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Characterization of the CRP^{CY} core formed after treatment with carboxypeptidase Y

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ABSTRACT

CRP is resistant to attack by carboxypeptidase Y at 37°C, whereas cAMP-CRP is digested yielding a core terminating at Thr-202 and lacking the seven carboxyl-terminal amino acid residues. A similar core (CRP^{CY}) is formed when CRP is incubated with carboxypeptidase Y at 47°C in the absence of cAMP. CRP^{CY} has a more open conformation than CRP at 37°C. While unliganded CRP is resistant to trypsin, CRP^{CY} is sensitive to tryptic attack. Dithionitrobenzoic acid-mediated intersubunit disulfide crosslinking of CRP requires cAMP, CRP^{CY} subunits are crosslinked in the absence of cAMP. The carboxyl-terminal region of unliganded CRP is conformationally restricted at 37°C. The CRP^{CY} retains cAMP binding activity. The CRP^{CY} which terminates at Thr-202, no longer binds *lac* P^+ DNA nor stimulates abortive initiation by RNA polymerase from the *lac* P^+ promoter. The results indicate that the C-terminal region of CRP participates in the conformational stability of the closed form of CRP and indirectly in DNA binding by the open cAMP-CRP conformer.

INTRODUCTION

In *E. coli* the expression of over 20 genes is regulated by the binding of the cAMP receptor protein (CRP) to promoter-associated sites (1). CRP is a cAMP-dependent, sequence-specific DNA binding protein. The mechanism by which cAMP-CRP stimulates transcriptional initiation appears to involve direct contact between CRP and RNA polymerase on the promoter (2–4). CRP consists of two identical subunits of 209 amino acids (5). Each subunit of the CRP dimer is folded into two domains connected by a nine-residue hinge region (6). The larger N-terminal domain binds cAMP and is involved in subunit-subunit contacts; the smaller C-terminal domain is involved in site-specific DNA binding (6–8). The helix-turn-helix motif implicated in site-specific-DNA binding for several prokaryotic regulatory factors (9) resides in the C-terminal domain of CRP.

Binding of cAMP results in a conformational change in CRP. The allosteric effect of cAMP binding has been demonstrated by the increased sensitivity of cAMP-CRP to attack by endoproteases (7,8,10,11). In the presence of cAMP, the reaction of the two available sulfhydryl groups (Cys-178) mediated by reaction with dithionitrobenzoic acid results in the formation of a disulfide bond linking the subunits within the CRP dimer (12). It has also been shown that cAMP enhances CRP dimer stability (13) and that conformational change in secondary structure involves some conversion of α-helix to β-strand (14). A class of CRP mutants, designated CRP*, is able to function in the absence of cAMP (2,29,30). Unliganded CRP*598 is susceptible to endoprotease cleavage indicating that its conformation is related to that of cAMP-CRP (2). In this paper we report on the results obtained using carboxypeptidase Y digestion as a probe of structure and function of the C-terminal region of CRP.

MATERIALS AND METHODS

Materials

Reagents were obtained as follows: cAMP, cGMP, carboxypeptidase Y, trypsin, DNase I, bovine serum albumin, and pepstatin, Sigma; sodium dodecyl sulfate, TEMED, and bisacrylamide, Bio-Rad; acrylamide, Serva; [^{3}H]cAMP, [^{3}H]UTP, [α-^{32}P]dATP, ICN; HPLC grade water and acetonitrile, Burdick and Jackson Laboratories Inc.; sequence grade phenylisothiocyanate and pyridine, Pierce Chemical Co.; DNA polymerase I Klenow fragment, Boehringer Mannheim; Ecolume, Isolab.

Proteins

CRP was isolated by a modification of the method of Ren *et al.* (15) from *E. coli* strain pp47 containing the recombinant plasmid pHA7 (5) donated by Dr. H. Aiba (University of Tsukuba, Japan). RNA polymerase was isolated from *E. coli* K12 by a modification of the method of Burgess and Jendrisak (16). Protein concentrations were determined using the extinction coefficients: CRP, $E^{1\%}_{280nm}$ = 8.8 (17); RNA polymerase holoenzyme, $E^{1\%}_{280nm}$ = 6.7 (18).

DNA Fragments

E. coli containing the *lac* P^+ promoter cloned into pMB9 (19) was obtained from Dr. A. Revzin (Michigan State University, East Lansing). The 203-base pair *lac* P^+ fragment was prepared

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as described in Li and Krakow (3). ^{32}P-*lac* P$^+$ was prepared by labeling with [α-^{32}P]dATP by DNA polymerase I Klenow fragment.

Carboxypeptidase Y Digestion of CRP

Mixtures containing (final volume, 40 μL): 10 mM MES (4-morpholineethanesulfonic acid) (pH 5.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 8 μg CRP, 0.24 μg carboxypeptidase Y, 0.024 μg pepstatin, and where indicated 1 mM cAMP or cGMP were incubated at the indicated temperatures for the indicated time periods; the reactions were terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. The samples were then subjected to polyacrylamide gel electrophoresis (20) on a 15% polyacrylamide gel with a 4.75% stacking gel.

Larger scale preparation of CRP^{CY}: CRP (500 μg/mL) in 10 mM MES (pH 5.5), 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 1.5 μg/mL pepstatin and 15 μg/mL carboxypeptidase Y was incubated at 47°C for 40 min; the CRP^{CY} was kept at 0°C until used. For incubations with CRP*598 or cAMP-CRP the reactions were carried out at 37° with 10 mM Tris (pH 8.0) replacing the MES buffer.

Amino Acid Analysis

Incubation of CRP with carboxypeptidase was carried out as described above. The samples to be used for determination of the released amino acids were dried in pyrolyzed and siliconized 6×50 mM Corning glass tubes and redissolved in 100 μL of fresh derivitization buffer (ethanol:triethylamine:H_2O = 15:2:3) and dried under vacuum. 100 μL of derivitization buffer plus 1 μL PITC was added to each tube and the reaction was carried out for 5 min at room temperature. The samples were dried under vacuum and dissolved in 100 μL of a solution containing 49 mM sodium acetate, pH 5.4 plus 28% acetonitrile and loaded on an Applied Biosystems C-18 PTC-amino acid column for analysis.

Intersubunit Crosslinking

Mixtures (final volume, 40 μL): 20 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (pH 8.0), 4 μg CRP^{CY} or CRP and 1 mM cAMP or cGMP where indicated. The mixtures were incubated at 30°C for 15 min, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to a final concentration of 20 μM and the incubation was continued 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 (2).

Binding Assays

Binding of [^{3}H]cAMP to CRP^{CY} or CRP was assayed by the method of Anderson *et al.* (21). For binding of CRP^{CY} and CRP to the *lac* P$^+$ fragment the mixtures contained (final volume, 15 μL): 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.4 μM cAMP, 2 nM ^{32}P-*lac* P$^+$ fragment, 90 nM CRP or CRP^{CY}, and where indicated, 80 nM RNA polymerase and 0.5 μg tRNA. The mixtures were incubated for 15 min at 37°C and binding was assayed by polyacrylamide gel electrophoresis with TBE buffer (22). The DNA bands were visualized by autoradiography.

Abortive Initiation Assay

A modification of the abortive initiation assay of Malan *et al.* (23) was used to determine the activity of CRP^{CY} for CRP-dependent transcription from *lac* P$^+$ promoter. The reaction mixtures contained (final volume, 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1mM dithiothreitol, 50 μM cAMP, 2 nM *lac* P$^+$ fragment, 40 nM RNA polymerase, 1% glycerol, and the indicated amount of CRP or CRP^{CY}. The mixtures were incubated for 15 min at 37°C. After addition of 500 μM ApA and 50 μM [^{3}H]UTP, the mixtures were incubated for 15 min at 37°C. The reaction was terminated by addition of 10 μL of 0.5 M EDTA. The radioactive products were resolved by paper chromatography in WASP solvent (24). After cutting the chromatography strips into 1 cm segments, the amount of ApApUpU synthesized was determined by assaying appropriate segments in Ecolume.

RESULTS

Previous studies showed that the unliganded CRP is relatively resistant to attack by a variety of endoproteases (7,8). In contrast cAMP-CRP is digested resulting in N-terminal cores which bind cAMP and retain the dimeric structure of native CRP. The results presented in Fig. 1 indicate that at 37°C, cAMP-CRP is rapidly attacked by carboxypeptidase Y yielding a slightly smaller subunit fragment. At 37°C the C-terminal arm of unliganded CRP is folded in a manner preventing attack by carboxypeptidase Y.

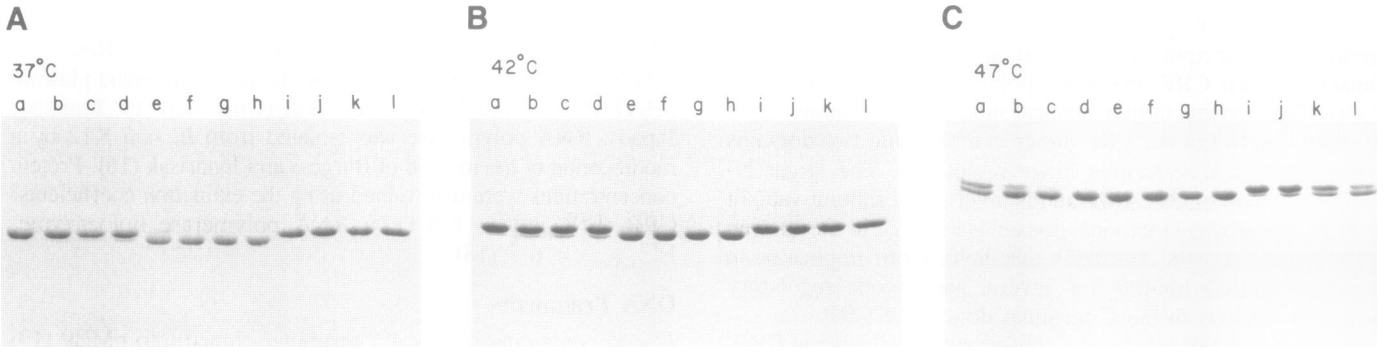

Figure 1. Time course of digestion of CRP by carboxypeptidase Y. The reaction conditions are described in Materials and Methods. The reactions were run at 37°C (A), 42°C (B) and 47°C (C) for the indicated times and then terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.8 mM. Lanes a, b, c, and d incubated for 10, 20, 40, and 80 min respectively in the absence of cyclic nucleotide; lanes e, f, g, and h incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cAMP; lanes i, j, k, and l incubated for 10, 20, 40, and 80 min respectively in the presence of 1 mM cGMP.

Even after 80 minutes at 42°C only a trace amount of the CRP^{CY} core is produced after incubation of unliganded CRP with carboxypeptidase Y. When incubated at 47°C the C-terminal domain melts allowing access to carboxypeptidase Y; by 40 minutes complete conversion to CRP^{CY} results. In contrast, cGMP-CRP is attacked at a much slower rate so that even after 80 minutes at 47°C residual intact CRP subunit is still evident.

The apparent impediment to continued exoproteolytic hydrolysis made the determination of the number of C-terminal amino acid residues removed fairly straightforward. Following incubation of CRP with carboxypeptidase Y for 40 minutes at 47°C, the released amino acids were determined by the PITC method. The data presented in Table I indicate that seven residues were removed; CRP^{CY} terminates at Thr-202. Similar results were obtained after analysis of the amino acids released from cAMP-CRP and unliganded CRP*598 following incubation with carboxypeptidase Y at 37°C.

Table I. Amino acids released from the C-terminus of CRP after incubation with carboxypeptidase Y.

Amino acid Released	CRP (47°C) (pH 5.5)	cAMP-CRP (37°C) (pH 8.0)	CRP*598 (37°C) (pH 8.0)
		pmol	
Arg	291	277	236
Thr	350	295	360
Gly	368	397	287
Tyr	331	323	311
Val	584	582	508
Ile	418	402	303
Lys	40	12	0
His	0	0	0

Determination of the amino acids released following incubation of 330 pmol CRP or CRP*598 with carboxypeptidase Y was carried out as indicated in Experimental Procedures. The result shown for CRP (47°) is the average of three experiments. The C-terminal sequence of CRP (5) is shown below:

199 200 201 202 203 204 205 206 207 208 209

CRP--His-Gly-Lys-Thr-Ile-Val-Val-Tyr-Gly-Thr-Arg-COOH

In the closed structure of unliganded CRP, it appears that the C-terminal domain must interact with a proximal segment of CRP. Trypsin was used to probe whether loss of the C-terminal oligopeptide segment affects CRP conformation. The results shown indicate that in contrast to CRP, unliganded CRP^{CY} is sensitive to trypsin (Fig. 2). Native CRP complexed with cAMP is attacked by trypsin with the formation of a 19,000 Da intermediate and the accumulation of a 14,000 Da core. The cAMP-CRP^{CY} shows a similar pathway for tryptic attack but appears to be more sensitive than cAMP-CRP. CRP^{CY} in the presence of cGMP is attacked by trypsin at a rate similar to that seen for unliganded CRP^{CY}. However, there does not appear to be an accumulation of the 19,000 Da intermediate; the final product appears to be identical to that formed from cAMP-CRP or cAMP-CRP^{CY}. The unliganded CRP^{CY} is further degraded yielding smaller fragments. The results indicate that the conformation of CRP^{CY} differs markedly from that of native CRP.

Reaction of the two available sulfhydryl groups (Cys-178) of cAMP-CRP with DTNB results in formation of an intersubunit bond within the CRP dimer (12). Under the conditions used, CRP and cGMP-CRP do not show the DTNB-mediated subunit crosslinking (Fig. 3). In contrast the subunits of CRP^{CY} are crosslinked in the presence or absence of cyclic nucleotide. The results provide additional evidence for an altered conformation of the C-terminal domain of CRP^{CY}.

The CRP^{CY} is prepared by incubation at pH 5.5 with carboxypeptidase Y at 47°C. The data presented in Fig. 4 indicate that the incubation temperature and pH used do not affect cAMP binding by CRP. The CRP^{CY} prepared under these conditions retains cAMP binding activity.

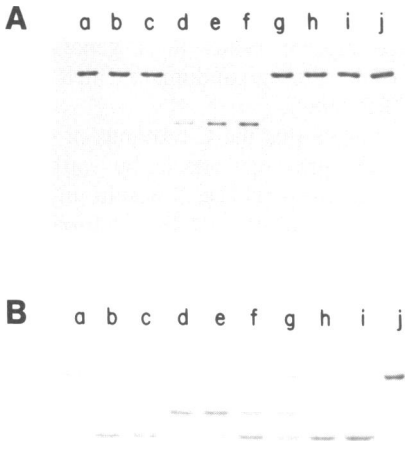

Figure 2. Sensitivity of CRP^{CY} to trypsin attack in the presence and absence of cyclic nucleotide. CRP^{CY} was prepared by carboxypeptidase Y digestion of CRP at 47°C. The reaction mixtures contained (final volume, 50 μL): 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10 μg CRP or CRP^{CY}, 1 mM cAMP or cGMP and 0.1 μg trypsin. The reactions were incubated at 37°C for the times indicated. Panel A: CRP + trypsin incubated for: lane a, 10 min; lane b, 20 min; lane c; 40 min; lane d, + cAMP 10 min; lane e, + cAMP 20 min; lane f; + cAMP 40 min; lane g, + cGMP 10 min; lane h, + cGMP 20 min; lane i, + cGMP, 40 min; lane j, omit trypsin. Panel B: CRP^{CY} incubated for: lane a, 5 min; lane b, 10 min; lane c, 20 min; lane d, + cAMP 5 min; lane e, + cAMP 10 min; lane f; + cAMP 20 min; lane g, + cGMP 5 min; lane h, + cGMP 10 min; lane i, + cGMP, 20 min; lane j, omit trypsin.

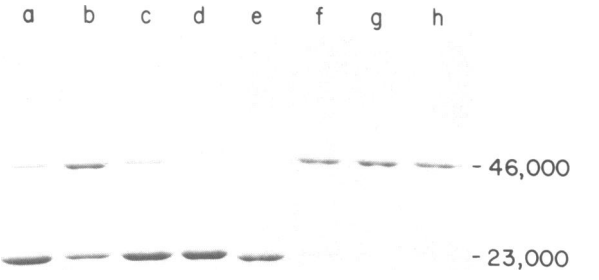

Figure 3. Effect of DTNB on intersubunit crosslinking of CRP and CRP^{CY}. CRP^{CY} was prepared by incubation with carboxypeptidase Y at 47°C. The reaction mixtures contained (final volume, 40 μL): 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 8.0), 20 μM DTNB, 4 μg CRP^{CY} or CRP and 1 mM cAMP or cGMP where indicated. The mixtures were incubated at 30°C for 15 min, DTNB was then added and the incubation was continued for 15 min at 30°C. Lane a, CRP + DTNB; lane b, CRP + cAMP + DTNB; lane c, CRP + cGMP + DTNB; lane d, CRP; lane e, CRP^{CY}; lane f, CRP^{CY} + DTNB; lane g, CRP^{CY} + cAMP + DTNB; lane h, CRP^{CY} + cGMP + DTNB.

The CRP core formed after cutting with the *Staph. aureus* V8 protease at Glu-171 is unable to support *lac* P^+-directed abortive initiation (unpublished results). The data presented in Fig. 5 demonstrate that the truncated CRP^{CY} which terminates at Thr-202 also does not activate RNA polymerase in the abortive initiation reaction.

It is possible that the observed inactivity of CRP^{CY} in supporting abortive initiation is a consequence of the loss of the contact site or conformation required for interaction with RNA polymerase or that CRP^{CY} is unable to bind to the CRP site on the *lac* promoter. The data presented in Fig. 6 show that CRP^{CY} does not bind to the *lac* P^+ fragment. Virtually all of the DNA migrates as the free *lac* fragment in the presence of cAMP-CRP^{CY} (Fig. 6, lanes e, f). The incubation of cAMP-CRP^{CY} plus RNA polymerase with the *lac* fragment results in a complex which is dissociated by tRNA. The pattern obtained is comparable to that observed with RNA polymerase in the absence of CRP.

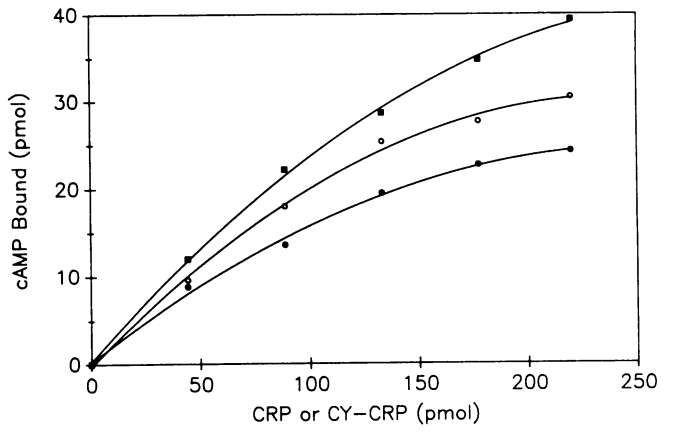

Figure 4. Binding of cAMP by CRP and CRP^{CY}. Reaction mixtures contained (final volume, 100 μL): 50 mM Tris-HCl (pH 8.0), 400 mM KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, 50 μg casein, 2 μM [^{3}H]cAMP and the indicated amount of CRP or CRP^{CY} (prepared at 47°C in the absence of cAMP). After 15 min at 0°C, 500 μL of saturated ammonium sulfate was added. The precipitates were collected by centrifugation at 10,000 rpm. CRP, ●-●; CRP preincubated at 47°C, ○-○; CRP^{CY}, ■-■.

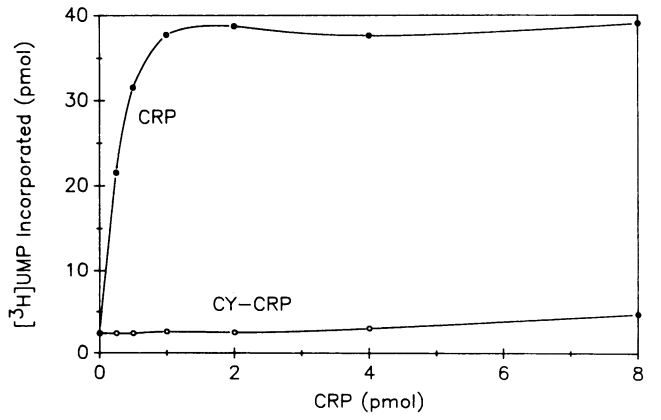

Figure 5. Ability of CRP and CRP^{CY} to support abortive initiation from the *lac* P^+ promoter. Reaction mixtures contained (final volume, 50 μL): 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM cAMP, 2 nM *lac* P^+ fragment, 40 nM RNA polymerase, 1% glycerol and the indicated amount of CRP or CRP^{CY} (prepared by incubation at pH 8.0 of cAMP-CRP with carboxypeptidase Y at 37°C). The mixtures were incubated for 15 min at 37°C; after addition of 500 μM ApA and 50 μM [^{3}H]UTP the incubation was continued for 15 min at 37°C.

DISCUSSION

Limited proteolysis has proven to be a useful probe of the conformation of CRP and CRP* mutants. Unliganded CRP is resistant to a variety of endoproteases (7,8). Conversion to a protease-sensitive state can be effected in several ways. cAMP-CRP is degraded to yield N-terminal cores which retain cAMP-binding activity and the dimeric structure of CRP (8,25). The DNA-CRP complex formed at low ionic strength is attacked by trypsin while remaining resistant to attack by subtilisin, chymotrypsin and the *Staph. aureus* V8 protease (26). Two fragments are formed after incubation of DNA-CRP with trypsin and cAMP binding activity is lost. The CRP within the monoclonal antibody 64D1-CRP complex becomes sensitive to attack by all four of the endoproteases used (27). The 9,000 Da fragment formed by tryptic hydrolysis of the mAb 64D1-CRP complex appears to be identical to the N-terminal fragment formed after tryptic attack on the DNA-CRP complex. The results presented in this paper indicate that facile attack of CRP at 37°C by carboxypeptidase Y occurs in the presence of cAMP. Unliganded CRP is resistant to carboxypeptidase Y at 37°C. At 47°C the C-terminal arm appears to melt allowing for attack by carboxypeptidase Y. The scanning calorimetric study of the thermal unfolding of CRP by Ghosaini *et al.* (28) indicates that CRP structure is not markedly affected at temperatures below 60°C. In contrast to the insensitivity of unliganded CRP, CRP* mutants are inherently sensitive to attack by both endoproteases (2,29,30) and carboxypeptidase Y. The effect of the mutation near the hinge region results in a general effect on the conformation of the C-terminal domain which extends to the C-terminus of CRP*598.

The results indicate that the C-terminus of CRP is folded in a manner which prevents attack by carboxypeptidase Y. Hydrolysis by carboxypeptidase Y results in a core, CRP^{CY}, which terminates at Thr-202. CRP^{CY} is formed from cAMP-CRP at 37°C or CRP at 47°C. Weber and Steitz (6) propose that interaction between the β 11 (residues 195–199) and β 12

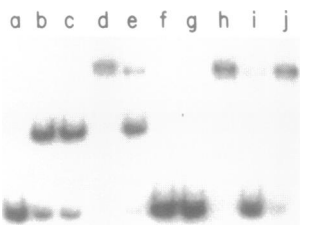

Figure 6. Binding of CRP and CRP^{CY} to the *lac* P^+ fragment. Reaction mixtures contained (final volume, 15 μL): 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 μg tRNA, 0.4 μM cAMP, 2 nM ^{32}P-labeled *lac* P^+ fragment (0.1 μg), 80 nM RNA polymerase and 90 nM CRP or CRP^{CY}. The mixtures were incubated for 15 min at 37°C. Lane a, free *lac* P^+; lane b, CRP; lane c, CRP + tRNA; lane d, CRP + RNA polymerase; lane e, CRP + RNA polymerase + tRNA; lane f, CRP^{CY}; lane g, CRP^{CY} + tRNA; lane h, CRP^{CY} + RNA polymerase; lane i, CRP^{CY} + RNA polymerase + tRNA; lane j, RNA polymerase.

(residues 201–205) strands may occur in cAMP-CRP. This should not result in a conformational impediment preventing continued hydrolysis by carboxypeptidase Y. An alternative hypothesis is that the block is due to an unfavorable amino acid sequence which prevents further hydrolysis by carboxypeptidase Y. The resistance of unliganded CRP to attack by carboxypeptidase suggests that stabilization of the C-terminal region is effected by interaction with a proximal region of CRP.

As was shown previously with much shorter N-terminal cores (7,8), the CRP^{CY} retains cAMP-binding activity. Removal of the 7 amino acid residues from the C-terminus results in the complete loss of the ability of CRP^{CY} to activate *lac* P^+ transcription and to bind to the *lac* DNA fragment. This is probably not due to the loss of amino acid residues directly involved in DNA binding beyond the intact helix-turn-helix region. In an analysis of the binding of CRP to a consensus DNA site for CRP, Ebright *et al.* (31) proposed that Lys-201 is one of the residues of CRP involved in ion-pair formation with DNA. Based on this model, CRP^{CY} should retain DNA-binding activity. A direct role of the C-terminal Arg-209 in DNA binding has been ruled out. Site specific mutation of Arg-209→Leu results in a mutant CRP which retains *lac* DNA binding activity (32).

The N-terminal seven amino acid arm of the λ repressor is considered to be involved in making contacts with the operator site. The N-terminal arm of the λ repressor is accessible to attack by trypsin suggesting that this region may be less compactly folded than the rest of the N- terminal domain (33). The crystal structures indicate that the C-terminal arm of cAMP-bound CRP (6) and the N-terminal arm of the λ repressor (34) should be flexible in solution. The accessibility of the C-terminus of cAMP-CRP to attack by carboxypeptidase Y and the N-terminal arm of the λ repressor to attack by trypsin are in keeping with these regions being less tightly folded. The truncated CRP^{CY} is sensitive to attack by endoproteases and subject to intersubunit crosslinking mediated by DTNB in the absence of cAMP. These properties are also seen with the conformationally relaxed CRP* mutants which, however, are active for DNA binding and transcription activation (2,29,30). The loss of the C-terminal arm results in a major effect on the conformation of the resultant CRP^{CY}. This appears to be a consequence of the ensuing disruption of the small β-sheet present in the C-terminal domain of CRP (6). Loss of critical amino acid residues in the C-proximal β-strand 12 would prevent hydrogen-bonding with the β 11 and β 10 strands. The consequent effects on the conformational orientation of the helix-turn-helix domain would explain the observed loss of DNA binding by CRP^{CY}. The results presented in this paper indicate that the C-terminal region of CRP participates in the conformational stability of the closed form of CRP and indirectly in DNA binding by the open cAMP-CRP conformer.

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