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NADP, corepressor for the *Bacillus* catabolite control protein CcpA

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ABSTRACT **Expression of the α-amylase gene (*amyE*) of *Bacillus subtilis* is subject to CcpA (catabolite control protein A)-mediated catabolite repression, a global regulatory mechanism in *Bacillus* and other Gram-positive bacteria. To determine effectors of CcpA, we tested the ability of glycolytic metabolites, nucleotides, and cofactors to affect CcpA binding to the *amyE* operator, *amyO*. Those that stimulated the DNA-binding affinity of CcpA were tested for their effect on transcription. HPr-P (Ser-46), proposed as an effector of CcpA, also was tested. In DNase I footprint assays, the affinity of CcpA for *amyO* was stimulated 2-fold by fructose-1,6-diphosphate (FDP), 1.5-fold by oxidized or reduced forms of NADP, and 10-fold by HPr-P (Ser-46). However, the triple combinations, CcpA/NADP/HPr-P (Ser-46) and CcpA/FDP/HPr-P (Ser-46) synergistically stimulated DNA-binding affinity by 120- and 300-fold, respectively. NADP added to CcpA specifically stimulated transcription inhibition of the *amyE* promoter by 120-fold. CcpA combined with HPr (Ser-46) inhibited transcription from the *amyE* promoter, but it also inhibited several control promoters. FDP did not stimulate transcription inhibition by CcpA nor did the triple combinations. The finding that NADP had little effect on CcpA DNA binding but increased the ability of CcpA to inhibit transcription suggests that catabolite repression is not simply caused by CcpA binding *amyO* but rather a result of interactions with the transcription machinery enhanced by NADP.**

Catabolite repression in microorganisms is a wide spread, global regulatory phenomenon, but different molecular mechanisms have evolved to accomplish it (1, 2). In Gram-positive bacteria including *Bacillus subtilis* (3), *Bacillus megaterium* (4), *Clostridium acetobutylicum* (5), *Staphylococcus xylosus* (6), and *Lactobacillus pentosus* (7), the system involves negative regulation by the catabolite regulatory protein A (CcpA) and homologs. CcpA, a member of the LacI repressor family (8), is a bifunctional protein that acts as a repressor or an activator of transcription in response to the availability of glucose and other carbohydrates in the growth medium (9–11). Transcription units subject to CcpA-mediated catabolite repression or glucose activation contain CcpA-binding sites, known as catabolite responsive elements (*cre*s) (12). CcpA specifically binds *amyO*, the cre of the *amyE* (13), as well as to the cres of *gnt*, *xyl*, *hut*, *lev*, and *ackA*, but with affinities differing >30-fold between the strongest (*amyO*) and the weakest (*hut cre*) (14). The binding pattern of a *cre*–CcpA complex follows that of typical bacterial repressor–operator interactions (15). However, CcpA has a dissociation constant (K_d) of ≈28 nM for *amyO* (14), an affinity that is a 100- to 1,000-fold less than that of other bacterial repressors such as LacI (16) or TrpR (17) for their cognate operators. The relatively weak affinity of CcpA for its DNA-binding sites, the observation that CcpA is expressed constitutively in the presence and absence of glucose (18), and the presence of a conserved effector-binding domain in CcpA (3) together strongly suggest the necessity of an effector(s) such as a corepressor to activate CcpA. To date, HPr, a phosphocarrier protein in the phosphoenolpyruvate-:sugar phosphotransferase system, and two glycolytic metabolites, fructose-1,6-diphosphate (FDP) and glucose-6-phosphate, have been proposed as effectors of CcpA (19–22).

HPr is phosphorylated in two different fashions: ATP-dependent phosphorylation at Ser-46 and phosphoenolpyruvate-dependent phosphorylation at His-15 (23). In the *ptsH*1 mutant strain, the serine residue at position 46 of HPr is replaced with an alanine, which eliminates phosphorylation of HPr at that position (19). Catabolite repression of the *gnt* operon was reported to be relieved in a strain carrying the *ptsH*1 mutation (19). In addition, a mutation at His-15 of HPr also conferred catabolite repression resistance to expression of the *gnt* operon (24), indicating that the two residues at Ser-46 and His-15 are linked to catabolite repression. It also reported that HPr-P (Ser-46) is essential for CcpA binding to the *gnt* and *xyl cre*s *in vitro* (20, 21). FDP, a key glycolytic intermediate, was reported to stimulate the phosphorylation of HPr at Ser-46 by an ATP-dependent HPr kinase (23). In addition, an *in vitro* interaction between CcpA and HPr-P (Ser-46) was found to require FDP (24). Therefore, it was suggested that FDP could stimulate CcpA binding to DNA through an interaction between FDP and HPr-P (Ser-46), not directly between CcpA and FDP. But, it was not clear whether FDP would stimulate CcpA binding to *cre*s even in the presence of HPr-P (Ser-46) because the CcpA binding to the *gnt* and *xyl cre*s triggered by HPr-P (Ser-46) did not require FDP nor was it stimulated by it (20–22).

Distribution of *cre*s in some catabolite repressible operons is multipartite, consisting of a major *cre* with a high similarity to the consensus *cre* sequence and a minor *cre*(s) with less similarity to the consensus sequence (12). In the *gnt* operon, CcpA binds to the major *cre* (+148 region) in the presence of HPr-P (Ser-46) (20), whereas CcpA binds both the major and minor *cre* (−35 region) in the presence of glucose-6-phosphate (22). Similarly, in the *xyl* operon, CcpA combined with HPr-P (Ser-46) binds the major *cre* (+130.5 region), whereas CcpA combined with glucose-6-phosphate binds cooperatively the major *cre* as well as the minor auxiliary *cre*s (−35.5 and +219.5 regions) (21). However, CcpA binding triggered by glucose-6-phosphate in the auxiliary *xyl cre*s required very low pH, below 5.4 (21).

Given the above results, the picture with regard to *in vivo* effectors of CcpA is far from clear. The *ptsH*1 mutant strain does not relieve catabolite repression of *amyE* (25) and only does so partially for the *xyl* operon (26). Furthermore, Miwa, *et al*. (22) recently reported that catabolite repression of gluconate kinase synthesis, first reported to be HPr-P (Ser-46)-dependent (19), is partially independent of HPr-P (Ser-46). To date, there has been no demonstration that HPr-P

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Abbreviations: CcpA, catabolite control protein A. NF, N-terminal fragment of CcpA; FDP, fructose-1,6-diphosphate; cre, catabolite responsive element; RNAP, RNA polymerase.

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(Ser-46), glucose-6-phosphate, or FDP directly stimulate CcpA to inhibit transcription. Our results have demonstrated that although HPr-P (Ser-46), FDP, and glucose-6-phosphate may stimulate the affinity of CcpA for *cre*s, they are not required for CcpA binding to *cre*s of *amyE*, *gnt*, *xyl*, *hut*, *lev*, and *ackA in vitro* (13, 14).

We used both DNA binding and *in vitro* transcription to examine potential effectors of CcpA. Our results show that an effector or combination of effectors exhibiting maximum stimulation of DNA-binding affinity poorly stimulated inhibition of transcription, suggesting that the two activities are independently affected by different effectors. Of the compounds examined, NADP(H) caused the greatest stimulation in the transcription inhibition by CcpA, but it had minimal effect on the affinity of CcpA for DNA.

MATERIALS AND METHODS

Proteins. CcpA was expressed in *Escherichia coli* BL21(λDE3) pLysS and purified according to the method of Kim *et al.* (13). It was important that freshly prepared, highly active CcpA was used in all experiments. To prepare the N-terminal fragment of CcpA (NF), CcpA was digested with trypsin, and the NF was purified by gel filtration chromatography by using Sephadex G-100 as described (15). RNA polymerase (RNAP) was purified as described in ref. 27, with final purification using a Sephacryl S-300 column (28). Sigma A was purified as described in ref. 29 from the *E. coli* strain containing pLC2. HPr and HPr-P (Ser-46) were kindly provided by J. Deutscher (Centre National de la Recherche Scientifique, France).

Plasmids and DNA Labeling. The *amyO* (−27 to +27) fragment from pAMYO was used as the template for the DNA-binding assays and pAMYR bearing the whole regulatory region (−111 to +96) of *amyE* was used for the *in vitro* transcription assays (13, 15). In addition, pPH9 containing the *veg* promoter (30) was used as an internal control of transcription inhibition by CcpA. End labeling of the *amyO* fragment at 3′ using [α-^{32}P]dATP and the isolation of the labeled DNA fragment were performed as described in Kim *et al.* (15).

DNase I Protection. After CcpA was incubated with combinations of HPr-P (Ser-46) and 45 gylcolytic metabolites, nucleotides and cofactors in TGED buffer (50 mM Tris·HCl, pH 8.0/10% glycerol/0.1 mM EDTA/0.1 mM DTT) for 5 min, 1 ng of *amyO* (0.5 nM final concentration) was added, allowing formation of the *amyO*–CcpA complex. Two units of DNase I (Promega) were added to the 30 μl of reaction at room temperature. The DNase I digests were resolved in a 8% sequencing gel and bands were detected by autoradiography (13). Fractional occupancy of *amyO* by CcpA with or without effectors was obtained by the quantification and normalization of band intensities to reference bands. The fractional occupancy was plotted as a function of CcpA concentration. DNA band intensities were quantitated by phosphorimager analysis with IMAGEQUANT software (Molecular Dynamics) (15).

***In Vitro* Transcription.** Run-off transcription reactions were carried out with linearized pAMYR and pPH9 plasmids digested with *Eco*RI and *Bam*HI, respectively. After CcpA was combined with the various effector molecules in transcription buffer (40 mM Tris·HCl, pH 7.9/10 mM NaCl/5 mM $MgCl_2$/0.1 mM EDTA/7 mM DTT), the plasmids were added to the mixture and preincubated at room temperature for 10 min. Transcription reactions were allowed to initiate by adding RNAP plus a 2-fold molar excess sigma A and 0.1 mM each ATP, CTP, and GTP and 0.025 mM [α-^{32}P]UTP. After transcription elongation proceeded for 15 min, the transcripts were precipitated with 0.3 M sodium acetate and cold ethanol. The RNA samples were dried and resolved in a 6% sequencing gel containing 8 M urea. The relative amount of transcription (%) was obtained by quantification and normalization of the band intensities of *amyE* transcripts to those of the *veg* transcripts and were plotted as a function of CcpA concentration. The pH in both DNA-binding and run-off transcription reactions was monitored and maintained between 7 and 7.5 because some compounds caused acidification of the reactions.

RESULTS

Stimulating Factors of CcpA DNA Binding. To examine the ability of HPr-P (Ser-46) to stimulate CcpA-binding affinity for *amyO*, CcpA (28 nM) was incubated with the different amounts of HPr or HPr-P (Ser-46) and then allowed to bind *amyO*. Specific binding of CcpA to *amyO* was monitored by DNase I footprinting (Fig. 1). Nonphosphorylated HPr had little effect on CcpA binding, even at high concentration (3.4 μM). HPr-P (Ser-46) greatly stimulated DNA binding with 0.68 μM HPr-P (Ser-46) being required for one-half of maximum stimulation. Neither form of HPr bound *amyO* in the absence of CcpA. These findings would indicate that phosphorylation of serine 46 is required for HPr to stimulate the binding of CcpA to *amyO*. *E. coli* GalR binds amyO *in vitro*, but HPr-P (Ser-46) did not stimulate the binding affinity of GalR for amyO, indicating that the effect of HPr-P (Ser-46) on CcpA is specific (data not shown). HPr-P (Ser-46) changed the affinity of CcpA for *amyO*, but it did not change the general pattern of bands protected within the *amyO* region. However, in the presence of HPr-P (Ser-46) DNase I hypersensitive bands were observed ≈1.5 helix turns beyond the ends of the *amyO* sequence in both strands. The appearance of hypersensitive sites would suggest that the combination of CcpA and

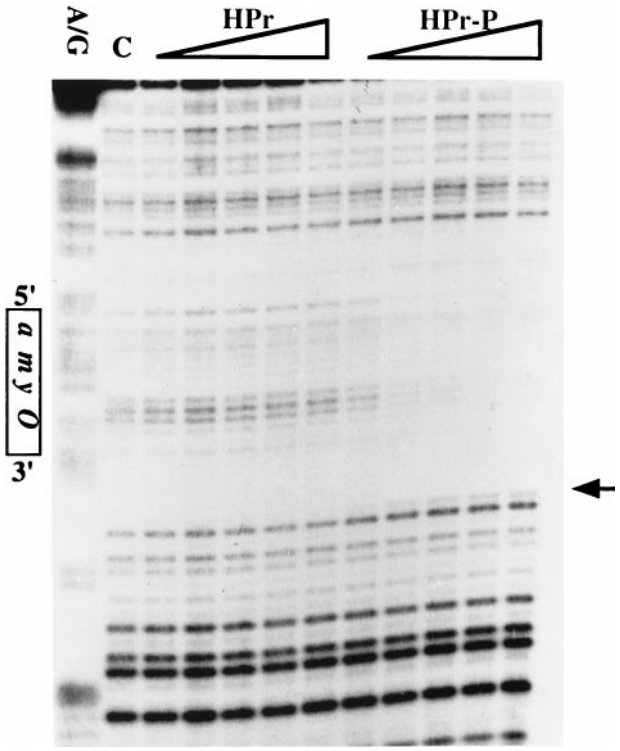

FIG. 1. Effect of HPr and HPr-P (Ser-46) on CcpA binding to *amyO*. Various concentrations of the HPr or HPr-P (Ser-46) (0.68–3.4 μM) were combined with the amount of CcpA required for one-half saturation of *amyO* (28 nM). After the *amyO* fragment was added to the binding buffer containing CcpA (C), CcpA + HPr, or CcpA + HPr-P (Ser-46) and allowed to form complexes; DNase I was added to digest unprotected DNA. A/G represents A+G ladder of coding strand of the *amyO* fragment and the arrow indicates a band showing hypersensitivity to DNase I digestion. The location of the *amyO* site is indicated by the box on the *Left*.

HPr-P (Ser-46) induces a conformational change in the DNA not caused by the binding of CcpA alone, in addition to increasing the affinity of CcpA for its target site.

Quantitation of Factors Effect on CcpA DNA-Binding Affinity. Of the glycolytic metabolites and related compounds examined, only FDP and NADP enhanced the DNA-binding activity of CcpA. Glucose-6-phosphate had no effect. The amounts of FDP and NADP required for maximum activation of CcpA were 2 mM and 0.4 mM, respectively. DNase I protection patterns by CcpA combined with FDP or NADP exhibited no apparent differences from those produced by CcpA alone, nor were DNase I hypersensitive bands found (data not shown).

The DNA-binding affinity of CcpA was titrated with various factors and observed by using DNase I footprinting. CcpA binds to *amyO* with a K_d of ≈28 nM, whereas the CcpA combined with HPr-P (Ser-46), FDP, or NADP bound to the DNA with K_d values of ≈2.8 nM, 11 nM, and 17 nM, indicating stimulations of 10-, 2.5-, and 1.6-fold, respectively. To determine whether combinations of effectors would be more effective than the individual effectors alone, 0.68 μM HPr-P (Ser-46) was tested with 3 mM FDP or 1 mM NADP at different concentrations of CcpA. Surprisingly, the triple combinations, CcpA–HPr-P (Ser-46)–FDP and CcpA–HPr-P (Ser-46)–NADP, showed very strong DNA-binding affinities with K_d values of 0.09 nM and 0.22 nM, respectively (Fig. 2). These are DNA-binding affinity stimulations of 300- and 120-fold over that of CcpA without effector, indicating synergistic rather than additive effects of the combinations. DNA-binding affinity by the CcpA–HPr-P (Ser-46)–FDP complex was 2.5-fold higher than that of the CcpA–HPr-P (Ser-46)–NADP complex. Considering the concentration of FDP used (2 mM), it is unlikely that the FDP effect on CcpA binding of DNA is caused simply by a nonspecific ionic effect. The combination of CcpA–FDP–NADP was no better in stimulating DNA-binding activity than were the combinations CcpA–FDP or CcpA–NADP.

The NF generated by treatment of CcpA with trypsin carries the helix-turn-helix motif responsible for specific binding to *amyO* (15). This truncated CcpA (≈4.5 kDa) was tested for its response to the factors identified above. HPr-P (Ser-46), FDP, NADP, and combinations thereof did not stimulate the DNA-binding activity of the NF (data not shown), indicating that the peptide (NF) is devoid of the portion of CcpA, which is recognized by these factors, and conversely it suggests that CcpA contains recognition sites for HPr-P (Ser-46) and FDP or NADP in the central or C-terminal region of the molecule.

Effect of Factors on Inhibition of Transcription by CcpA. Using an *in vitro* "run-off" transcription assay, we tested the ability of CcpA alone and combined with factors to inhibit transcription from the *amyE* promoter. The *veg* promoter, which is recognized by the *B. subtilis* RNAP but is not sensitive to catabolite repression (31), was used as an internal control to monitor specific CcpA-mediated inhibition of transcription. A transcript of 95 nt was produced from the *veg* promoter on pPH9 linearized with *Bam*HI. As reported previously, there are two overlapping promoters in the *amyE*-promoter region, but only one of these promoters is used *in vivo* (32). Two transcripts, 112 nt and 126 nt (the 112 nt transcript arises from the *in vivo* used promoter), are produced from the *amyE*-promoter region when plasmid pAMR is linearized with *Eco*RI. The *veg* and *amyE*-promoter transcripts are labeled in Fig. 3. The transcript common to all lanes in Fig. 3 arises from a plasmid promoter common to both pAMR and pPH9. Increasing concentrations of CcpA selectively inhibited production of the 112 nt transcript without reducing the other three transcripts.

To test the potential effectors in the transcription assay, 0.16 μM CcpA alone or in combination with different amounts of HPr-P (Ser-46), FDP, and NADP was preincubated with

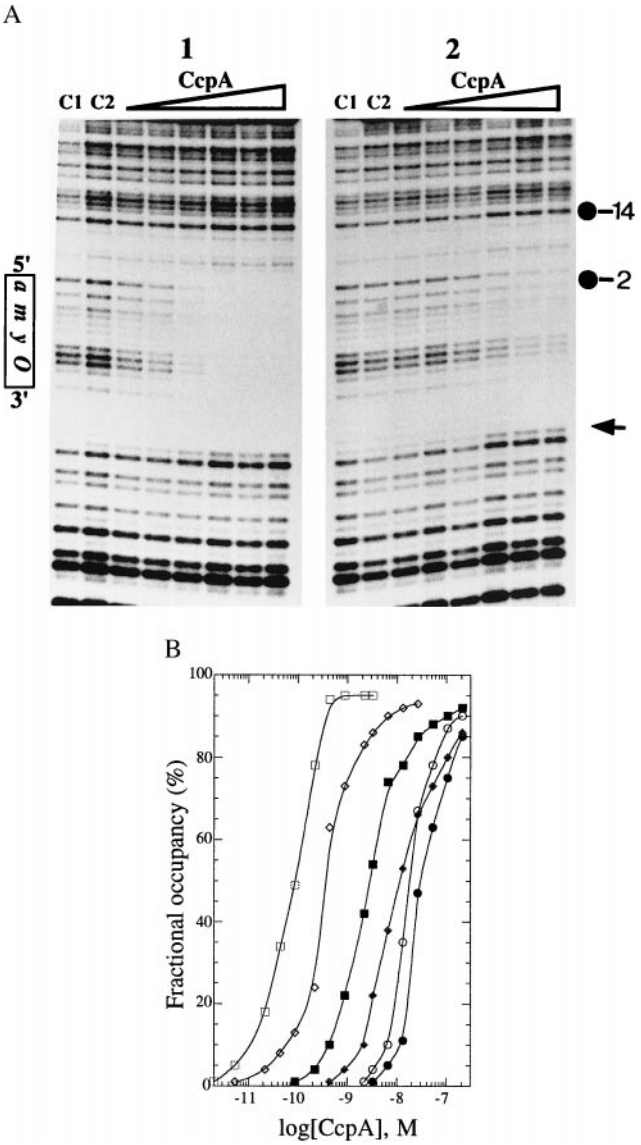

FIG. 2. Synergistic stimulation of combinations of HPr-P (Ser-46)/FDP and HPr-P (Ser-46)/NADP on CcpA binding to *amyO*. (*A*) DNase I footprints of *amyO* fragment bound by CcpA/HPr-P (Ser-46)/FDP (1) and CcpA/HPr-P (Ser-46)/NADP (2). After the various concentrations of CcpA (2.2 pM-2.2 nM) were incubated with HPr-P (Ser-46) (0.68 μM) and FDP (3 mM) or HPr-P (Ser-46) (0.68 μM) and NADP (1 mM), *amyO* fragments were added to the mixture to allow formation of *amyO*–CcpA complexes. The complexes were digested with DNase I. C1 and C2 represent the DNase I-digested *amyO* fragments without (C1) and with (C2) CcpA (28 nM), respectively. The DNase I hypersensitivity band is indicated by an arrow. (*B*) Titration of CcpA binding to *amyO* by CcpA alone (●) and CcpA combined with FDP (◆), NADP (○), HPr-P (Ser-46) (■), HPr-P (Ser-46) + FDP (□), or HPr-P (Ser-46) + NADP (◇). The DNA-binding isotherms were derived from original footprinting gels such as those shown in *A*. Fractional occupancy (%) was obtained by quantifying and normalizing the band intensity of diagnostic band (−2G) to that of the reference band (−14A) and plotted as a function of CcpA concentration. Both bands are shown by numbers in the figure.

plasmid DNA for 5 min and then added to the transcription mixture. HPr-P (Ser-46) stimulated transcription inhibition by CcpA, whereas unphosphorylated HPr had no effect (Fig. 4*A*). This result indicates that only the phosphorylated HPr-P is active in affecting CcpA activity as was observed with *amyO* binding. HPr-P (Ser-46) inhibited transcription from the all promoters, suggesting that HPr-P (Ser-46) causes nonspecific effects. FDP and NADP stimulated transcription inhibition by

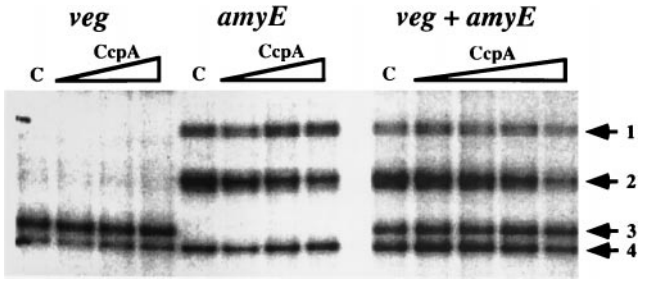

FIG. 3. Inhibition of transcription from *amyE* and *veg* promoter by CcpA. Run-off transcriptions were carried out by using the linearized plasmids containing the *veg* promoter (*veg*), the *amyE* promoter (*amyE*), and a mixture of the two plasmids (*veg* + *amyE*) in the absence (C) and presence of various concentrations of CcpA (28–560 nM; *veg* and *amyE*, 28–780 nM; *veg* + *amyE*). The *veg* promoter was used as a negative control to test specific inhibition of transcription by CcpA. Two transcripts from the *amyE*-promoter region, 126 nt (1) and 112 nt (2), and a transcript from the *veg* promoter, 95 nt (3), are labeled on the *Right*. The shortest transcript (4) detected at both plasmids was produced from a promoter in the common portion of the two plasmids.

CcpA from the *amyE* promoter but not from control promoters (Fig. 4*B*). Without CcpA neither NADP, FDP, nor HPr-P (Ser-46) at the concentrations test inhibited transcription (data not shown).

Quantitation of Factors Effect on Inhibition of Transcription by CcpA. Transcription inhibition of the *amyE* promoter by a range of CcpA concentrations was monitored in the presence of 2.72 μM HPr-P (Ser-46), 0.4 mM NADP, or 3 mM FDP and CcpA alone (Fig. 5). When HPr-P (Ser-46) or NADP was added, the amounts of CcpA required for one-half inhibition of the transcription from the *amyE* promoter were 42

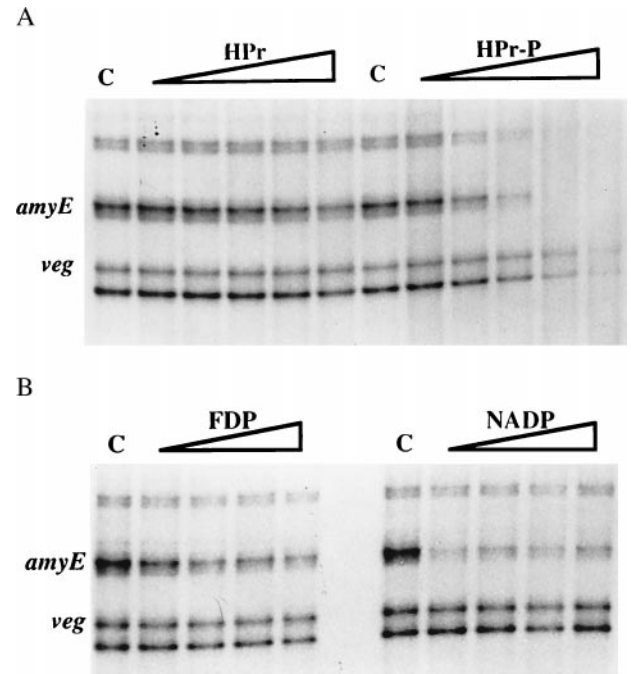

FIG. 4. Stimulation of CcpA-mediated inhibition of transcription by the HPr protein (*A*) and FDP or NADP (*B*). (*A*) After various amounts of the HPr or HPr-P (Ser-46) (0.68–10.8 μM) were combined with CcpA (0.18 μM), transcription buffer and template DNA were added. Transcription was started by adding RNAP to the mixture and incubating for 15 min at room temperature. (*B*) CcpA (0.18 μM) was combined with FDP (1.5–12 mM) or NADP (0.4–1.5 mM) and transcription was performed as described in *A*. The transcripts from the *amyE* and *veg* promoters were labeled on the *Left*, and lane C (control) in both *A* and *B* indicates transcription carried out in the absence of CcpA.

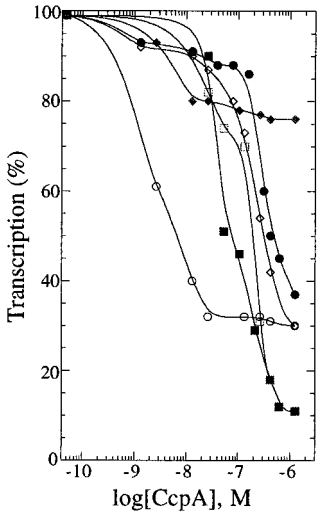

FIG. 5. Titration of the inhibition of transcription by CcpA alone and CcpA combined with effectors. In the presence of effectors individually or combinationally, inhibition of transcription of the *amyE* promoter was monitored by different concentrations of CcpA. The relative amount of transcription (%) was derived from run-off transcription gels by quantifying intensities of the transcripts and normalizing the amount of transcripts from the *amyE* promoter to those of the *veg* promoter and plotted as a function of CcpA concentration. CcpA alone, ●; HPr-P (Ser-46) (2.72 μM), ■; FDP (3 mM), ◆; NADP (0.4 mM), ○; HPr-P (Ser-46, 2.72 μM) + FDP (3 mM), ◇; HPr-P (Ser-46) (2.72 μM) + NADP (0.4 mM), □.

nM and 3.6 nM, respectively. These represent 10- and 120-fold greater inhibition, respectively, than by CcpA alone. FDP (3 mM) was almost without effect. The triple combinations, CcpA/HPr-P (Ser-46)/FDP or CcpA/HPr-P (Ser-46)/NADP, also were tested. Surprisingly, transcription inhibition by these was only slightly stimulated. In contrast to the results of DNA binding, FDP or NADP did not act cooperatively with HPr-P (Ser-46) to activate CcpA to inhibit transcription, rather HPr-P (Ser-46) apparently antagonized the transcription inhibition caused by CcpA activated by NADP alone.

DISCUSSION

The molecular mechanism by which CcpA mediates catabolite repression is unknown. Our results indicate that DNA-binding affinity does not necessarily correlate with transcription inhibition by CcpA. Greater DNA-binding affinity was obtained when CcpA was combined with either HPr-P (Ser-46) or FDP compared with CcpA with NADP, but maximum inhibition of transcription was obtained with the CcpA/NADP combination. The observation that the CcpA N-terminal fragment was not sensitive to the factors indicates that HPr-P (Ser-46) and FDP or NADP have specific binding sites in CcpA, but it is not clear whether they recognize the same site. These factors may modulate CcpA differently with the possibility that the HPr-P (Ser-46) induces a conformational change in CcpA as evidenced by the DNase I hypersensitve bands, whereas FDP or NADP could shift the orientation of the helix-turn-helix motif of CcpA as happens upon inducer binding to LacI (33). Our results suggest that CcpA can interact with at least two effectors at the same time to stimulate affinity for DNA, whereas the interaction of LacI with isopropyl β-D-thiogalactoside reduces affinity for DNA.

NADP stimulated the DNA-binding affinity of CcpA by only 1.6-fold but stimulated CcpA-mediated inhibition of transcription by 120-fold. This result would suggest that NADP does not stimulate the inhibition of transcription from the *amyE* promoter simply by increasing the binding affinity of CcpA for *amyO*. Although HPr-P (Ser-46) stimulated CcpA inhibition of

transcription, it did so in a nonspecific manner as the unregulated promoters were inhibited in addition to the *amyE* promoter. The finding that NADP is more effective than HPr-P (Ser-46) as a cofactor of CcpA for the catabolite repression of the *amyE* promoter is consistent with the *in vivo* study showing no effect on catabolite repression of α-amylase expression by the *ptsH*1 mutation (25). However, it may be that the *crh* gene product can compensate for the loss of HPr-P (Ser-46) in the *ptsH*1 strain (34). We have not tested *ptsH*1*crh* double mutants yet. HPr-P (Ser-46) does appear to assist CcpA in binding *amyO*, therefore HPr-P (Ser-46) may be involved in CcpA binding in some or all systems, but it does not appear to act directly in the inhibition of transcription of the *amyE* promoter.

In our experiments, NADP and NADPH were equally effective in stimulating DNA binding and inhibition of transcription by CcpA, whereas NAD and NADH had no effect on either activity. This observation would suggest that the phosphate of NADP not found in NAD or NADH is linked to the ability of CcpA to inhibit transcription. The intracellular levels of combined NADP and NADPH have been measured in *B. megaterium* spores, germinating spores, during exponential growth, and during sporulation (stationary phase) in three different culture media (35). NADP levels were low in dormant spores but increased rapidly during germination. In general, the NADP levels were maximal during growth and decreased during sporulation. The highest level of NADP (0.4 mM) (36) was attained in Spizizen minimal medium, which contains glucose as the carbon and energy source. These observations are consistent with the notion that the NADP + NADPH pool size might be connected to catabolite repression of the *amyE* gene; however, the *B. megaterium* experiments were not conducted in a manner that would allow any firm correlation between NADP + NADPH pool size and catabolite repression.

Our results implicate NADP as a corepressor of CcpA. Corepressors usually affect the DNA-binding capacity of repressors either through target site recognition or by elevating or reducing their affinity for cognate-binding sites (37, 38). That the NADP effect is primarily on the inhibition of transcription rather than on DNA binding by CcpA may, in part, be comparable with the role of D-galactose in the induction of the *E. coli gal* operon. Transcription from the *galP*1 promoter is repressed by GalR bound to its cognate operator (O_E), and the repression is relieved by the presence of D-galactose (39, 40). The role of the inducer in derepression may not be to reduce the affinity of GalR for the operator but to allosterically change GalR so that it no longer interacts with the α-subunit of RNAP to block transcription (41, 42). The action of NADP on *amyE* transcription may be similar to that of D-galactose on *galP*1 transcription in the sense that neither effector greatly changes the binding affinity of its repressor but may affect communication of the repressor with RNAP. However, the regulatory outcome in these two cases is opposite: D-galactose acts on GalR to derepress expression of the *gal* operon, whereas NADP appears to act on CcpA to repress expression of the catabolite repressible genes when glucose is present.

If both CcpA and RNAP can occupy the promoter region of *amyE* simultaneously then for inhibition of transcription to occur, CcpA must interact in a specific manner with RNAP and results presented in this paper indicate that this interaction depends on NADP. As has been reported, some repressors contact specific regions of RNAP such as the α-subunit (42, 43), and such interactions between a repressor and RNAP result in inhibition of transcription initiation by blocking isomerization to open complex formation (42, 44), or promoter clearance (43). A finding suggestive of an interaction between CcpA and RNAP is that catabolite repression of *amyE* (45) and *gnt* (46) is relieved in strains carrying the *crsA* mutation of *rpoD*.

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