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Cooperative DNA binding of heterologous proteins: Evidence for contact between the cyclic AMP receptor protein and RNA polymerase

(crp/Escherichia coli)*

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ABSTRACT **Four cAMP-independent receptor protein mutants (designated CRP* mutants) isolated previously are able to activate *in vivo* gene transcription in the absence of cAMP and their activity can be enhanced by cAMP or cGMP. One of the four mutant proteins, CRP*598 (Arg-142 to His, Ala-144 to Thr), has been characterized with regard to its conformational properties and ability to bind to and support abortive initiation from the *lac* promoter. In the absence of cGMP, CRP*598 shows a more open conformation than CRP, as indicated by its sensitivity to proteolytic attack and 5,5′-dithiobis(2-nitrobenzoic acid)-mediated subunit crosslinking. Binding of wild-type CRP to its site on the *lac* promoter and activation of abortive initiation by RNA polymerase on this promoter are effected by cAMP but not by cGMP. CRP*598 can activate *lacP*$^+$-directed abortive initiation in the presence of cAMP and less efficiently in the presence of cGMP or in the absence of cyclic nucleotide. DNase I protection ("footprinting") indicates that cAMP-CRP* binds to its site on the *lac* promoter whereas unliganded CRP* and cGMP-CRP* form a stable complex with the [^{32}P]*lacP*$^+$ fragment only in the presence of RNA polymerase, showing cooperative binding of two heterologous proteins. This cooperative binding provides strong evidence for a contact between CRP and RNA polymerase for activation of transcription. Although cGMP binds to CRP, it cannot replace cAMP in effecting the requisite conformational transition necessary for site-specific promoter binding. In contrast, the weakly active unliganded CRP*598 can be shifted to a functional state not only by cAMP but also by cGMP and RNA polymerase.**

The cAMP receptor protein (CRP) acts to modulate the expression of a large number of *Escherichia coli* genes (1–3). Binding of cAMP invokes a conformational change in CRP with a resultant increase in the affinity and specificity for specific promoter-associated sites; unliganded CRP binds nonspecifically to DNA. CRP is composed of two identical 23,619-Da subunits (4–6). The CRP monomer has a two-domain structure in which the large N-terminal domain is responsible for cAMP binding and subunit–subunit interaction and the smaller C-terminal domain is involved in DNA binding (7, 8). Various lines of evidence indicate that CRP undergoes a conformational transition on binding cAMP (7, 9–13). The conformational change elicited by cAMP binding minimally involves an alteration in the relative orientation of the large and small CRP domains. A hinge region lying between the C α-helix of the large domain and the D α-helix of the small domain connects the two domains of the CRP subunits (8). A class of *crp* mutations in *E. coli* (designated *crp**) has been shown to allow the product, CRP*, to function in the absence of endogenous cAMP (14–20). *In vitro* studies have shown that CRP* can support transcription from the *lacP*$^+$ promoter in the presence of cAMP and cGMP or in the absence of added cyclic nucleotide (16, 19). CRP* is also susceptible to protease cleavage in the absence of cAMP, indicating that its conformation differs from that of wild-type CRP (16, 19, 20).

In this study we have investigated the biochemical properties of one of the CRP* mutants. Garges and Adhya (18) proposed that CRP*598 assumes a conformation normally evoked only on binding of cAMP—one in which the relative orientation of three α-helices (C, D, and F) in both domains is altered. The experiments presented in this study demonstrate that the conformation of the CRP*598 differs from that of CRP in the absence of cAMP. Although CRP*598 can activate *in vitro lac* transcription in the presence of cAMP or cGMP as well as in the absence of added cyclic nucleotide, stable binding to the CRP site in *lacP*$^+$ by unliganded CRP* or cGMP-CRP* is seen only in the presence of RNA polymerase.

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: cAMP, cGMP, adenylyl(3′-5′)adenosine (ApA), bovine serum albumin, proteases, calf thymus DNA, and DNase I, Sigma; [^{3}H]cAMP, [^{3}H]UTP, and [^{32}P]dATP, ICN; DNA polymerase I Klenow fragment and *Eco*RI and *Pvu* II restriction endonucleases, Boehringer Mannheim; polymin P, Gallard Schlessinger (Carle Place, NY); Sephacryl 200, Pharmacia; Bio-Rex 70, *N,N,N′,N′*-tetramethylethylenediamine, and bisacrylamide, Bio-Rad; acrylamide, Serva (Heidelberg); and Scintisol, Isolab (Akron, OH).

Proteins. By using the method of Eilen and Krakow (12) CRP*598 (18) was purified from an *E. coli* strain containing the recombinant plasmid pZ598 and wild-type CRP was isolated from an *E. coli* strain containing the recombinant plasmid pHA7 (5) donated by H. Aiba (University of Tsukuba, Ibaraki, Japan). RNA polymerase was isolated from *E. coli* K-12 by a modification of the method of Burgess and Jendrisak (21). Protein concentrations were determined by using the following extinction coefficients: CRP, $A_{280}^{1\%} = 8.8$ (22); RNA polymerase holoenzyme, $A_{280}^{1\%} = 6.7$ (23).

DNA Fragments. *E. coli* containing the *lacP*$^+$ promoter cloned into pMB9 by S. Fuller was obtained from A. Revzin (Michigan State University, East Lansing, MI). Plasmid DNA was prepared by a modification of the method of Marko *et al.* (24). The 203-base-pair (bp) fragment containing the *lacP*$^+$ promoter was excised by digestion with *Eco*RI and

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Abbreviations: CRP, cAMP receptor protein; CRP*, cAMP-independent receptor protein.

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purified by polyacrylamide gel electrophoresis. DNA concentrations were determined fluorometrically using ethidium bromide by the method of Le Pecq and Paoletti (25).

Preparation of Labeled *lacP*$^+$ DNA. The reaction mixture contained (final volume, 50 μl) 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 100 μCi of [^{32}P]dATP (600 mCi/mmol; 1 Ci = 37 GBq), 4 μg of *lacP*$^+$ DNA fragment, and 5 units of DNA polymerase I Klenow fragment. After 15 min at room temperature, 10 μl of 0.65 mM dATP was added and the incubation was continued for an additional 15 min. The reaction was terminated by addition of 200 μl of a solution that contained 3 M ammonium acetate and 30 mM EDTA. The labeled DNA was precipitated by addition of 2 vol of 95% ethanol and the [^{32}P]*lac* DNA was dissolved in 50 μl of 10 mM Tris·HCl, pH 8.0/50 mM NaCl/10 mM $MgCl_2$/1 mM dithiothreitol. The labeled fragment was then restricted with 35 units of *Pvu* II to cut the DNA at −123, yielding promoter fragments uniquely labeled on the upper strand. The restricted [^{32}P]*lacP*$^+$ DNA was precipitated with ethanol and dissolved in 140 μl of TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA).

Abortive Initiation Assay. A modification of the abortive initiation assay of Malan *et al.* (26) was used to determine the effect of CRP* on CRP-dependent transcription from the *lac* promoter. 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 *lacP*$^+$ DNA fragment, and 40 nM RNA polymerase holoenzyme to which was added CRP or CRP* at the concentrations indicated. After incubation at 37°C for 10 min, 0.5 mM ApA and 50 nM [^{3}H]UTP (200 cpm/pmol) were added. The reaction was allowed to proceed for 15 min at 37°C and then was terminated by addition of 10 μl of 0.5 M EDTA. The radioactive products were resolved by paper chromatography in WASP solvent (27). After cutting the chromatography strip into 1-cm segments, the amount of ApApUpU synthesized was determined by assaying appropriate segments in Scintisol.

DNase I Protection ("Footprinting"). DNase I footprinting was carried out by using incubation conditions similar to those used for the transcription assay. The standard binding 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 (glycerol was added because it gave clearer protection patterns; to be consistent, this concentration of glycerol was also included in the abortive initiation assay), 3 nM [^{32}P]*lacP*$^+$ fragment, 120 nM RNA polymerase holoenzyme, and the indicated concentrations of cAMP or cGMP and CRP or CRP*. After formation of the complexes for 10 min at 37°C, 1 μl of a solution containing 20 ng of DNase I per ml in 20 mM potassium phosphate, pH 6.8/1 mM EDTA/50% glycerol was added and incubated for 30 sec at 37°C. The reaction was terminated by addition of a solution containing 3.1 M ammonium acetate (pH 7.6), 25 mM EDTA, and 63 μg of tRNA per ml; this was followed by phenol extraction, ethanol precipitation, and reprecipitation. After drying the pellets under vacuum, 10 μl of loading buffer containing 80% deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol was added. The resuspended samples were loaded on an 8% denaturing sequencing gel according to Maxam and Gilbert (28). After electrophoresis the gel was autoradiographed at −70°C using Kodak XAR-5 film and a Cronex H-Plus intensifying screen.

[^{3}H]cAMP Binding. Binding of [^{3}H]cAMP to CRP or CRP* was assayed by the method of Krakow and Pastan (7).

Proteolytic Cleavage of CRP and CRP*598. Mixtures contained (final volume, 50 μl) 10 mM Tris·HCl (pH 8.0), 40 mM NaCl, 7.5 μg of CRP or CRP*598, and 0.5 mM cAMP or cGMP as indicated. Following addition of the indicated protease, the mixtures were incubated at 37°C for the times specified. The reactions were terminated by addition of 2.5 μl of 20 mM phenylmethylsulfonyl fluoride. The resultant cleavage products were resolved by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (29) on a 15% polyacrylamide gel with a 4.75% stacking gel.

Intersubunit Crosslinking. Mixtures contained (final volume, 50 μl) 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 8.0), 0.01 mM 5,5′-dithiobis(2-nitrobenzoic acid), 10 μg of CRP or CRP*, and 0.1 mM cAMP or cGMP as indicated. The mixtures were incubated for 15 min at 30°C. After the addition of 0.1 mM *N*-ethylmaleimide, aliquots were added to sample buffer lacking mercaptoethanol and heated at 100°C for 2 min prior to electrophoresis.

RESULTS

Activation of abortive initiation by RNA polymerase with the *lacP*$^+$ promoter requires CRP complexed with cAMP (Table 1). In the absence of added cyclic nucleotide or in the presence of cGMP, wild-type CRP does not affect the basal level of ApApUpU synthesized in the absence of CRP. In the presence of cAMP, maximal levels of abortive synthesis are effected by 40 nM CRP and 40 nM CRP*598. The amount of ApApUpU synthesized decreased in the reaction mixtures containing cAMP and 400 nM CRP or 400 nM CRP*598. It is possible that this effect seen with the higher cAMP-CRP or cAMP-CRP* concentration used may be due to binding to the low-affinity CRP site 2, which overlaps the *lac* operator with a consequent inhibition of promoter binding by RNA polymerase. In the complete absence of cAMP, CRP*598 was able to effect a low-level activation of *lac* expression *in vivo* (18). The data presented in Table 1 show that unliganded CRP*598 at a low (40 nM) and a high concentration (400 nM) is able to support the abortive initiation reaction. In the presence of cGMP, a further stimulation of CRP*598 activity is obtained. At a concentration of 400 nM CRP*598, abortive synthesis in the absence of cyclic nucleotide or in the presence of cGMP approaches that of the maximal level seen in the presence of cAMP. The results demonstrate that CRP*598 differs from CRP in its ability to function in the unliganded state or as a cGMP-CRP*598 complex.

DNase I footprinting can provide a direct visualization of the interaction of CRP and CRP*598 with the CRP binding site in the *lacP*$^+$ promoter. CRP*598 and CRP bind to the −80 to −50 segment of the [^{32}P]*lacP*$^+$ fragment in the presence of 100 μM cAMP (Fig. 1, lanes c and g). The characteristic open promoter pattern is obtained with cAMP-CRP and cAMP-CRP*598 on the further addition of RNA polymerase (Fig. 1, lanes d and h). Unliganded CRP at a concentration of 400 nM does not show binding to the [^{32}P]*lacP*$^+$ fragment even in the presence of RNA polymerase (Fig. 1, lane f) or in the presence of cGMP and RNA polymerase (data not shown).

Table 1. Effect of cyclic nucleotides on CRP and CRP* in *lacP*$^+$-directed abortive initiation

	[^{3}H]UMP incorporated,* pmol	
CRP	40 nM	400 nM
CRP*598	46	128
+ cGMP	87	153
+ cAMP	163	115
CRP	14	11
+ cGMP	13	11
+ cAMP	180	166

*[^{3}H]UMP incorporated in the presence of CRP or CRP*598 at the indicated concentrations. Incorporation of [^{3}H]UMP in the absence of CRP or CRP*598 was 13 pmol.

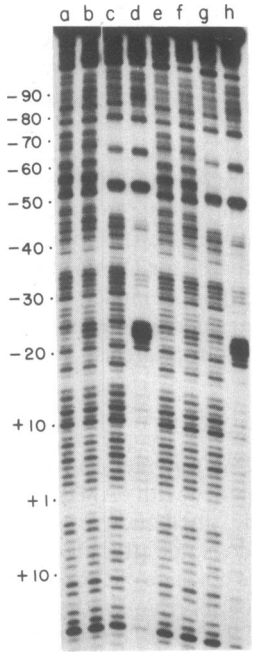

FIG. 1. Binding of CRP and CRP*598 to $lacP^+$ in the presence of cAMP. Three nanomolar [^{32}P]-$lacP^+$ fragment was used, and, where indicated, 120 nM RNA polymerase, 100 μM cAMP, and the specified concentration of CRP or CRP*598 were used. Lane a, [^{32}P]$lacP^+$; lane b, [^{32}P]$lacP^+$ + RNA polymerase; lane c, [^{32}P]-$lacP^+$ + cAMP-CRP*598 (40 nM); lane d, [^{32}P]$lacP^+$ + cAMP-CRP*598 (40 nM) + RNA polymerase; lane e, [^{32}P]$lacP^+$ + CRP (400 nM); lane f, [^{32}P]$lacP^+$ + CRP (400 nM) + RNA polymerase; lane g, [^{32}P]$lacP^+$ + cAMP-CRP (40 nM); lane h, [^{32}P]$lacP^+$ + cAMP-CRP (40 nM) + RNA polymerase.

The data presented in Table 1 indicate that CRP*598 supports abortive initiation in the absence of cAMP or cGMP. The footprint data presented in Fig. 2 indicate that CRP*598 at a concentration of 320 nM does not form a stable complex with the [^{32}P]$lacP^+$ fragment in the absence of cyclic nucleotide (Fig. 2, lane h) or in the presence of cGMP (Fig. 2, lane c). In the presence of cAMP the characteristic

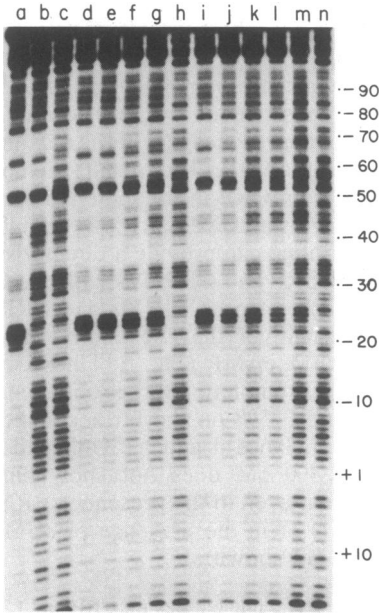

FIG. 2. Effect of CRP*598 concentration on promoter open complex formation in the absence of cAMP. Three nanomolar [^{32}P]$lacP^+$ fragment was used, and, where indicated, 120 nM RNA polymerase, 1 mM cGMP or 0.1 mM cAMP, and the specified concentration of CRP*598 were used. Lane a, [^{32}P]$lacP^+$ + cAMP-CRP*598 (40 nM) + RNA polymerase; lane b, [^{32}P]$lacP^+$ + cAMP-CRP*598 (40 nM); lane c, [^{32}P]$lacP^+$ + cGMP-CRP*598 (320 nM); lane d, [^{32}P]$lacP^+$ + RNA polymerase + cGMP-CRP*598 (320 nmol); lane e, [^{32}P]$lacP^+$ + RNA polymerase + cGMP-CRP*598 (160 nmol); lane f, [^{32}P]$lacP^+$ + RNA polymerase + cGMP-CRP*598 (80 nmol); lane g, [^{32}P]$lacP^+$ + RNA polymerase + cGMP-CRP*598 (40 nmol); lanes h–l, identical to lanes c–g except that cGMP is absent; lane m, [^{32}P]$lacP^+$ + RNA polymerase; lane n, [^{32}P]$lacP^+$.

footprint pattern is effected by CRP*598 (Fig. 2, lane b). As the concentration of CRP*598 is raised from 40 nM to 320 nM, the presence of RNA polymerase stabilizes binding of unliganded CRP*598 to the [^{32}P]$lacP^+$ fragment (Fig. 2, lanes i–l). A similar effect of RNA polymerase in stabilizing binding of cGMP-CRP*598 to the [^{32}P]$lacP^+$ fragment can also be seen (Fig. 2, lanes d–g). cGMP-CRP*598 shows a somewhat higher affinity than does the unliganded CRP*598 for RNA polymerase-mediated [^{32}P]$lacP^+$ fragment binding. In the presence of CRP*598 there is a concomitant stabilization of RNA polymerase binding to its site in the $lacP^+$ promoter.

Previous studies demonstrated that sensitivity to proteolytic attack is a useful approach for assessing the conformation of CRP (9, 13). Unliganded CRP and cGMP-CRP are resistant to a variety of proteases; cAMP-CRP is attacked, generating N-terminal cores that retain cAMP binding activity (9). The data presented in Fig. 3 show that CRP*598 differs markedly from CRP in its sensitivity to proteolytic attack in the absence of cyclic nucleotides and in the presence of cGMP. The protease-resistant fragments generated from cAMP-CRP and cAMP-CRP*598 are identical. cGMP-CRP*598 is attacked by chymotrypsin (Fig. 3, lane b) and subtilisin (Fig. 3, lane f), resulting in the formation of a major core fragment along with a slightly smaller fragment. Unliganded CRP*598 is more sensitive to attack by chymotrypsin (Fig. 3, lane d) and subtilisin (Fig. 3, lane h), and smaller fragments are produced than those formed in the presence of cAMP or cGMP. We suggest that the initial cutting sites are identical to those that are accessible in the cAMP-CRP and cAMP-CRP*598 complexes with additional sites subsequently attacked in cGMP-CRP*598 and more so in unliganded CRP*598. Earlier results showed that the α core formed by subtilisin digestion of cAMP-CRP was in a conformational state that was sensitive to further attack in the absence of cAMP or cGMP (30).

Trypsin digestion of unliganded CRP*598 results in the accumulation of a smaller fragment than that formed after digestion of cGMP-CRP*598, cAMP-CRP*598, or cAMP-CRP (Fig. 4). It is interesting to note that the cAMP-CRP*598 is more resistant to trypsin than the cGMP-CRP*598 complex. Assay for [^{3}H]cAMP binding activity after digestion of unliganded CRP*598 (Fig. 5) shows that the CRP*598 fragment formed does not bind cAMP. A similar loss of cAMP binding activity was found following trypsin digestion of DNA-CRP (31).

In the presence of cAMP, reaction of the two available sulfhydryl groups (Cys-178) with 5,5′-dithiobis(2-nitrobenzoic acid) results in the formation of a disulfide bond

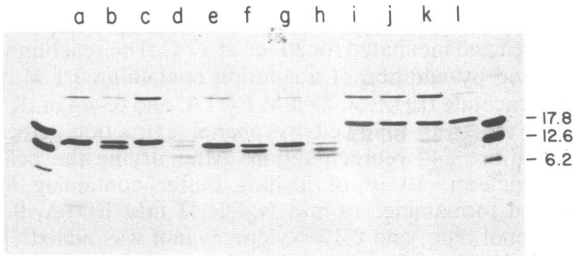

FIG. 3. Sensitivity of CRP*598 to proteolytic attack in the presence and absence of cyclic nucleotide. Lane a, cAMP-CRP + chymotrypsin (0.1 μg); lane b, cGMP-CRP*598 + chymotrypsin; lane c, cAMP-CRP*598 + chymotrypsin; lane d, CRP*598 + chymotrypsin; lane e, cAMP-CRP + subtilisin (0.1 μg); lane f, cGMP-CRP*598 + subtilisin; lane g, cAMP-CRP*598 + subtilisin; lane h, CRP*598 + subtilisin; lane i, cAMP-CRP + *Staphylococcus aureus* V8 protease (0.5 μg); lane j, cGMP-CRP*598 + *S. aureus* V8 protease; lane k, cAMP-CRP*598 + *S. aureus* V8 protease; lane l, CRP*598 + *S. aureus* V8 protease. Molecular mass markers (shown in kDa) are myoglobin, cytochrome *c*, and bovine trypsin inhibitor.

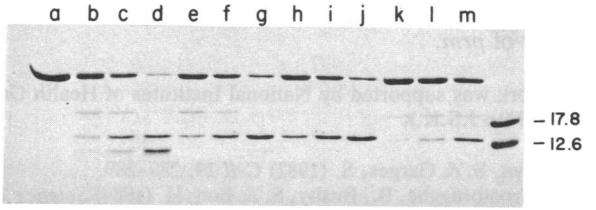

FIG. 4. Time course of trypsin digestion of CRP*598 in the presence and absence of cyclic nucleotide. Lane a, CRP*598; lanes b, c, and d, CRP*598 + trypsin (0.1 μg) incubated at 37°C for 10, 20, and 40 min, respectively; lanes e, f, and g, cGMP-CRP*598 + trypsin (0.1 μg) incubated at 37°C for 10, 20, and 40 min, respectively; lanes h, i, and j, cAMP-CRP*598 + trypsin (0.4 μg) incubated at 37°C for 10, 20, and 40 min, respectively; lanes k, l, and m, cAMP-CRP + trypsin (0.4 μg) incubated at 37°C for 10, 20, and 40 min, respectively. Molecular mass markers (shown in kDa) are myoglobin and cytochrome *c*.

linking the two subunits within the CRP protomer (12). The conformational change in CRP elicited by cAMP binding is required to bring the two cysteine residues into proximity. As shown in Fig. 6, 5,5′-dithiobis(2-nitrobenzoic acid)-mediated formation of the intersubunit disulfide bond occurs in cAMP-CRP (Fig. 6, lane e) but not in cGMP-CRP (lane f) or unliganded CRP (lane g). In contrast, CRP*598 shows significant crosslinking in the absence (lane d) or presence of cGMP (lane c) or cAMP (lane b). The data are consonant with an altered conformation in CRP*598 allowing the Cys-178 present between the E and F helices to approach within a distance required for the 5,5′-dithiobis(2-nitrobenzoic acid)-mediated intersubunit disulfide interchange reaction. Since the crosslinking has been demonstrated to occur within the CRP protomer, the results also indicate that the CRP*598 mutation does not affect the stability of the major subunit contacts in the N-terminal domain of CRP.

DISCUSSION

CRP*598 is a representative of a set of CRP mutants that were predicted to show a destabilization of the interaction between

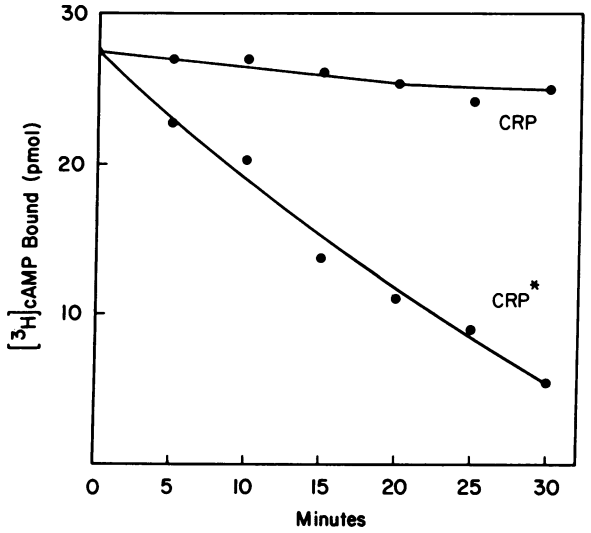

FIG. 5. Loss of cAMP binding activity after trypsin digestion of unliganded CRP*598. Reaction mixtures contained (final volume, 500 μl) 10 mM Tris·HCl (pH 8.0), 40 mM NaCl, 75 μg of CRP or CRP*598, and 1 μg of trypsin. After incubation of 37°C for the times indicated, aliquots (50 μl) were removed and the reaction was terminated by addition of 2.5 μl of 20 mM phenylmethylsulfonyl fluoride. Binding of [^{3}H]cAMP was assayed by using 20 μl of the sample removed at each time point.

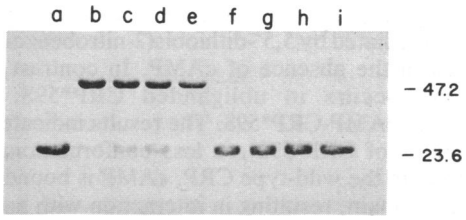

FIG. 6. Effect of 5,5′-dithiobis(2-nitrobenzoic acid) on intersubunit crosslinking of CRP*598 and CRP. Lane a, CRP*598; lane b, cAMP-CRP*598 + 5,5′-dithiobis(2-nitrobenzoic acid); lane c, cGMP-CRP*598 + 5,5′-dithiobis(2-nitrobenzoic acid); lane d, CRP*598 + 5,5′-dithiobis(2-nitrobenzoic acid); lane e, cAMP-CRP + 5,5′-dithiobis(2-nitrobenzoic acid); lane f, cGMP-CRP + 5,5′-dithiobis(2-nitrobenzoic acid); lane g, CRP + 5,5′-dithiobis(2-nitrobenzoic acid); lane h, cAMP-CRP; lane i, CRP. CRP dimer and monomer are indicated in kDa.

the large and small domains of CRP (18). Unliganded CRP is in a conformation that is unfavorable for site-specific DNA binding. A conformational change occurs in cAMP-CRP that can be monitored by an increased susceptibility to proteolytic attack (7, 9). The conformation established in cAMP-CRP enables binding to specific promoter-associated sites and the concomitant activation of transcription from CRP-dependent promoters. The CRP* mutants differ in their ability to activate transcription under conditions where CRP is inactive. CRP*598 can activate *in vivo lac* operon expression in the absence of endogenous cyclic nucleotides and in the presence of cGMP (18). The CRP*598 mutations have been mapped within the hinge region of CRP (18). Based on the chymotrypsin digestion patterns, the substitution in the D α-helix of histidine for arginine at position 142 and threonine for alanine at position 144 in the CRP*598 sequence appears to alter the stability within the N-terminal and C-terminal domains. The results obtained by using CRP*598 are comparable to those described by Harman *et al.* (20) with the related NCR91 CRP*, which has a single amino acid change: Ala-144 to Thr. The structure of the NCR91 CRP* crystallized with cAMP is similar but not identical to that of cAMP-CRP (32). Small changes in the mutant CRP were noted, which include concerted shifts in the small domains, in the hinge joining the two domains, and in an adjacent loop between the β strands 4 and 5. The distortion resulting from the amino acid substitution proximal to the hinge region apparently disturbs the interaction between the two domains in unliganded CRP*598, thereby opening up sites within each domain to attack by proteases. Unliganded CRP is relatively resistant to proteolytic attack. The proteases used generate N-terminal fragments from cAMP-CRP of differing sizes, indicating the presence of resistant folded regions of cAMP-CRP. In contrast, unliganded CRP*598 is sensitive to proteolysis and the limit digests generated under the conditions used in this study result in smaller fragments than those arising from cAMP-CRP or cAMP-CRP*598. The digestion patterns are indicative of the loss of important contacts between the large and small domains in unliganded CRP*598. Based on the protease study, it would appear that the conformations established in cAMP-CRP and cAMP-CRP*598 are similar. The binding of cGMP to CRP is without apparent effect, whereas cGMP-CRP*598 adopts a conformation that is similar to but not identical with that established in cAMP-CRP*598.

A similar conclusion obtains from the results of the 5,5′-dithiobis(2-nitrobenzoic acid)-mediated disulfide crosslinking experiment. Crosslinking of the CRP subunits requires that the small domains adopt a conformation allowing the Cys-178 residues to move within a distance required for the formation of the disulfide bond. The Cys-178 is present in the small linker joining the E and F α-helices present in the

C-terminal domain. In wild-type CRP, the subunit crosslinking reaction mediated by 5,5'-dithiobis(2-nitrobenzoic acid) is not favored in the absence of cAMP. In contrast, subunit crosslinking occurs in unliganded CRP*598, cGMP-CRP*598, and cAMP-CRP*598. The results indicate that the small domains of CRP*598 are less conformationally constrained than in the wild-type CRP. cAMP is bound to a site in the large domain, resulting in interaction with amino acid side chains from both subunits of the CRP dimer. McKay *et al.* (8) stress the important role of the 6-amino group of cAMP in the allosteric activation of CRP. In the cAMP-CRP complex hydrogen bonding is believed to occur to the Thr-127 and the Ser-128 of the other subunit. It was proposed that the binding of cAMP alters the relative orientation of the two subunits, thereby affecting the shape of the DNA binding sites. The absence of a 6-amino group in cGMP accordingly would not allow for the trans-subunit allosteric transition evoked by cAMP binding. The lower protease resistance found for cGMP-CRP*598 may reflect the lack of second subunit contacts by bound cGMP and a consequent lower stability of subunit–subunit interactions.

The data clearly indicate that the conformations of unliganded CRP*598 and cGMP-CRP*598 differ from that established in cAMP-CRP*598. Each of these conformational variants of CRP*598 is able to activate $lacP^+$ abortive initiation, whereas, in the absence of RNA polymerase, only cAMP-CRP*598 can bind to the CRP site in the *lac* promoter. DNase I footprinting demonstrates that on addition of RNA polymerase the characteristic open promoter pattern is established with cAMP-CRP*598 and higher concentrations of cGMP-CRP*598 or unliganded CRP*598. The fact that unliganded CRP* and cGMP-CRP* bind to $lacP^+$ only when RNA polymerase is present indicates a cooperative binding of heterologous proteins. Although other explanations are possible, the simplest interpretation of such cooperativity is direct contact between the two DNA-bound proteins. With the *lac* L8UV5 promoter mutant, cAMP-CRP forms a stable complex at the L8 site only in the presence of RNA polymerase (33). Thus, these results provide strong evidence in support of a model whereby activation of transcription from CRP-dependent operons involves contact between CRP and RNA polymerase. RNA polymerase alone does not bind to the $lacP^+$ site (referred to as *lacP1* in ref. 34) and CRP*598 does not bind to the CRP site in the absence of cAMP. The conformation of CRP*598 and cGMP-CRP*598 is not optimal for site-specific binding to the $lacP^+$. According to the model proposed by Malan and McClure (34), RNA polymerase binds to an upstream promoter, *lacP2*, in the absence of cAMP-CRP. Addition of cAMP-CRP results in the coordinate repression of *lacP2* and activation of *lacP1*. The coordinate repression–activation must also be effected by unliganded CRP*598 and cGMP-CRP*598. Since CRP*598 and cGMP-CRP*598 cannot by themselves bind optimally to the *lac* CRP site, the displacement of RNA polymerase present in the closed complex at *lacP2* may first involve CRP* contact with the bound RNA polymerase followed by movement of the RNA polymerase into the P1 site. Accompanying this shift into the *lacP1* site would be the concomitant binding of CRP* to the CRP site in the *lac* promoter.

Recently, Hwang and Gussin (35) have shown that a similar situation of cooperativity between heterologous proteins exists in the bacteriophage λ *prm* promoter. In that system, formation of open complexes at *prm* by RNA polymerase enhances binding at O_R by λ repressor, which functions as an activator of *prm*.

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