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

Yun Ling Ren, Susan Garges[1], Sankar Adhya[1] and Joseph S. Krakow*
Department of Biological Sciences, Hunter College of CUNY, New York, NY 10021 and [1]Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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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, 1.9×10^4 M^{-1}; CRP*598, 3.8×10^5 M^{-1}. The *K* values obtained for cGMP binding are: CRP, 2.9×10^4 M^{-1}; CRP*598, 2.7×10^4 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^6 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 α 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 evince 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)

* To whom correspondence should be addressed

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 [^{3}H]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 Eilen *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: $A^{1\%}_{278nm}$ = 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: $E^{1\%}_{280nm}$ = 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 $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, the indicated concentration of cAMP or cGMP, 2 nM *lac* P$^+$ DNA fragment and 40 nM 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 [^{3}H]UTP (9200 cpm/pmol) were added. The reaction was allowed to proceed for 15 minutes at 37°C when it 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 [^{3}H]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 [^{3}H]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. [^{3}H]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.

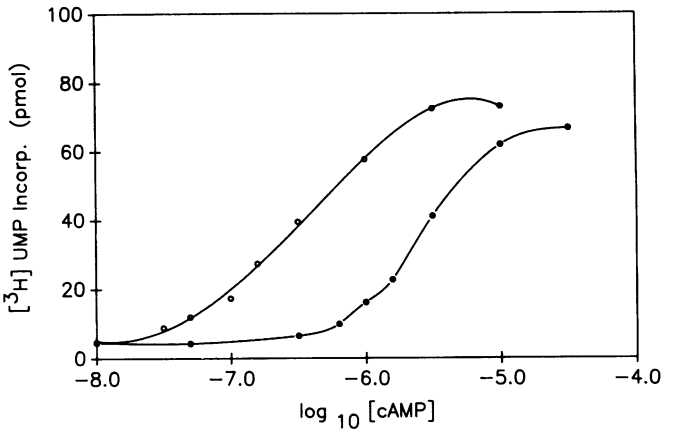

Figure 1. Effect of cAMP concentration on the ability of CRP and CRP*598 to support *lac* P$^+$-directed abortive initiation. Conditions for the abortive initiation reaction are described in Materials and Methods. Incorporation of [^{3}H]UMP in the absence of CRP or CRP*598 was 4 pmol. CRP, ●−●; CRP*598, ○−○.

(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 [^{3}H]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 [^{3}H]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 [^{3}H]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 H_2O and the radioactivity determined in Ecolume. The nucleotide concentrations were varied as follows: cAMP, N^6-butyryl cAMP, 8-bromo cAMP, 8-ethylamino cAMP, N^6-$O^{2'}$-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.

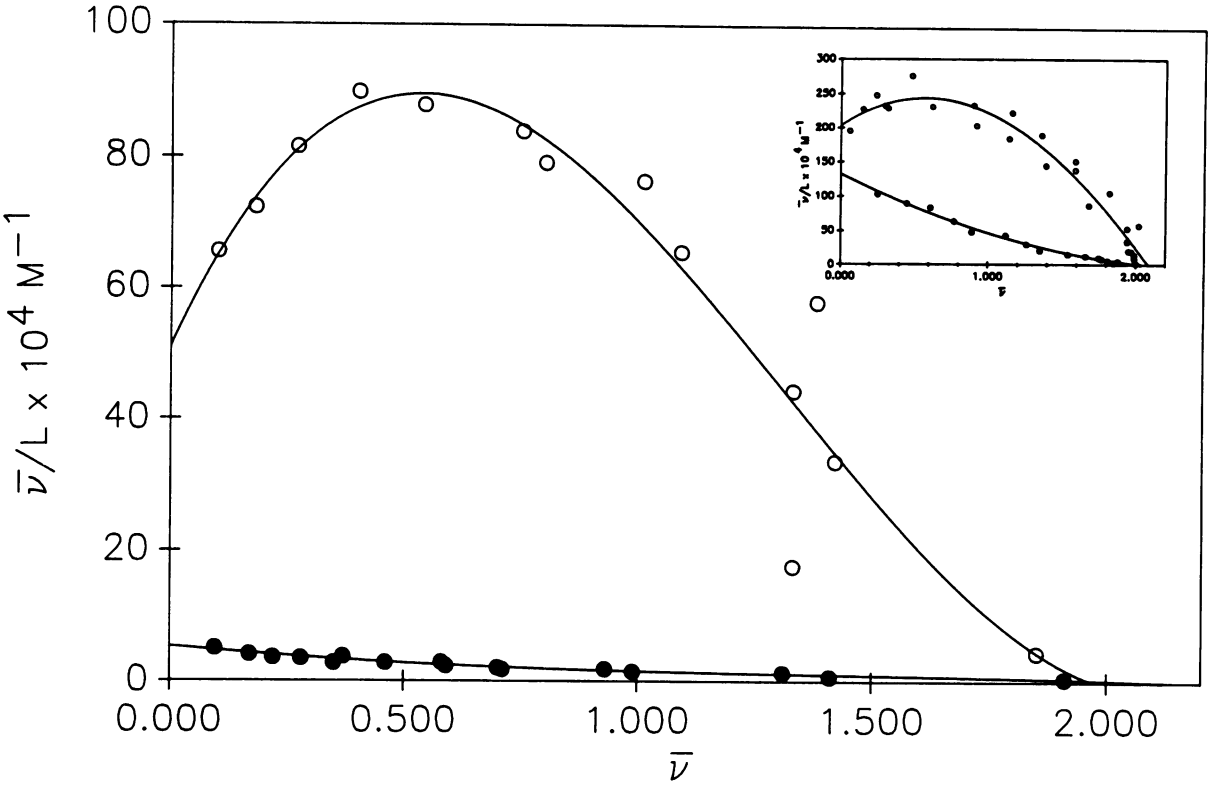

Figure 2. Scatchard representation of the binding of cAMP to CRP and CRP*598 determined by equilibrium dialysis and ammonium sulfate precipitation. The conditions used for equilibrium dialysis are presented in Materials and Methods with 4 μM CRP or CRP*598; the insert graph shows the results obtained 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 $1.9\times10^4\ M^{-1}$ for the intrinsic affinity constant, K, for binding of cAMP by CRP (Table I) is comparable to that obtained by Takahashi *et al.* (19): $K = 3.9\times10^4\ M^{-1}$. The data obtained for CRP*598 indicate a higher affinity for cAMP, $K = 3.8\times10^5\ M^{-1}$. 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

	K	α	n_H
Equilibrium Dialysis Method			
cAMP			
CRP	$(1.9 \pm 0.23)\times10^4\ M^{-1}$	0.84 ± 0.22	0.82 ± 0.07
CRP*598	$(3.8 \pm 0.56)\times10^5\ M^{-1}$	3.74 ± 1.11	1.94 ± 0.26
cGMP			
CRP	$(2.9 \pm 0.13)\times10^4\ M^{-1}$	0.61 ± 0.13	0.97 ± 0.02
CRP*598	$(2.7 \pm 0.35)\times10^4\ M^{-1}$	1.00 ± 0.25	0.94 ± 0.01
Ammonium Sulfate Method			
cAMP			
CRP	$(6.8 \pm 0.53)\times10^5\ M^{-1}$	0.45 ± 0.06	0.9 ± 0.06
CRP*598	$(8.4 \pm 1.2)\times10^5\ M^{-1}$	6.9 ± 1.9	1.4 ± 0.08
cGMP			
CRP	$(1.7 \pm 0.13)\times10^4\ M^{-1}$	0.45 ± 0.06	0.71 ± 0.07
CRP*598	$(1.9 \pm 0.13)\times10^4\ M^{-1}$	0.32 ± 0.04	0.79 ± 0.06

The intrinsic association constant K and the cooperativity parameter α were determined by the method of Takahashi *et al.* (19). The Hill coefficient n_H 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.

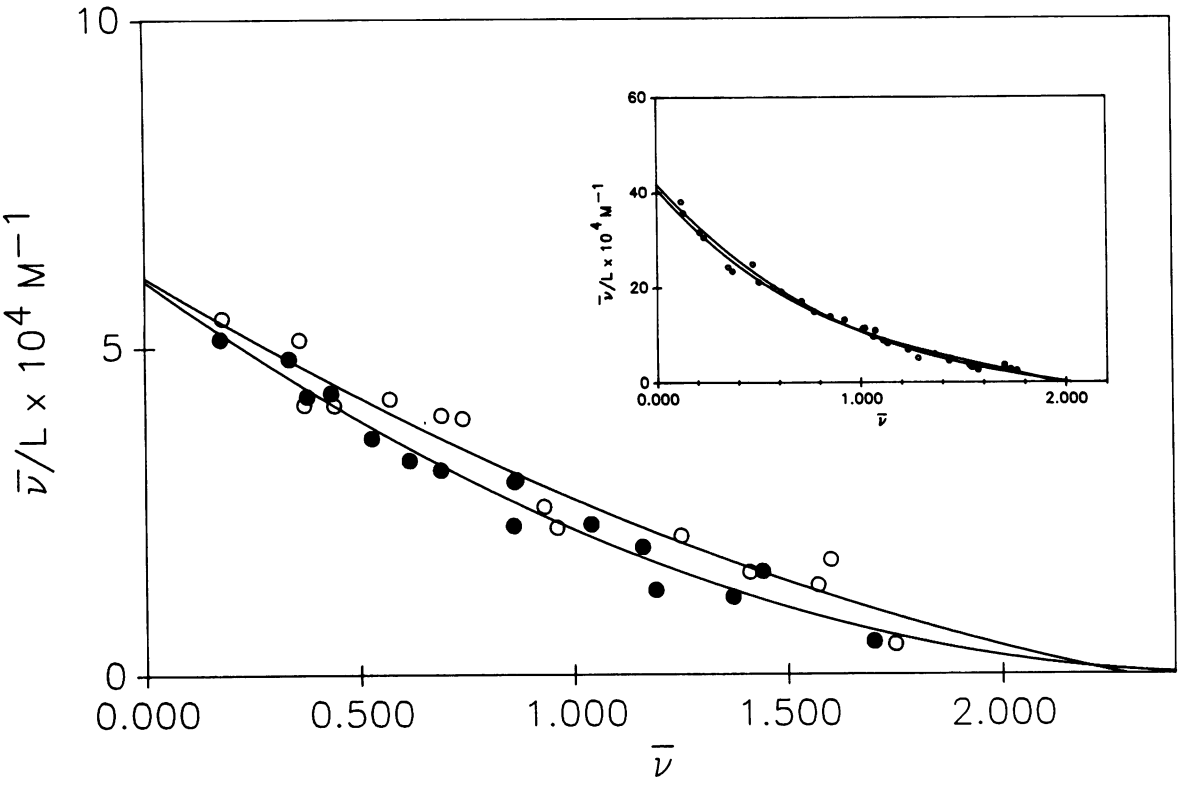

Figure 3. Scatchard representation of the binding of cGMP to CRP and CRP*598 as determined by equilibrium dialysis and ammonium sulfate precipitation. The conditions used for equilibrium dialysis are presented in Materials and Methods with 6 μM CRP or CRP*598; the insert graph shows the results obtained using the ammonium sulfate precipitation method. The conditions are presented in Materials and Methods with 2 μM CRP or CRP*598. CRP, ●–●; CRP*598, ○–○.

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

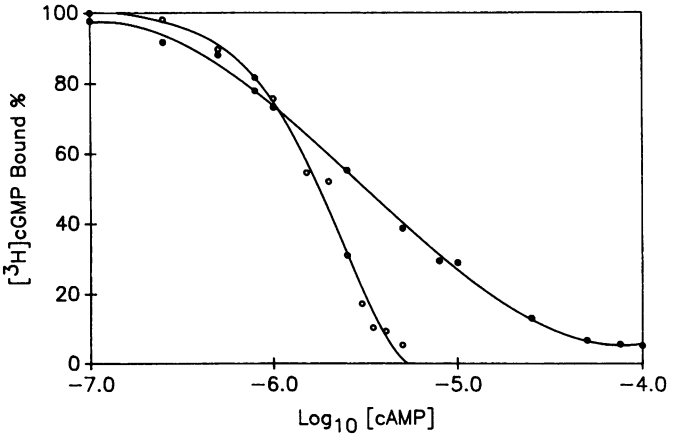

Figure 4. Displacement of $[^3H]$cGMP binding by cAMP with CRP or CRP*598. The incubation conditions are those presented in Materials and Methods using 2×10^{-6} M $[^3H]$cGMP and the cAMP concentrations indicated in the Figure. CRP, ●–●; CRP*598, ○–○.

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

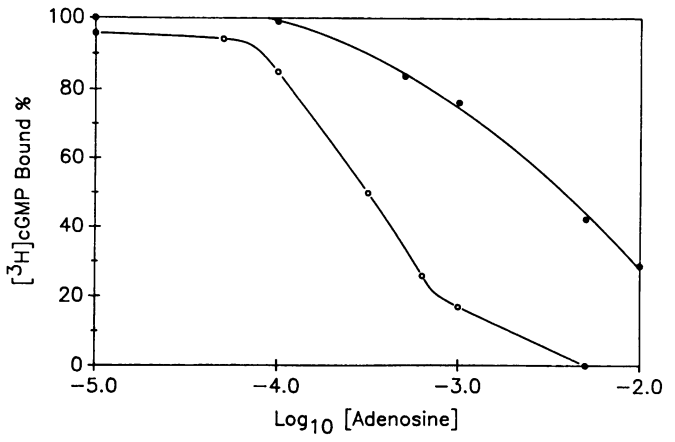

Figure 5. Displacement of [^{3}H]cGMP binding by adenosine with CRP or CRP*598. The incubation conditions are those presented in Materials and Methods using 2×10^{-6} M [^{3}H]cGMP and the adenosine concentrations indicated in the Figure. CRP, ●−●; CRP*598, ○−○.

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

	Concentration of cNMP resulting in 50% inhibition of [^{3}H]cGMP binding	
Competitor	CRP	CRP*598
		μM
cAMP	3.2	1.5
adenosine	3300	320
8-bromo cAMP	3.2	4.2
8-methylamino cAMP	2.8	2.4
N^6-butyryl cAMP	2.8	2.4
N^6,$O^{2'}$-dibutyryl cAMP	42	56
cIMP	63	63
cCMP	23	28
2'-deoxy cAMP	NE	NE
2'-deoxy cGMP	NE	NE
5' AMP	NE	NE
ATP	NE	NE

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).

property of such mutants is that the addition of exogenous cAMP or cGMP further stimulates the utilization of a variety of sugars. Subsequent characterization of the biochemical properties of the CRP* proteins demonstrated *in vitro* responses comparable to those seen *in vivo*. High concentrations of the CRP*598 are able to support *lac* P^+ transcription in the absence of cyclic nucleotide. At protein concentrations where cAMP-CRP effectively supports abortive initiation from the *lac* P^+ promoter the concentration of cAMP required by CRP*598 is approximately 11% of that required by CRP. The properties described for CRP*598 (17) and other CRP* forms (13−16) indicate that this class of protein has an intrinsically different conformation than that seen for CRP. Unliganded CRP is resistant to attack by a variety of proteases, cAMP-CRP is attacked with the resultant formation of an N-terminal core whose length varies with the protease used (10). In contrast CRP* forms are sensitive to protease attack in the absence of cAMP. The CRP*598 which functions in the absence of cAMP or in the presence of low concentrations of cAMP appears to already exist in a more open conformation than that evinced by cAMP-bound CRP.

The available data do not allow one to distinguish whether the ability of CRP* to support transcription at low cAMP levels is directly attributable to an inherent property of the mutated protein or whether this is due to a secondary consequence of the interaction of CRP* with its site on the *lac* P^+ promoter and/or contact with RNA polymerase. The data presented in this study demonstrate that CRP* differs from CRP in its mode of cAMP binding. Binding of cAMP by CRP has been shown by Takahashi *et al.* (19) to vary from negative to positive cooperativity depending on the ionic strength of the binding buffer. Under the conditions that we have used the binding of cAMP by CRP is negatively cooperative. In contrast, the binding of cAMP by CRP*598 shows strong positive cooperativity. Binding of cGMP by CRP and CRP*598 is virtually identical showing negative cooperativity and comparable association constants. Takahashi *et al.* (19) found that the binding of CRP to double stranded DNA resulted in an increased affinity for cAMP together with the reversal of cooperativity from negative (in the absence of DNA) to positive. In a detailed study on the effect of DNA on cAMP binding by CRP, Takahashi *et al.* (25) showed that the affinity of CRP for cAMP increases with NaCl concentration between 0.01 M and 0.2 M while cooperativity progressively changes from positive to negative. It appears likely that the CRP* mutant is shifting the equilibrium to favor the CRP conformation active in DNA binding away from that of the unliganded CRP. CRP bound to DNA at 10 mM NaCl becomes sensitive to trypsin attack with the formation of a N-terminal 9.7 kD fragment (28). Tryptic attack on CRP*598 results in an apparently identical fragment (17) suggesting that the CRP* dimer may be inherently unstable, in the same way CRP-DNA is unstable. Brown and Crothers (29) found that under low ionic strength conditions DNA destabilizes the CRP dimer while cAMP has an opposite effect on dimer stability.

CRP is able to tolerate large substitutions at the N^6 position of cAMP without adverse effect on either binding or conformation. Ebright *et al.* (11) identified several analogues, including N^6-butyryl cAMP, that are able to elicit conformational change in CRP but are unable to activate transcription. CRP*598 is able to bind N^6-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, N^6-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 N^6 position of the adenine moiety. The N^6 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 N^6 atom of cAMP which is required for CRP (or CRP*598) to bind to DNA.

The CRP*598 mutation results in 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

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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