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Specificity of DNA Binding Activity of the *Bacillus subtilis* Catabolite Control Protein CcpA

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CcpA was purified from *Escherichia coli* BL21(λDE3)/pLysS carrying plasmid pTSC5, which was constructed by inserting the *ccpA* gene into the polycloning site of pGEM4. The purified protein migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent mass of 38 kDa but was eluted from a calibrated Bio-Gel P-100 column with an apparent mass of 75 kDa. Western blot (immunoblot) analysis revealed the presence of CcpA in *E. coli* BL21(λDE3)/pLysS/pTSC5, which carries *ccpA*, and in wild-type *Bacillus subtilis* 168 but not in *E. coli* BL21(λDE3)/pLysS/pGEM4 or in *B. subtilis* WLN-29, in which *ccpA* is inactivated by transposon Tn*917* insertion. Purified CcpA bound to DNA containing *amyO* and retarded its mobility in electrophoretic mobility shift analysis. Complete retardation of the DNA required 75 ng of CcpA per assay. In DNase protection analysis, CcpA bound to DNA containing *amyO* and protected a region spanning *amyO* when either DNA strand was labeled. Mutant forms of *amyO* not effective in catabolite repression were not retarded by CcpA.

Catabolite repression is an important global regulatory system found in both procaryotic and eucaryotic microorganisms (2, 26, 45). It serves to prioritize the utilization of carbon and energy sources as well as to regulate the synthesis of extracellular enzymes (2, 5) and toxins (14, 17, 30, 40, 41) and the initiation of bacterial sporulation (5, 11, 35). The molecular mechanism by which catabolite repression operates has been well studied in *Escherichia coli* (23–25, 45), in which it is a positive regulatory system involving catabolite repression protein (CRP) and cyclic AMP (cAMP). The mechanisms by which it operates in gram-positive bacteria and yeasts are just beginning to be elucidated, and they appear not to involve cAMP (2, 15, 38, 44), nor are they positive controls.

To begin the elucidation of the mechanism by which catabolite repression operates in *Bacillus subtilis*, we have concentrated our studies on α-amylase synthesis, which is subject to catabolite repression and temporal control but is not inducible in the classical sense (28). This lack of inducibility eliminates inducer exclusion as a consideration. We have shown that catabolite repression and temporal regulation are separable regulatory mechanisms (28) that function at the level of transcription (29).

Catabolite repression of *B. subtilis* α-amylase synthesis involves a *cis*-acting 14-bp sequence, designated *amyO*, that flanks the transcription start site of the α-amylase structural gene, *amyE* (29). Mutation of specific base pairs in the sequence alleviates catabolite repression of *amyE* expression (29, 46). Mutagenic analysis of *amyO* revealed a recognition sequence of TGT/AANC ↓ GNTNA/TCA where the underlined bases are the most important and N is any base (46). Sequences similar to this recognition sequence are found near the promoters of a number of *B. subtilis* genes that are known to be or suspected of being subject to catabolite repression (46). In the *hut* (48) and *xyl* (19) operons, similar sequences are found within the first structural genes of the operons. Mutations of the *amyO*-like sequences in the *hut* (48) and *xyl* (19) operons and the *acsA* and *acuABC* genes (8, 10) alter catabolite repression of those operons and genes. Sites homologous to *amyO* are required for glucose activation of the *ackA* gene of *B. subtilis* (9). A further demonstration of the importance of the *amyO* structure is that the *amyO* homologs from the *xyl* and *hut* operons render unregulated promoters sensitive to catabolite repression when fused to them (19). Sequences with great homology to *amyO* mediate catabolite repression in *Bacillus megaterium* (33) and *Staphylococcus xylosus* (39).

The similarity of the *amyO* sequence to those of *lacO* and *galO* operators in *E. coli* suggests that *amyO* might be the binding site of a repressor (29). The fact that mutations of *amyO* and its homologs relieve catabolite repression (19, 46, 48) further supports the contention that *amyO* is the binding site of a repressor involved in catabolite repression. To identify genes that might encode such a repressor, mutants resistant to glucose repression of α-amylase synthesis as a result of a *trans*-acting mutation (13) were isolated. In one such mutant, WLN-29, glucose repression of α-amylase synthesis was relieved (13). The wild-type allele of the *trans*-acting mutation in WLN-29 was cloned and sequenced (13). The protein, CcpA, deduced from the nucleotide sequence has 31% similarity to GalR and 25% to LacI of *E. coli*.

The objective of this study was the examination of the biochemical properties of purified CcpA to determine if it is a DNA-binding protein that binds *amyO* specifically.

MATERIALS AND METHODS

Materials. Polymin P (polyethylenimine) and calf thymus DNA were purchased from Sigma Chemical Company (St. Louis, Mo.). Sephacryl S-300, poly(dI-dC) · poly(dI-dC), DNase I, and heparin-Sepharose were purchased from Pharmacia; the Bradford assay reagent was purchased from Bio-Rad; *Taq* polymerase was purchased from Epicenter (Madison, Wis.); and restriction endonucleases and the Klenow fragment of DNA polymerase were purchased from

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TABLE 1. Genetic alterations of *amyO* mutants that relieve catabolite repression of α-amylase synthesis[a]

amyO	Sequence[b]	Repression ratio[c]
	↓	
Wild type	TGTAAGCGTTAACA	14.5
M10	-------T--G-T-	1.5
M113	--C---T-------	1.6
M115	---CG---------	1.7
M165	Δ-------Δ--T--	2.1
M192	---C----G-----	1.9

[a] From reference 46.
[b] Arrow, transcription start site. Δ, deletion.
[c] Amylase specific activity (4 h after the end of exponential growth) in the absence of glucose divided by that in the presence of glucose.

Promega (Madison, Wis.). The rabbit polyclonal anti-CcpA serum was prepared for us by the University of Wisconsin Medical School Animal Care Unit.

Bacterial strains and growth conditions. *E. coli* BL21(λDE3)/pLysS/pTSC5 (42), which carries the *B. subtilis ccpA* gene, was grown in Luria-Bertani medium. To maximize CcpA production, transcription from the T7 promoter was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when the turbidity of the culture reached 40 Klett units. The culture was harvested 3 h later. After centrifugation, the cell paste was stored at −75°C until use.

Protein concentration determination. To measure protein concentration, the Bio-Rad Bradford dye binding assay, with which polymin P does not interfere, was used. Bovine serum albumin was used as the protein standard.

Probe DNAs. A double-stranded oligonucleotide extending from position −27 to +27 surrounding the transcription start site of *amyE* was synthesized by the University of Wisconsin Biotechnology Center. At each end of this oligonucleotide, an *Xba*I site was engineered to allow the fragment to be cloned into the *Xba*I site in the multiple cloning sequence of plasmid pGEM3Zf(+), creating pAMYO. The insert DNA in pAMYO was sequenced to confirm that the construct was correct and also to ascertain the orientation of the insert.

To end label the coding strand of *amyO*, the insert fragment was removed from pAMYO by digestion with *Eco*RI and *Sph*I. The 5′ overhang generated by *Eco*RI digestion was filled in by using the Klenow fragment of DNA polymerase I, deoxynucleoside triphosphates (dNTPs), and [α-^{32}P]dATP (34). The end-labeled fragment is 104 bp in length.

To label the template strand, the *amyO* fragment was removed from pAMYO by digestion with *Hin*dIII and *Sma*I. The *Hin*dIII site was filled in by using the Klenow fragment and [α-^{32}P]dATP as the labeled nucleotide (34).

The labeled *amyO*-containing fragment was purified from 2% agarose gels by electroelution followed by phenol extraction before use in footprinting experiments.

Routinely, for gel retardation analysis, the labeled 104-bp *Eco*RI-*Sph*I fragment containing *amyO* was the probe DNA. To examine the binding of CcpA to mutated *amyO*'s, the 157-bp *Alu*I-*Hin*fI fragment which extends from position −77 to +80 of the amylase gene and includes *amyO* (positions −3 to +11) was isolated from the wild type and each of the *cis*-acting glucose-resistant mutants in Table 1. The fragments were labeled by filling in at the *Hin*fI end with Klenow polymerase, dNTPs, and [α-^{32}P]dATP.

Electrophoretic mobility retardation assay. Labeled probe DNA (1,000 cpm or approximately 1 ng of DNA) containing *amyO* or a mutation of *amyO* was incubated with the indicated amounts of CcpA in 10 mM Tris-HCl (pH 7.4)–1 mM dithiothreitol–1 mM EDTA–50 mM KCl–5% glycerol–50 μg of bovine serum albumin per ml–0.05% Nonidet P-40 (12) for 10 min at 37°C. After incubation, samples were loaded directly onto a prerun 5% polyacrylamide gel prepared in 6.7 mM Tris-HCl (pH 7.9)–1 mM EDTA–2.5% glycerol (3). Gels were run at 100 V for 1 h in a Tall Mighty Small vertical slab gel electrophoresis unit (Hoefer Scientific Co.). Gels were dried and exposed to X-ray film (X-Omat) for autoradiography.

DNase I protection. DNase I protection experiments were performed as suggested by Schmitz and Galas (37) with modifications. The 3′-end-labeled DNA (50,000 cpm or 50 ng of DNA) bearing *amyO* was incubated with CcpA under the binding conditions used for gel retardation except that no EDTA was present and each reaction contained 2.5 μg of poly(dI-dC). Two units of DNase I (Promega Co.) was added directly to the binding reaction mixture of 30 μl and incubated at room temperature for 20 s. The DNase I digestion was stopped by adding 60 μl of a DNase stop solution (10 mM EDTA and 100 μg of yeast tRNA per ml) and 1% sodium dodecyl sulfate (SDS). DNA was collected after phenol extraction and ethanol precipitation and then analyzed in an 8% polyacrylamide sequencing gel.

Western blotting (immunoblotting) and immunodetection. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (BA85; Schleicher and Schuell) by the method of Towbin et al. (43). CcpA was detected with rabbit polyclonal antibodies produced in response to immunization with purified CcpA and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega) according to the manufacturer's instructions.

Purification of CcpA. (i) Preparation of cell crude extract from *E. coli*. *E. coli* BL21(λDE3) carrying pLysS and pTSC5 was grown in a Luria-Bertani broth (34) containing ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml) at 37°C. When the culture reached 60 Klett units, IPTG was added (to 4 mM), and growth was continued for 3 h. Cells were harvested by centrifugation, frozen immediately, and kept at −75°C until use. Crude cell extracts were prepared as described by Studier et al. (42) with modification. The frozen cell pellets were thawed at room temperature in TGED buffer (50 mM Tris-HCl [pH 8.0], 10% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol) containing 50 mM NaCl, 0.2 mM diisopropylfluorophosphate, and 1 mM phenylmethylsulfonyl fluoride. The supernatant produced by removal of unlysed cells and cellular debris by centrifugation was mixed with streptomycin sulfate (1%, wt/vol) to remove nucleic acids. After centrifugation, the nucleic-acid-free supernatant was brought to 80% saturation with ammonium sulfate. The precipitated protein was collected by centrifugation and dissolved in TGED buffer. Desalting and concentration of the protein were performed by ultrafiltration using Diaflo membrane YM-30 (Amicon Co.) at 4°C. The concentrated protein solution was referred to as the crude cell extract.

(ii) Heparin-agarose column chromatography. The crude cell extract protein was loaded onto a heparin-agarose (Sigma Co.) column previously equilibrated with TGED buffer containing 50 mM NaCl. The loaded column was washed with TGED buffer containing 0.1 M NaCl to remove loosely bound protein, and then a linear gradient of 0.1 to 0.5 M NaCl in TGED buffer at a flow rate of 1 ml/min was applied. Fractions containing *amyO* binding activity as measured by gel retardation and by DNase I footprinting were pooled, desalted, and concentrated.

(iii) Single-stranded-DNA column chromatography. Single-stranded DNA–agarose was prepared by the method of Schaller et al. (36) and Lowe et al. (22). DNA-agarose beads were suspended in TGED buffer containing 1 M NaCl and packed into a column with the same buffer solution. The column, which usually contained 3 to 4 mg of DNA bound per ml of packed resin, was equilibrated with TGED buffer containing 50 mM NaCl. The pool of CcpA-containing fractions which normally were eluted at 0.4 M NaCl from the heparin-agarose column was loaded onto the single-stranded-DNA–agarose column. Bound protein was eluted with a linear gradient of 0.1 to 0.4 M NaCl in TGED buffer at a flow rate of 1 ml/min. Fractions were tested for the CcpA activity, and the active fractions were pooled and dialyzed.

(iv) DEAE (FPLC) chromatography. The CcpA pool from the single-stranded-DNA–agarose column was applied to a MemSep-HP1010 DEAE (ConSep LC100 fast protein liquid chromatography [FPLC] system; Millipore) ion exchange column preequilibrated with TGED buffer containing 50 mM NaCl. The column was washed with TGED buffer containing 0.1 M NaCl after protein adsorption, and the proteins were then eluted with a linear salt gradient of 0.1 to 0.5 M NaCl in TGED buffer at a flow rate of 2.6 ml/min.

RESULTS

Overproduction of CcpA. To facilitate the purification of CcpA, the 1.37-kb *Eco*RI fragment of *B. subtilis* DNA containing *ccpA* (13) was inserted into the *Eco*RI site in the polycloning region of plasmid pGEM4, creating plasmid pTSC5. In pTSC5, the 1.37-kb fragment is in the proper orientation for the transcription of *ccpA* from the T7 promoter of pGEM4. For the overproduction of CcpA, pTSC5 was introduced into *E. coli* BL21(λDE3)/pLysS (42). A protein of 38 kDa was present in extracts of cells carrying pTSC5 but not in extracts of cells carrying pGEM4 (data not shown). The relative amount of this protein increased when expression from the T7 promoter was induced by IPTG, but it was significant even without induction. The 38-kDa protein represented a significant fraction of the total protein of the cell extract after 3 h of induction.

Purification of CcpA. Purification of CcpA followed the scheme described in Materials and Methods. Presence of CcpA in fractions collected from the chromatographic columns was monitored by SDS-PAGE as well as gel retardation and footprinting. In Fig. 1, CcpA-containing fractions from various stages of purification are shown. With some exceptions, the material in Fig. 1, lane 5, served as the source of CcpA for subsequent studies.

To determine the native molecular weight of CcpA, the material in lane 5 of Fig. 1 was passed through a calibrated

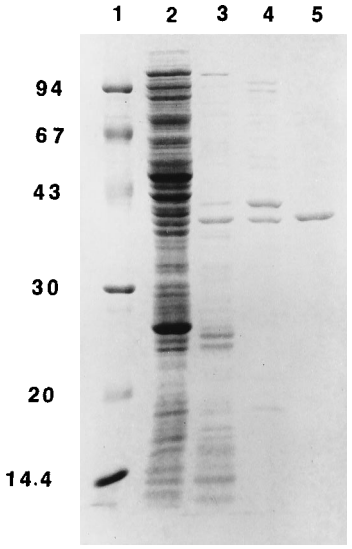

FIG. 1. Purification of CcpA produced by *E. coli* BL21(λDE3)/pLysS/pTSC5. Samples taken at various stages of purification were subjected to SDS-PAGE. Gel was stained with Coomassie brilliant blue G. Lane 1, molecular weight standards; lane 2, crude cell lysate; lane 3, pool of CcpA-containing material from a heparin-agarose column; lane 4, CcpA-containing material from a single-stranded-DNA–agarose column; lane 5, CcpA-containing material from DEAE chromatography. Sizes (in thousands) of molecular weight markers are indicated on the left.

Bio-Gel P-100 column. CcpA was eluted with an apparent molecular mass of 75,000, which would indicate that it is a dimer of two 38-kDa subunits (data not shown). This is very similar to what was observed previously (27).

Western blotting. Polyclonal antiserum was prepared by injecting rabbits with the purified protein in lane 5 of Fig. 1. In Western blots, the antiserum reacted with the purified CcpA protein preparation to give two bands: a major band corresponding in size to CcpA and a second band of lower intensity and greater mobility (Fig. 2, lane 1). Crude cell extract of *E. coli* BL21(λDE3)/pLysS/pGEM4 gave no detectable cross-reacting material (Fig. 2, lane 2), while *E. coli* BL21(λDE3)/pLysS/pTSC5 extract yielded bands very similar to those of the purified CcpA preparation. Extract of *B. subtilis* 168 gave a single band corresponding to a protein of 38 kDa (lane 5), while the WLN-29 ($CcpA^-$) extract contained no cross-reacting material (lane 4).

The lower-molecular-weight band in the purified CcpA preparation and in the *E. coli* BL21(λDE3)/pLysS/pTSC5 extract is apparently a CcpA degradation product, since the band was not present in the *E. coli* BL21(λDE3)/pLysS/pGEM4 extract. The absence of a band in the WLN-29 extract corresponding in size to CcpA was predictable because *ccpA* of WLN-29 is disrupted by the insertion of transposon Tn*917* at position 775 of the DNA sequence, interrupting the *ccpA* open reading frame at codon 148 (13). The absence of any cross-reacting material at all would indicate that any translation product of this disrupted gene must be unstable and rapidly degraded.

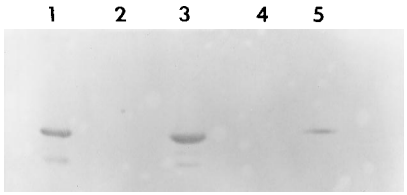

FIG. 2. Western blot analysis of cell extracts. Polyclonal anti-CcpA rabbit serum was used to examine extracts of *E. coli* and *B. subtilis* cells for CcpA. Lane 1, purified CcpA (0.2 μg); lane 2, *E. coli* BL21(λDE3)/pLysS/pGEM4 extract (50 μg); lane 3, *E. coli* BL21(λDE3)/pLysS/pTSC5 extract (50 μg); lane 4, *B. subtilis* WLN-29 ($CcpA^-$) extract (50 μg); lane 5, *B. subtilis* 168 ($ccpA^+$) extract (50 μg).

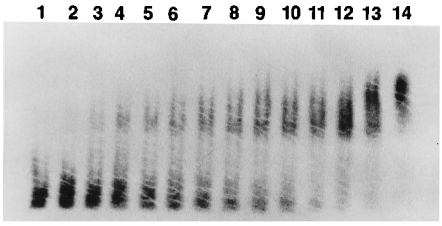

FIG. 3. Binding of CcpA to *amyO*. Increasing concentrations of CcpA were tested for retardation of the 104-bp *amyO*-containing *Eco*RI-*Sph*I fragment from pAMYO (see Materials and Methods) in a 5% polyacrylamide gel. Lanes 2 to 14, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 25, 50, and 75 ng of CcpA, respectively (3.3, 6.6, 9.9, 13.2, 16.5, 19.8, 23, 26, 30, 33, 33, 66, and 99 nM, respectively). Lane 1, no protein.

Electrophoretic mobility retardation. The similarity, especially in the helix-turn-helix domain, of CcpA to the GalR and LacI proteins of *E. coli* (13, 47) suggests that CcpA should be a DNA-binding protein. Its ability to bind DNA was initially tested in the gel retardation assay (3, 12) using the 104-bp *Eco*RI-*Sph*I fragment of pAMYO which contains *amyO*. The fragment contains the sequence extending from position −27 to +27 with respect to the transcription start site of the amylase gene. With 2.5 ng of CcpA in the binding reaction, the migration of only a very small fraction of the labeled DNA was retarded (Fig. 3, lane 2). As the concentration of CcpA was increased, the migration of a greater portion of the probe DNA was retarded until the migration of all of it was retarded at 75 ng of CcpA per reaction.

DNase I protection. The results from the gel retardation experiments above demonstrate that CcpA does bind DNA. To gauge the specificity of the DNA binding activity of CcpA, DNase I protection analysis was performed. The *amyO*-containing 104-bp DNA fragment from pAMYO labeled at the 3′ end of the coding strand was subjected to limited DNase I digestion (Fig. 4A). Binding of CcpA to the DNA fragment prior to DNase I treatment protected a region extending from position −7 to +17 from digestion. The exact boundaries of the protected region are not clear because of the lack of DNase I cut sites at each end of the protected region. Even with this caveat, it is evident that *amyO*, which extends from position −3 to position +11, is included in the protected region.

With the template DNA strand labeled, CcpA protected the region bounded by positions −14 and +16. As above, the lack of DNase I cut sites in the control DNA between positions −5 and −15 and between positions +9 and +21 makes it difficult to determine precisely the outer bounds of the CcpA protected region, but it is clear that the protected region on the template DNA strand overlaps that of the coding strand and includes *amyO* near its center.

Binding of mutant *amyO*'s by CcpA. As a further test of the specificity with which CcpA binds *amyO*, the ability of CcpA to bind mutated forms of *amyO* was tested. The mutant *amyO*'s chosen for testing were among those that resulted in the greatest relief of catabolite repression (46). Their sequences are given in Table 1. With 10 ng of CcpA per assay, wild-type *amyO* DNA was partially retarded (Fig. 5). The only mutant

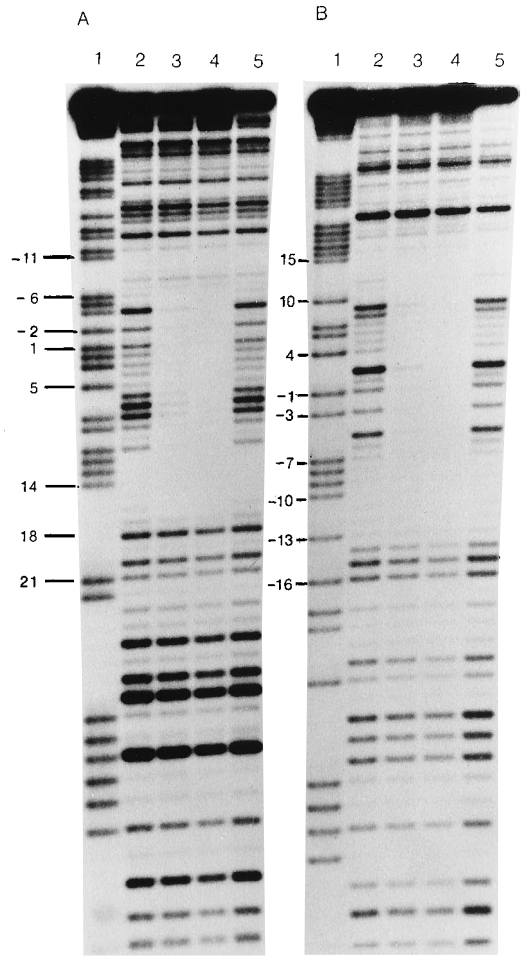

FIG. 4. CcpA protection of *amyO* DNA from digestion by DNase I. (A) Protection of the coding strand. Lane 1, G+A sequencing ladder; lane 2, no CcpA; lane 3, 0.5 μg of CcpA (0.66 μM); lane 4, 2.0 μg of CcpA (2.6 μM); lane 5, no CcpA. (B) Binding of CcpA to the template strand. Lane 1, G+A sequencing ladder; lane 2, no CcpA; lane 3, 0.5 μg of CcpA (0.66 μM); lane 4, 2.0 μg of CcpA (2.6 μM); lane 5, no CcpA. Base pair positions relative to the transcription start site are indicated.

DNA that demonstrated any degree of retardation was mutant M10, and for it only a very slight amount of DNA was retarded.

DISCUSSION

Inactivation of *ccpA* by transposon insertion (13) results in relief of catabolite repression of *amyE* (13), the *hut* operon (48), the *xyl* operon (16, 19), the *gnt* operon (7), *bgl* (20), *acsA* (8), *acuABC* (8), glucitol dehydrogenase (4), inositol dehydrogenase (4), and mannitol-1-P dehydrogenase (4). This pleiotropic phenotype demonstrates that CcpA has a role, either directly or indirectly, in catabolite repression. The similarity of CcpA to known bacterial regulatory proteins (13, 47) suggests that it might be the repressor that binds to catabolite repression operators similar to *amyO*. If this is true, CcpA would be expected to selectively bind the catabolite repression operators. In this study, we examined the ability of CcpA to bind *amyO*, the first of the *B. subtilis* catabolite repression operators to be identified (29).

Electrophoretic mobility shift (gel retardation) is one technique used to assay DNA binding activity. DNA-binding proteins bind DNA both at specific sites and nonspecifically; however, binding at specific sites occurs with much greater affinity than does nonspecific binding (6, 21).

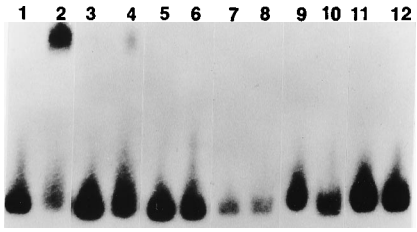

FIG. 5. Binding of CcpA to mutant forms of *amyO*. The ability of CcpA to bind to mutant forms of *amyO* (Table 1) that confer catabolite repression resistance to amylase synthesis in *cis* was tested by the electrophoretic mobility shift assay. The probe DNA contained wild-type *amyO* (lanes 1 and 2), mutant M10 (lanes 3 and 4), mutant M113 (lanes 5 and 6), mutant 115 (lanes 7 and 8), mutant 165 (lanes 9 and 10), or mutant 192 (lanes 11 and 12). Lanes 2, 4, 6, 8, 10, and 12, 10 ng of CcpA (13 nM); lanes 1, 3, 5, 7, 9, and 11, no CcpA.

Several approaches have been utilized to estimate the affinity of LacR for *lacO* (1, 31). Visual inspection of the autoradiograms of gel retardation of *amyO* by CcpA shows that 15 ng of CcpA per assay retarded about half of the probe DNA. This would correspond to an apparent binding constant of roughly 20 nM. On the basis of data from gel shift assays similar to those used in this study, a constant for the binding of LacR to *lacO* was calculated to be 24 nM (1), and similarly, for PurR binding to the *purF* operator, a value of 3.4 nM was determined (32). These values, which were based on the monomeric forms of the proteins, would suggest that the affinity of CcpA for *amyO* is comparable to those of other members of the LacI family of repressors for their operators.

We have observed that long purification protocols result in poorly active CcpA, similar in activity to the protein reported by Miwa et al. (27). To counter this, we used a rather short purification scheme. Secondly, we have observed that dilution of purified CcpA causes inactivation which can be prevented or greatly reduced by inclusion of bovine serum albumin (50 μg/ml) and Nonidet (0.05%) in the dilution solutions. Possibly, neither of these countermeasures is totally effective in preventing CcpA inactivation during purification and assay; consequently, only a fraction of the CcpA molecules may be fully active in *amyO* binding.

To determine if CcpA binds to a specific area of the *amyO*-containing DNA, DNase I protection was carried out. CcpA protected the region surrounding the start site of transcription of the amylase gene. Although the precise boundaries of the region protected by CcpA were not well defined, primarily because of a lack of DNase I cleavage sites, it is clear that CcpA protects overlapping regions on the two DNA strands and that the protected regions include *amyO*.

It has been long established that O^c mutations reduce the affinity with which the cognate repressor binds the mutated operator (18). In view of this, one would predict that mutations in *amyO* that relieve catabolite repression of α-amylase synthesis should result in reduced binding of CcpA to the mutated *amyO* if *amyO* is the CcpA target site. To test this prediction, mutations in *amyO* that caused the greatest relief of catabolite repression in vivo were selected for testing CcpA binding in vitro. It was assumed that these mutant forms of *amyO* would be the least likely to bind CcpA and, therefore, should reduce the ambiguity in interpreting the results. As can be seen in Fig. 5, CcpA does not bind the mutant DNAs under conditions in which wild-type DNA is bound, with the possible exception of mutant M10 DNA, which shows slight binding. In comparison

with binding to wild-type *amyO*, the binding of CcpA to M10 DNA is greatly diminished. These findings further establish that *amyO* is the CcpA binding site.

CcpA has strong similarity to the LacI-GalR family of bacterial repressors (13, 47). It possesses the conserved α-helix–turn–α-helix motif near the amino terminus of the protein and has homology with the other repressor proteins in the carboxy-terminal region important in effector binding. These similarities would suggest that CcpA, like the other repressors of the LacI-GalR family, is a repressor the operator-binding activity of which is determined by an effector molecule that either activates or inactivates operator binding. Typically, effectors are inactivators (inducers) of these repressors; however, some, as in the case of PurR, are activators (corepressors). To date, it is not possible to predict from sequence data whether a repressor will be activated or inactivated by its effector; thus, knowing the amino acid sequence of CcpA does not tell us whether it will be activated or inactivated by an effector. One might expect that a repressor requiring a corepressor for activation either would not bind its operator in vitro or would do so poorly. This does not appear to be the case for PurR, which binds its operator very tightly in the absence of a corepressor in vitro (32). In view of this, the strong, highly specific binding of CcpA to its operator, *amyO*, that we have demonstrated in vitro does not rule out the possibility that CcpA could require the binding of a corepressor to be an active repressor in vivo.

From our current, preliminary knowledge of CcpA, an outline of a mechanism by which carbon source-mediated catabolite repression in *B. subtilis* may operate can be proposed. When a repressing carbohydrate, such as glucose, is present in the culture medium, CcpA is in an active form and binds catabolite repression operators, thus repressing transcription of catabolite-sensitive genes and operons. Similarly, it should bind the *amyO* homologs located upstream of glucose-activated promoters such as *ackA* (9), leading to their transcription. As discussed above, activation of CcpA could result from either an increased level of an activator molecule (corepressor) or a decreased concentration of an inactivator (inducer). Currently, neither possibility can be ruled out. In the absence of a repressing carbohydrate, CcpA is predicted to be inactivated either as the result of a decreased concentration of a corepressor or an increased concentration of an inducer. The inactivated CcpA would not bind the *amyO*-like operators, thus relieving catabolite repression of the relevant genes and operons and deactivation of glucose-activated promoters.

Currently, the nature of the proposed effector is not known. We have tested a number of compounds including glucose, catabolic intermediates, and nucleotides, etc., and have yet to identify an effector, either positive or negative. It is possible that a low-molecular-weight effector is not involved; maybe the operator binding activity of CcpA is modulated by covalent modification or by another protein, as has been suggested by Deutscher et al. (3a). Future studies will address these possibilities.

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