Catabolite repression of a number of catabolic operons in bacilli is mediated by the catabolite control protein CcpA, the phosphocarrier protein HPr from the phosphoenolpyruvate-dependent sugar transport system (PTS), and a cis-acting DNA sequence termed the catabolite-responsive element (cre). We present evidence that CcpA interacts with HPr that is phosphorylated at Ser46 (Ser(P) HPr) and that these proteins form a specific ternary complex with cre DNA. Titration experiments following the circular dichroism signal of the cre DNA indicate that this complex consists of two molecules of Ser(P) HPr, a CcpA dimer, and the cre sequence. Limited proteolysis experiments indicate that the domain structure of CcpA is similar to other members of the LacI/GalR family of helix-turn-helix proteins, comprised of a helix-turn-helix DNA domain and a C-terminal effector domain. NMR titration of Ser(P) HPr demonstrates that the isolated C-terminal domain of CcpA forms a specific complex with Ser(P) HPr but not with unphosphorylated HPr. Based upon perturbations to the NMR spectrum, we propose that the binding site of the C-terminal domain of CcpA on Ser(P) HPr forms a contiguous surface that encompasses both Ser(P)46 and His15, the site of phosphorylation by enzyme I of the PTS. This allows CcpA to recognize the phosphorylation state of HPr, effectively linking the process of sugar import via the PTS to catabolite repression in bacilli.

Catabolite repression (CR)4 in Escherichia coli has provided a general paradigm for understanding the regulation of the synthesis of various catabolic enzymes in response to the availability of rapidly metabolizable carbon sources. The mechanism for CR in bacteria such as E. coli involves the cyclic AMP-dependent action of the catabolite gene activator protein, CAP (1). In Bacillus subtilis, however, the mechanism is completely different, because no cyclic AMP or CAP homologue is present (2). CR in some bacilli and a few other Gram-positive organisms has been shown to be dependent on the catabolite control protein A (CcpA), which is a member of the LacI/GalR family of regulators (3, 4), and a cis-active operator DNA sequence, termed the catabolite-responsive element (cre), which has been identified in the promoter or in the 5’ region of 29 B. subtilis genes (5).

There is growing evidence that CR is mechanistically linked to the phosphoenolpyruvate-dependent sugar transport system (PTS), which is responsible for the import of various sugars (1). Mutation of Ser46 to Ala in the PTS phosphocarrier protein HPr results in resistance to CR for several catabolic genes in B. subtilis (6). Ser46 in HPr is known to be phosphorylated by an ATP-dependent kinase that is activated by glycolytic intermediates such as fructose-1,6-diphosphate (7). Confirming the link between CR and the PTS, recent DNase I protection experiments have shown that cre sequences are specifically protected by CcpA only in the presence of HPr phosphorylated at Ser46 (Ser(P) HPr) (8). Other mechanisms for CR have been established; CcpA is known to bind to the cre sequence of the amyE operon in the absence of any corepressor (9, 10), and CR of the B. subtilis lev and bgl operons appear to be regulated by a mechanism involving Ser(P) HPr and the transcriptional activator LevR or the antiterminator LicT but not CcpA (11, 12). CcpA-mediated CR is therefore not the only mechanism for CR, although it is probably the dominant mechanism (13).

Based upon these data, a general model for CR in Gram-positive bacteria has been proposed. The availability of a rapidly metabolizable carbon source results in elevated levels of glycolytic intermediates, which leads to the phosphorylation of Ser46 of HPr. Ser(P) HPr is proposed to interact with CcpA only in the presence of Ser(P) HPr phosphorylated at Ser46 (Ser(P) HPr) (8). Other mechanisms for CR have been established; CcpA is known to bind to the cre sequence of the amyE operon in the absence of any corepressor (9, 10), and CR of the B. subtilis lev and bgl operons appear to be regulated by a mechanism involving Ser(P) HPr and the transcriptional activator LevR or the antiterminator LicT but not CcpA (11, 12). CcpA-mediated CR is therefore not the only mechanism for CR, although it is probably the dominant mechanism (13).

We have therefore used a variety of biophysical methods to characterize CcpA and its interactions with DNA and Ser(P) HPr. The results demonstrate the formation of a ternary complex between CcpA, Ser(P) HPr, and cre DNA. Binding of the binary CcpA-Ser(P) HPr complex to cre DNA is clearly distin-
guishable from the nonspecific complex formed between CcpA and cre DNA in the absence of Ser(P) HPr. Additionally, the binding site for CcpA on Ser(P) HPr allows CcpA to recognize the phosphorylation state of HPr, effectively linking the process of sugar import via the PTS to catabolite repression.

EXPERIMENTAL PROCEDURES

Protein Purification—His6-CcpA from Bacillus megaterium was purified using a Ni2+-NTA resin (Qiagen) as described previously (13, 15). Following elution with imidazole, CcpA was dialyzed in 10 mM Tris-HCl (pH 7.5). Protein was quantified using the method of Gill and von Hippel (16). B. subtilis HPr was purified as described previously (17). Ser(P) HPr was generated by phosphorylation and purified as described previously (18). 15N isotopic labeling of HPr was accomplished by growing bacteria in minimal medium with [15N]NH4Cl (Isotec) as the sole nitrogen source.

Synthetic oligonucleotides (Macromolecular Resources, Colorado State University) were purified as described previously (19). The single-stranded DNA was annealed in 10 mM MgCl2 by heating to 70 °C and slowly cooling to room temperature. Exact ratios of each strand for annealing reactions were determined by polyacrylamide gel analysis of multiple annealing reactions and silver staining. The double-stranded DNA was quantified assuming A260 nm = 1.0 corresponds to 33 μg/ml.

CD Experiments—CD experiments were performed on an Aviv 82DS Circular Dichroism Spectropolarimeter. Typically, experiments were performed with a 1.5 nm band width, and data points were taken every 1 nm with a 15-s averaging time. DNA concentrations ranged from 5 to 40 μM, and either 1- or 10-mm path length cuvettes were used. The buffer used in the CD experiments was 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl.

Limited Proteolysis Experiments—Limited proteolysis experiments were carried out with trypsin, chymotrypsin, or clostripain (Worthington Enzymes) in 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl. For clostripain digests, the buffer also contained 1 mM CaCl2 and 1 mM dithiothreitol. Typically, the CcpA concentration was 1 mg/ml, and the ratio of substrate to protease was 100:1 (w/w). Time course reactions were quenched with 1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone (for trypsin), 1 mM phenylmethylsulfonyl fluoride (for chymotrypsin), or 10 mM EDTA (for clostripain) before analysis by either SDS-polyacrylamide gel electrophoresis (20) or MALDI-TOF mass spectrometry. For samples analyzed by mass spectrometry, trifluoroacetic acid was added to a final concentration of 0.1%. MALDI-TOF mass spectra were recorded on a Perceptive Biosystems Voyager Elite (University of Washington Mass Spectrometry Facility) using 3,5-dimethoxy-4-hydroxy-cinnamic acid as the matrix (21).

Purification of CcpA-C was accomplished by limited proteolysis with clostripain (1 mg/ml CcpA concentration and 100:1 (w/w) ratio of CcpA to clostripain) for 90 min. The reaction was quenched by the addition of 10 mM EDTA. Samples were lyophilized, resuspended in one-tenth the original volume with H2O, and purified by size exclusion chromatography on a 50 × 2-cm Sephacryl S-100 column (Pharmacia Biotech Inc.).

NMR Experiments—Titrations of uniformly 15N-labeled HPr (0.5 mM) or Ser(P) HPr (0.4 mM) with CcpA-C were carried out at 30 °C. The appropriate amounts of protein were dialyzed extensively in 5 mM sodium phosphate buffer (pH 6.9) and lyophilized. The HPr or Ser(P) HPr was dissolved in 500 μl of 10% H2O/90% H2O; the CcpA-C was dissolved in 100 μl of 10% D2O. Typically, experiments were performed in 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl.

RESULTS

Circular Dichroism Studies—Near-UV CD has been demonstrated to be sensitive to changes in either the environment or conformation of DNA (for review see Ref. 23). Therefore, we used CD studies to monitor a DNA oligonucleotide containing a consensus cre sequence in the presence of CcpA with and without Ser(P) HPr. Fig. 1 shows various CD spectra of a 26-base pair duplex oligonucleotide in the presence and the absence of CcpA and Ser(P) HPr. The DNA sequence is 5'-GGGCTGATT-TGAAAGCGCTAACAGGG-3' and its complement, where the underlining denotes the 14-base pair consensus cre sequence (5, 24). The presence of an equimolar amount of CcpA dimer (30 μM DNA duplex and 30 μM CcpA dimer) results in a moderate change in the DNA CD spectrum compared with free DNA; there is a slight decrease in ellipticity near 265 nm and a slight increase in ellipticity at ~285 nm. However, the presence of Ser(P) HPr (60 μM) results in much more pronounced changes, with a maximal decrease in ellipticity occurring at ~267 nm.

The observation that the CD spectrum of DNA in the presence of CcpA is different from the free DNA spectrum suggests that there is some nonspecific interaction between the protein and DNA, even at 150 mM NaCl. However, it is clearly different from what is observed in the presence of Ser(P) HPr. Identical results are obtained under low ionic strength conditions; although the spectrum of the free DNA is slightly different, the spectra of DNA + CcpA and DNA + CcpA + Ser(P) HPr are superimposable with the spectra shown in Fig. 1 (data not shown).

We took advantage of the large change in CD signal observed upon the formation of the protein-DNA complex to determine an apparent binding constant. The results of titration of cre DNA (5 μM) with CcpA in the presence of excess Ser(P) HPr (40 μM) are shown in Fig. 2. The CD titration showed a clear isodichroic point (at 278 nm), indicating that a simple two-state equilibrium can be used to obtain an approximate affinity. The observation of an isodichroic point, which occurs at the same wavelength observed in the comparison of the spectra of DNA alone compared with the ternary complex (Fig. 1) and which is different from the isodichroic point (~275 nm) for the comparison of the spectra of DNA in the presence of CcpA and the ternary complex, strongly suggests that the CD titration data are monitoring the formation of the ternary complex from the DNA and a CcpA:Ser(P) HPr complex. The change in CD signal at 267 nm is well fit to a single binding event with an apparent $K_d$ of 4.5 ± 0.5 μM. The determined $K_d$ represents the apparent dissociation constant for the formation of the ternary complex;
because the CD signal is only sensitive to the formation of the ternary complex, it is not possible to measure the true dissociation constants directly for each of the possible binary complexes.

The measured affinity of CcpA binding to DNA is in a range that allowed us to determine the stoichiometry for the interaction between Ser(P) HPr and the CcpA dimer from the change in the CD signal. The oligonucleotide used contains only one cre sequence and is therefore sufficient for only one CcpA dimer. The change in CD signal during a titration of 40 \( \text{mM} \) cre DNA and 40 \( \text{mM} \) CcpA dimer was essentially linear up to 80 \( \text{mM} \) Ser(P) HPr (data not shown); further additions had no further effect on the CD spectrum, indicating that saturation has been reached. These results indicate that the ternary complex contains two molecules of Ser(P) HPr.

The CD results demonstrate the existence of a ternary complex formed between CcpA, Ser(P) HPr, and cre DNA. This supports the hypothesis that Ser(P) HPr is necessary for CcpA to bind specifically and with high affinity to cre sequences. Although these results do not directly rule out the possibility that the function of Ser(P) HPr is to increase the affinity of CcpA for DNA, specific or otherwise, the differences in the CD spectra for CcpA + DNA and CcpA + DNA + Ser(P) HPr suggest that Ser(P) HPr does alter the mode of DNA binding by CcpA. Other evidence also indicates that the interaction is specific to cre sequences; DNase I protection studies of CcpA in the presence of Ser(P) HPr show protection only within cre sequences (8, 9, 13), suggesting that a ternary complex is specifically formed with the cre sequence only.

**Domain Structure of CcpA by Limited Proteolysis**—In an effort to characterize CcpA and its interaction with either DNA or Ser(P) HPr further, we performed limited proteolysis experiments. Full-length CcpA (monomeric mass of \( \sim 39 \text{kDa} \)) was subjected to proteolysis using trypsin, chymotrypsin, or clostripain, and the time points were analyzed by SDS-polyacrylamide gel electrophoresis. In each case, a fragment of \( \sim 31 \text{kDa} \) was generated within the first minute and remained relatively resistant to further proteolysis (Fig. 3). Samples from each digest were analyzed by MALDI-TOF mass spectroscopy to determine the prominent cleavage sites. Some fragments whose identity could be unambiguously assigned based upon their molecular mass and cleavage sites for each enzyme are indicated in representative mass spectra for proteolysis samples (Fig. 4). The \(-31\text{kDa}\) fragments observed as protease-resistant bands on the SDS gels all correspond to a large C-terminal fragment that begins between residues 54 and 59 and extends to the C terminus of the protein, Lys332.

The domain structure of CcpA has been predicted based on its strong sequence homology to other helix-turn-helix regulators such as LacI, GalR, and PurR (3, 25) and is shown schematically in Fig. 4. The initial proteolytic fragments are consistent with this proposal; for each protease, cleavage occurs in the region between the DNA binding helix-turn-helix domain and the C-terminal domain, producing a stable C-terminal fragment. The observation that all three proteases cleave be-
between residues 54 and 59 suggests that this region is most likely the separation point between the DNA binding domain and the C-terminal domain.

Limited proteolysis experiments were also performed in the presence of Ser(P) HPr, cre DNA, or both to determine whether the formation of a binary or ternary complex would lead to protection of CcpA from cleavage. Under the conditions used for these experiments (typically 20–30 mM CcpA monomer, 100 mM Ser(P) HPr, and 60 mM DNA), no change in resistance to proteolysis was observed; this is in contrast to results of similar experiments with PurR, its corepressor, and its cognate DNA sequence (26, 27). These results indicate that the susceptibility of the linker between the DNA binding helix-turn-helix domain and the C-terminal domain is not greatly altered by the formation of any of the possible complexes or that the dissociation of any complex with CcpA occurs sufficiently fast to allow proteolysis to occur.

**NMR Studies of the CcpA-Ser(P) HPr Complex**—The CD results demonstrate the formation of a ternary complex between the cre-DNA, CcpA, and Ser(P) HPr. Previous elution-retardation experiments indicated that Ser(P) HPr interacts directly with CcpA (14); however, the question remains as to which regions on CcpA and Ser(P) HPr are important for this interaction. Interactions between HPr and its PTS protein partners have been characterized by NMR spectroscopy (28–31). We employed a similar approach, in which [1H-15N]HSQC spectra of 15N-labeled HPr (or Ser(P) HPr) are monitored as a function of added CcpA. Because CcpA was not isotopically 15N-labeled, its effect on HPr can be observed directly in these spectra.

Based upon sequence similarities to proteins with known three-dimensional structures such as LacI (32) and PurR (33), we reasoned that the C-terminal effector domain is likely responsible for binding Ser(P) HPr; the N-terminal helix-turn-helix domain presumably binds DNA, as has been observed for a proteolytic fragment of LacI corresponding to the helix-turn-helix DNA binding domain (34). To test this hypothesis, the C-terminal domain was generated by limited proteolysis and purified. Quantities of the C-terminal domain (CcpA-C) sufficient for NMR studies were isolated by treatment of CcpA with clostripain, followed by purification by gel filtration. Similar to intact CcpA, which is a dimer (3, 15), CcpA-C elutes on a gel filtration column with an apparent molecular mass of 60 kDa, indicating that this domain, isolated from the DNA binding domain, also exists as a dimer in solution (data not shown).

Aliquots of purified CcpA-C were titrated into a sample of 0.4 mM 15N-labeled Ser(P) HPr, and NMR spectra were collected following each addition. Fig. 5 shows [1H-15N]HSQC spectra at various points in the titration. Upon the addition of 0.25 mol equivalents CcpA-C, a subset of resonances (indicated in Fig. 5A) either broaden dramatically or disappear completely. As additional CcpA-C is added, most peaks in the spectrum completely disappear, suggesting line broadening of resonances whose environment is altered by the interaction between Ser(P) HPr and CcpA-C.
nances for Ser(P) HPr in the complex are not observed. The gradual disappearance of nonperturbed resonances, in contrast to the very rapid disappearance of a subset of resonances, suggests that exchange between free and bound is occurring in the fast to intermediate time regime (relative to the chemical shift differences of resonances in the free and bound states). Therefore, resonances that are perturbed most and consequently have the largest chemical shift changes due to the formation of the complex should disappear early in the titration, allowing for the determination of which portions of Ser(P) HPr are most affected in its interaction with CcpA-C.

Unfortunately, similar experiments with intact CcpA were impossible due to insufficient solubility of the intact protein. A similar titration of unphosphorylated HPr showed neither changes in intensity nor line width changes, indicating that CcpA-C does not bind unphosphorylated HPr, even at millimolar concentrations (Fig. 5D). Thus, the NMR results confirm that the C-terminal domain is responsible for the formation of a binary complex with Ser(P) HPr and that this interaction absolutely requires that Ser46 be in its phosphorylated state.

The residues that are selectively broadened at low concentrations of CcpA form a contiguous surface on Ser(P) HPr (Fig. 6). The binding surface comprises two α-helices in HPr; Helix A, which includes residues within the active site of HPr (residues near His115), and Helix B, which includes Ser46. Interestingly, this surface is very similar to the surface recognized by both enzyme I (30) and enzyme IIA (29, 31) of the PTS. The observation that the binding site for CcpA-C encompasses His15 suggests that CcpA can also discriminate whether HPr is phosphorylated at His15, consistent with previous results suggesting that CcpA does not interact with HPr phosphorylated at His15 (14) or mutated at His15 (35). Interestingly, the interaction of HPr with enzyme IIA and probably enzyme I is diminished upon phosphorylation at Ser46, allowing the PTS to sense the phosphorylation state of Ser46 in HPr. Thus, the regulatory phosphorylation of Ser46 in HPr effectively coordinates the functions of the import of sugars and the synthesis of catabolic enzymes.

DISCUSSION

The results presented here shed light on the general mechanism for CcpA-mediated CR and suggest a particular order of events from the initial phosphorylation of HPr at Ser46 to the repression of transcription by CcpA. The observation that a specific complex is formed between Ser(P) HPr and CcpA in the absence of DNA suggests that this interaction must precede the recognition of cre sequences. The apparent increase in affinity for cre DNA by CcpA in the presence of Ser(P) HPr and the observation of the ternary complex also support this sequence of events.

It is remarkable to consider the effect of Ser46 phosphorylation on the binding of CcpA. Considering the apparent Kd for the formation of the ternary complex (4.5 μM), phosphorylation of Ser46 on HPr leads to an increase in affinity for CcpA by at least 3 orders of magnitude over unphosphorylated HPr (no binding is observed at 0.5 mM HPr and CcpA, Fig. 5D). NMR studies have revealed that phosphorylation of Ser46 does not induce a conformational change in HPr (36). Thus, CcpA must specifically recognize the phosphoserine moiety, suggesting that the interaction has a large electrostatic component.

It is important to note that the two proteins are from different species; the CcpA used is from B. megaterium, and the HPr is from B. subtilis. However, the HPrs from these two species exhibit 81% sequence identity, and the CcpAs exhibit 76% sequence identity. More importantly, however, there are only three amino acid differences between the two species of HPr in the binding site for CcpA as identified by the present results (Fig. 6); residues Thr221, Ala225, and Gln266 in B. megaterium are replaced with Val221, Thr225, and Ala266 in B. subtilis. Therefore, the apparent Kd measured in this heterologous system is probably similar to either homologous system.

The crystal structure of another family member, the PurR-DNA-hypoxanthine ternary complex (33) provides insight into the mode of DNA binding by CcpA. Upon binding to DNA, a leucine (Leu56) from each monomer of PurR is inserted between bases through the minor groove of the DNA, resulting in a bend in the DNA of ~45°. A sequence comparison between CcpA and PurR indicates that this leucine is conserved, as are a number

**Fig. 6. Location of the binding site for CcpA-C on Ser(P) HPr.** Stereoview of HPr showing the residues that are selectively broadened by CcpA-C as a dot surface; these residues are: 14–17, 21–27, 43, 44, 46–56, 78, 81, and 82. The location of Ser46 and the active site residue His15 are also indicated. The residues not shaded blue in Helix A are Pro18 (which there is no amide proton to detect in the NMR spectra), Ala19, and Thr20, the latter two residues exhibit some broadening but not to the extent that is observed with the other shaded residues. This figure was made using MidasPlus (37) and the coordinates for the refined solution structure of B. subtilis HPr (Protein Data Bank entry 2HID) (38).

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2 In the fast to intermediate exchange regime, the observed line width for a peak will be the sum of the population-weighted average of the line widths of the free and bound species and an additional chemical exchange contribution that scales as $f_a f_b \Delta \omega_{ex}$, where $f_a$ and $f_b$ are the mole fractions of the free and bound species and $\Delta \omega_{ex}$ is the difference in chemical shift (in Hz) between the free and bound species. This predicts that all resonances that do not undergo a change in chemical shift upon complex formation will disappear (broaden) from the spectrum as a function of mole fraction of bound species, whereas resonances that undergo a change in chemical shift will broaden and disappear early in the titration due to the squared dependence on the chemical shift perturbation. This is precisely the behavior observed in the titration of CcpA-C into Ser(P) HPr.
of residues preceding this side chain that make contacts to the DNA phosphate backbone in the PurR-DNA complex structure. The large changes in the DNA CD spectrum we observe are consistent with a significant structural change in the DNA, suggesting that CcpA, in the presence of Ser(P) HPr, may interact with DNA in a manner similar to PurR. This conclusion is supported by nearly identical CD spectral changes observed with PurR and its cognate DNA sequence.3

The apparent structural similarity of CcpA to other helix-turn-helix proteins is interesting in light of its mode of action. Other helix-turn-helix proteins require small molecules for regulation, which upon binding cause the protein to bind to specific DNA sequences and affect transcription in some manner. Although the end result is the same, CcpA must bind another protein, namely Ser(P) HPr, to manifest a change in its DNA binding ability. A structural insight as to how this might occur can be gained from the three-dimensional structures of LacI and its complex with the co-factor isopropyl-1-thio-β-D-galactopyranoside (32). In this case, binding of isopropyl-1-thio-β-D-galactopyranoside to LacI results in a conformational change within the C-terminal effector domain that is proposed to reposition the DNA binding domains of each monomer relative to one another. Whether Ser(P) HPr performs its regulatory role in an analogous manner must await future characterization of this system.

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