate concentration range. After 30 min at 0°C 600 μL of saturated ammonium sulfate was added. After an additional 10 min at 0°C the mixture was centrifuged at 10,000 rpm for 5 min. The pellet was dissolved in 500 μL H₂O and the radioactivity determined in Ecolume. The nucleotide concentrations were varied as follows: cAMP, N6-butyryl cAMP, 8-bromo cAMP, 8-ethylamino cAMP, N5-O2'-butyryl cAMP from 1×10^{-7} M to 1×10^{-4} M; adenosine 1×10^{-5} M to 5×10^{-3} M; 2' deoxy cAMP, 2' deoxy cGMP, 5' AMP and ATP, 2×10^{-2} M.

![Figure 1. Effect of cAMP concentration on the ability of CRP and CRP*598 to support lac P' directed abortive initiation. Conditions for the abortive initiation reaction are described in Materials and Methods. Incorporation of [3H]UMP in the absence of CRP or CRP*598 was 4 pmol. CRP, • • •; CRP*598, ○ ○ ○.](image-url)
Figure 2. Scatchard representation of the binding of cAMP to CRP and CRP*598 determined by equilibrium dialysis and ammonium sulfate precipitation. The conditions used for equilibrium dialysis are presented in Materials and Methods with 4 μM CRP or CRP*598; the insert graph shows the results obtained using the ammonium sulfate precipitation method. The conditions are presented in Materials and Methods with 2 μM CRP or CRP*598. CRP, ■ ■; CRP*598, ○ ○.

RESULTS

Formation of the open complex with the lac P⁺ promoter by RNA polymerase requires the coincident binding of cAMP-CRP to its promoter-associated site. The response of CRP and CRP*598 to cAMP concentration in the abortive initiation reaction is shown in Figure 1. The data indicate that both forms of CRP respond to increasing cAMP concentration. However, the concentration of cAMP required to achieve a half-maximal response for supporting abortive initiation by RNA polymerase differs by about 11 fold: CRP, 3.2 × 10⁻⁶ M cAMP; CRP*598, 2.8 × 10⁻⁷ M cAMP.

In order to determine whether the apparent increased affinity for cAMP is an intrinsic property of CRP*598, direct cAMP binding assays were carried out using equilibrium dialysis and ammonium sulfate precipitation procedures. Scatchard plots for binding of cAMP by CRP*598 (Figure 2) obtained by both methods are indicative of strong positive cooperativity. Under the same conditions wild type CRP shows a deviation from linearity indicative of negative cooperativity. Takahashi et al. (19, 23) found that at low salt CRP bound cAMP with negative cooperativity; as the salt concentration was increased the cooperativity became progressively positive. The binding mixture used in the present study contains 0.4 M KCl where cooperativity was not observed by equilibrium dialysis (19). The value of 1.9 × 10⁴ M⁻¹ for the intrinsic affinity constant, K, for binding of cAMP by CRP (Table I) is comparable to that obtained by Takahashi et al. (19): K = 3.9 × 10⁴ M⁻¹. The data obtained for CRP*598 indicate a higher affinity for cAMP, K = 3.8 × 10⁵ M⁻¹. In addition, the strong positive cooperativity found for the binding of cAMP by CRP*598 is in distinct contrast with the binding properties of wild type CRP.

Table I. Parameters for binding of cAMP and cGMP to CRP and CRP*598

<table>
<thead>
<tr>
<th>Method</th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
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<tbody>
<tr>
<td><strong>Equilibrium Dialysis</strong></td>
<td></td>
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</tr>
<tr>
<td>CRP</td>
<td>(1.9 ± 0.23) × 10⁴ M⁻¹</td>
<td>(2.9 ± 0.13) × 10⁴ M⁻¹</td>
</tr>
<tr>
<td>CRP*598</td>
<td>(3.8 ± 0.56) × 10⁵ M⁻¹</td>
<td>(2.7 ± 0.35) × 10⁵ M⁻¹</td>
</tr>
<tr>
<td><strong>Ammonium Sulfate Method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>(6.8 ± 0.53) × 10⁵ M⁻¹</td>
<td>(1.7 ± 0.13) × 10⁴ M⁻¹</td>
</tr>
<tr>
<td>CRP*598</td>
<td>(8.4 ± 1.2) × 10⁵ M⁻¹</td>
<td>(1.9 ± 0.13) × 10⁵ M⁻¹</td>
</tr>
</tbody>
</table>

The intrinsic association constant K and the cooperativity parameter α were determined by the method of Takahashi et al. (19). The Hill coefficient nH was calculated using the EZ-FIT program (30).

Donoso-Pardo et al. (26) have presented evidence indicating that the addition of the ammonium sulfate solution does not markedly disturb the binding equilibrium. In this study we have found using ammonium sulfate precipitation indicate that the high ionic strength and aggregation alter the binding properties of CRP. The apparent affinity for cAMP is increased to a value similar to that observed with CRP*598. This increased affinity for cAMP by CRP is not paralleled by a concomitant change in cooperativity. Under the same assay conditions the binding of cAMP by CRP*598 shows strong positive cooperativity.
The differential response seen for cAMP binding by CRP and CRP*598 is not observed for cGMP binding. Binding of cGMP by both forms of the protein showed negative cooperativity and similar values for $K$ were obtained (Figure 3) using either equilibrium dialysis or ammonium sulfate precipitation. The results obtained for the binding of cAMP and cGMP by CRP and CRP*598 are summarized in Table I.

It has been reported that high concentrations of adenosine were able to support in vitro transcription from the lac $P^+$ promoter by another CRP* (16). The binding of cGMP to CRP or CRP*598 is similar. This provides a convenient assay for a comparison of the ability of cAMP analogues to bind to CRP and CRP*598. The data presented in Figure 4 and Figure 5 show the results of assays for the ability of cAMP and adenosine to displace cGMP from CRP and CRP*598. The results indicate that displacement of cGMP from CRP*598 occurs at lower concentrations of cAMP than are required for CRP (Figure 4). In a similar experiment using adenosine as the competitor the results indicate a much greater differential affinity of adenosine for CRP*598 versus CRP. The results establish the importance of the adenine moiety for the apparent enhanced affinity for CRP*598.

The binding properties of a series of cAMP analogues for CRP and CRP*598 are shown in Table II. The only ligands which show a greater apparent affinity for CRP*598 relative to CRP are cAMP and adenosine. Vaney et al. (27) showed that 1 mM adenosine can support in vitro lac transcription by another CRP* mutant, CAP91. The differential binding is not a function of the intrinsic affinity of the ligand for CRP or CRP*598 since 8-bromo cAMP, 8-methylamino cAMP and N$^6$-butyryl cAMP show apparent affinities which are comparable to that observed for cAMP. It is of interest that N$^6$-butyryl cAMP which has been shown to elicit a conformational change in CRP (11) does not show any differential affinity for CRP*598.

**DISCUSSION**

A series of CRP* mutants have been described which are able to function in *E. coli* lacking adenylate cyclase (12-16). Another
approximately 11% of that required by CRP. The properties to protease attack in the absence of cAMP. The CRP which conformation than that seen for CRP. Unliganded CRP is resistant with the protease used (10). In contrast CRP* forms are sensitive the resultant formation of an Nominal core whose length varies described for CRP* (17) and other CRP* forms (13-16) the concentration of cAMP required by CRP*598 is effectively supports abortive initiation from the P lac + promoter. The CRP*598 mutation results in amino acid replacements in position 6 of the CRP dimer while cAMP has an opposite effect on dimer stability. CRP is able to tolerate large substitutions at the N6 position of cAMP without adverse effect on either binding or conformation. Ebright et al. (11) identified several analogues, including N6-butyryl cAMP, that are able to elicit conformational change in CRP but are unable to activate transcription. CRP*598 is able to bind N6-butyryl cAMP with an apparent affinity similar to that observed for cAMP. However this analogue does not show the enhanced binding to CRP*598 shown by cAMP and adenosine. Furthermore, N6-butyryl cAMP does not support abortive transcription from the lac P + promoter under conditions where CRP*598 is stimulated by cAMP (data not shown). Enhanced binding of cAMP and adenosine by CRP*598 requires the unsubstituted N6 position of the adenine moiety. The N6 of cAMP interacts with both subunits of CRP (6). Ebright et al. (11) have proposed that there must be an event taking place in proximity to the N6 atom of cAMP which is required for CRP (or CRP*598) to bind to DNA. The CRP*598 mutation results in altered amino acid replacements in the D α helix close to the hinge connecting the large and small domains of the subunit. The CRP*598 mutant shows an altered conformation, activates transcription at high CRP* concentration in the absence of cAMP, activates transcription at low CRP* concentration in the presence of a much lower cAMP concentration then required by CRP, and binds to lac P + DNA in the absence of cAMP in a RNA polymerase-dependent mode (17). The property of positive cooperativity for the binding of cAMP by CRP*598 can be added to this list. The mutations in CRP*598 lie close to the hinge region and relatively far from

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**Table II. Displacement of [3H]cGMP binding by cyclic nucleotides with CRP or CRP*598.**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Concentration of cAMP resulting in 50% inhibition of [3H]cGMP binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>μM</td>
</tr>
<tr>
<td>adenine</td>
<td>CRP 3.2 CRP*598 1.5</td>
</tr>
<tr>
<td>8-bromo cAMP</td>
<td>CRP 3.2 CRP*598 4.2</td>
</tr>
<tr>
<td>8-methylamino cAMP</td>
<td>CRP 2.8 CRP*598 2.4</td>
</tr>
<tr>
<td>N6-butyryl cAMP</td>
<td>CRP 2.8 CRP*598 2.4</td>
</tr>
<tr>
<td>N6,O2-dibutyryl cAMP</td>
<td>CRP 42 CRP*598 56</td>
</tr>
<tr>
<td>cGMP</td>
<td>CRP 63 CRP*598 63</td>
</tr>
<tr>
<td>cAMP</td>
<td>CRP 23 CRP*598 28</td>
</tr>
<tr>
<td>2′-deoxy cAMP</td>
<td>NE CRP*598 NE</td>
</tr>
<tr>
<td>2′-deoxy cGMP</td>
<td>NE CRP*598 NE</td>
</tr>
<tr>
<td>5′ AMP</td>
<td>NE CRP*598 NE</td>
</tr>
<tr>
<td>ATP</td>
<td>NE CRP*598 NE</td>
</tr>
</tbody>
</table>

The conditions used for the assays are presented in Materials and Methods. The concentration required to give the 50% displacement was determined graphically. NE: no effect seen at the highest concentration used (20 mM).

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Figure 5. Displacement of [3H]cGMP binding by adenosine with CRP or CRP*598. The incubation conditions are those presented in Materials and Methods using 2x 10^{-8} M [3H]cGMP and the adenosine concentrations indicated in the Figure. CRP, •-•; CRP*598, O-O.
the cAMP binding site. It is clear that there is an effect on the conformation of the CRP*598 C-terminal domain based on its sensitivity to protease cleavage in the absence of cAMP. Binding of cAMP has been proposed to alter the conformation of CRP by altering the intersubunit contacts between the two large C± helices and also by affecting interdomain contacts (6,7). The positive cooperativity observed for binding of cAMP by CRP*598 may be a consequence of effects modulated by altered subunit contacts and/or interdomain contacts.

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