Phosphoprotein Crh-Ser46-P Displays Altered Binding to CcpA to Effect Carbon Catabolite Regulation*

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In Gram-positive bacteria, the catabolite control protein A (CcpA) functions as the master transcriptional regulator of carbon catabolite repression/regulation (CCR). To effect CCR, CcpA binds a phosphoprotein, either HPr-Ser46-P or Crh-Ser46-P. Although Crh and histidine-containing protein (HPr) are structurally homologous, CcpA binds Crh-Ser46-P more weakly than HPr-Ser46-P. Moreover, Crh can form domain-swapped dimers, which have been hypothesized to be functionally relevant in CCR. To understand the molecular mechanism of Crh-Ser46-P regulation of CCR, we determined the structure of a CcpA-(Crh-Ser46-P)-DNA complex. The structure reveals that Crh-Ser46-P does not bind CcpA as a dimer but rather interacts with CcpA as a monomer in a manner similar to that of HPr-Ser46-P. The reduced affinity of Crh-Ser46-P for CcpA as compared with that of HPr-Ser46-P is explained by weaker Crh-Ser46-P interactions in its contact region I to CcpA, which causes this region to shift away from CcpA. Nonetheless, the interface between CcpA and helix a2 of the second contact region (contact region II) of Crh-Ser46-P is maintained. This latter finding demonstrates that this contact region is necessary and sufficient to throw the allosteric switch to activate cre binding by CcpA.

Carbon catabolite repression/regulation (CCR)2 is a global regulatory mechanism utilized by bacteria to select, out of a mixture of compounds, the carbon source providing the optimal growth advantage (1–3). CCR is mediated largely at the level of transcription. The master transcriptional regulator of CCR in bacilli and other Gram-positive bacteria with low GC content is the catabolite control protein A (CcpA) (4–10). CcpA binds to catabolite responsive elements (cre) to mediate its effect (11, 12). Approximately 10% of the Bacillus subtilis genome is under regulation by CcpA, underscoring its vital metabolic role (13).

CcpA is a member of the LacI-GalR family of transcription regulators encoding the Mg2+–citrate transporter, is specifically repressed by Crh under regulation by CcpA, underscoring its vital metabolic role (13).

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2 The abbreviations used are: CCR, carbon catabolite repression; cre, catabolite responsive element; CcpA, catabolite control protein A; Crh, catabolite responsive HPr; HPr, histidine containing protein; CRI, contact region I; CRII, contact region II; r.m.s., root mean square; HTH, helix turn helix.

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4 Section 1734 solely to indicate this fact.

5 The atomic coordinates and structure factors (code 1ZVV for CcpA-(Crh-Ser46-P)-cre ternary complex) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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FIGURE 1. Structure of the CcpA-(Crh-Ser^{46}-P)-cre complex. A, ribbon diagram of the CcpA-(Crh-Ser^{46}-P)-cre complex. The CcpA subunits are colored blue and cyan. The Crh-Ser^{46}-P subunits are colored red and the DNA is shown as sticks with oxygen, nitrogen, carbon, and phosphorus atoms colored red, dark blue, light blue, and magenta, respectively. Also shown as sticks are CcpA residues Arg^{324}, Lys^{307}, and Arg^{324} and Crh-Ser^{46}-P residue Gln^{15}. Crh residues Ser^{46}-P are shown as red sticks. This figure and Figs. 2, 3, 8, and C, 4, A and B, and 5 were made with SwissPdb Viewer (46) and rendered with POV-Ray (Persistence of Vision Raytracer, version 3.1). B, simulated annealing composite omit electron density map of the CcpA Arg^{324}-Cre (Crh-Ser^{46}-P) Gln^{15} contact region. The electron density is shown as a blue mesh and contoured at 1.5 σ. Labeled are CcpA residues Arg^{324} and Asp^{296} and Crh-Ser^{46}-P residues Gln^{15} and Ala^{16}. Carbon, nitrogen, and oxygen atoms are colored yellow, blue, and red, respectively. Hydrogen bonds are represented as white dashed lines. This figure was made with O (38).

suggested that Crh residue Gln^{15} would be able to interact with CcpA residue Asp^{296} in a manner similar to the interaction observed between His^{15} and Asp^{296} in the CcpA-(HPr-Ser^{46}-P)-cre structure (27). Interestingly, biochemical studies have revealed that Crh-Ser^{46}-P binds CcpA with up to 10-fold reduction in affinity as compared with HPr-Ser^{46}-P (34), thereby indicating differences in their binding mechanisms.

Previous structural studies on Crh reveal that it has essentially the same fold as HPr. However, a recent crystal structure of Crh showed a domain-swapped dimer and NMR studies have provided evidence that, at high concentrations, CcpA can form a mixture of monomers and dimers (35, 36). From these studies a dimer-dimer type of interaction between Crh and CcpA was suggested. By contrast, the CcpA-(HPr-Ser^{46}-P)-cre structure, which showed two monomers of HPr-Ser^{46}-P bound per CcpA dimer, indicates that dimerization is not likely relevant in the binding of Ser^{46}-P-phosphorylated, HPr-like corepressors to CcpA (27). Moreover, recent biochemical studies, which examined the interaction of CcpA with HPr-Ser^{46}-P and Crh-Ser^{46}-P, indicate that both proteins bind CcpA as a monomer (34). The possible relevance of a Crh-Ser^{46}-P dimerization in its binding to CcpA remains unclear and whether dimerization or other structural alterations of Crh explains the reduced binding affinity of Crh-Ser^{46}-P for CcpA is not known. Therefore, to determine the molecular basis for Crh-Ser^{46}-P binding to CcpA and thus, gain insight into its reduced affinity for CcpA as compared with HPr-Ser^{46}-P, we determined the crystal structure of a CcpA-(Crh-Ser^{46}-P)-cre complex to 2.96 Å resolution.

MATERIALS AND METHODS

Protein Preparation, crystallization, and data collection—Bacillus subtilis and Bacillus megaterium CcpA and B. subtilis Crh-Ser^{46}-P proteins were overexpressed and purified as described (27, 34). Both B. subtilis and B. megaterium His-tagged CcpA were used in crystallization screens as sequence alignment of CcpA proteins revealed that the HPr-Ser^{46}-P interacting surfaces of these proteins are identical (27). The B. megaterium and B. subtilis Crh proteins (both 88 residues) share 64% sequence identity but their predicted CcpA interacting surfaces share 100% sequence identity. Data quality crystals were obtained using only B. megaterium CcpA, B. subtilis Crh-Ser^{46}-P, and the 16-bp cre duplex with the sequence of one strand 5’-CTGTTAGCGCTTTCAG-3’.

Crystals were grown at 298 K using the hanging drop vapor diffusion method by mixing the stoichiometric CcpA(dimer)-(Crh-Ser^{46}-P)(2 monomers)-cre duplex complex 1:1 with a reservoir solution of 22% PEG MME 3350, 0.2 M sodium iodide and sealing the drop over 1 ml of the reservoir. The crystals are monoclinic, space group C2, with a = 83.69 Å, b = 158.10 Å, c = 125.47 Å, and β = 100.73°. For cryoprotection, glycerol was added to a final concentration of 35%. X-ray intensity data were collected at the Advanced Light Source beamline 8.2.1 at 100 K, processed with MOSFLM, and scaled with SCALA (Table 1).

Structure Determination—The CcpA-(Crh-Ser^{46}-P)-cre structure was determined by molecular replacement using the CcpA-(HPr-Ser^{46}-P)-cre structure, with the solvent removed (27), as a search model and the program EPMR (37). Searching with a single subunit of CcpA, its bound HPr-Ser^{46}-P corepressor, and a cre half-site produced three clear solutions: two of which formed complex CcpA-(Crh-Ser^{46}-P)-cre dimer, and the third, a monomer, that when the crystallographic symmetry was applied produced a dimer. This starting model was first subjected to rigid body refinement, in which each CcpA subunit, Crh-Ser^{46}-P molecule and DNA half-site, were treated as rigid units (38, 39). This was followed by multiple cycles of simulated annealing and positional/thermal parameter refinement in CNS and rebuilding in O (38, 39).

RESULTS AND DISCUSSION

Structure of the CcpA-(Crh-Ser^{46}-P)-cre Complex—The CcpA-Crh-Ser^{46}-P-cre DNA complex was crystallized using equimolar amounts of CcpA monomer, Crh-Ser^{46}-P (monomer), and the 16-bp cre site (with one strand of the sequence 5’-CTGTTAGCGCTTTCAG-3’). This sequence was also used in the determination of the CcpA-(HPr-Ser^{46}-P)-cre structure to allow comparison of the CcpA-DNA contacts when bound by the different CcpA-phosphoprotein corepressor complexes (27). The structure was solved by molecular replacement using the CcpA-(HPr-Ser^{46}-P)-cre structure as a search model (Materials and Methods, Fig. 1, A and B, and Table 1). The crystallographic asymmetric unit contains one dimeric CcpA-(Crh-Ser^{46}-P)-cre complex and one monomeric complex in which crystallographic symmetry generates the dimer. Thus, the structure provides three independent views of CcpA-(Crh-Ser^{46}-P) and CcpA-cre interactions. The final model includes residues 1–42, 46–332 of one CcpA subunit and residues 1–332 of the other.
Flexible DNA Binding by the CcpA HTH Elements—The CcpA-(Crh-Ser46-P)-cre structure provides three crystallographically independent views of the interaction between CcpA and the 16-bp cre. CcpA, like other LacI-GalR proteins, kinks its DNA binding site to allow formation of operator-specific, HTH-major groove contacts (41, 42). As expected, these contacts are essentially identical to those observed in the CcpA-(HPr-Ser46-P)-cre structure, in which the same oligodeoxynucleotide was used in crystallization (27) (Fig. 2A). The global DNA bend angle, induced by partial interaction of the dyad-related hinge helix residues Leu55, the "leucine levers," 31°, is the smallest bend angle observed thus far for a LacI-GalR protein; the bend angles of the DNA bound by PurR and LacI are, respectively, 2.96 Å resolution and a subsequent crystal structure revealed a domain-swapped dimer in which the N-terminal β strand, β1, exchanges subunits (35). Superimposition of the Ca atoms of our CcpA-Ser46-P crystal structure onto the corresponding Ca atoms of a Crh subunit in the domain-swapped structure, excluding residues 1–12, which are involved in domain exchange, results in r.m.s. deviation of 1.1 Å. Intriguingly, the crystal structure of CcpA-Ser46-P is also identically domain swapped. What role, if any, CcpA dimerization may play in vivo is unclear. However, our structure, obtained under high protein concentrations (200 μM) clearly reveals that the monomer of CcpA-Ser46-P functions as a corepressor for CcpA, a finding consistent with recent studies examining CcpA binding to Crh-Ser46-P and HPr-Ser46-P by surface plasmon resonance (34).

Comparison of the CcpA-bound HPr-Ser46-P and Crh-Ser46-P Molecules—The three crystallographically independent CcpA-Ser46-P proteins bound to CcpA have the same structures as evidenced from the average r.m.s. deviation of 0.51 Å for the pairwise superimpositions of all 84 Crh Ca atoms. CcpA-Ser46-P contains three α-helices (α1, residues 18–27; α2, residues 47–52; α3, residues 70–81) and four β-strands (β1, residues 2–6; β2, residues 32–37; β3, residues 41–44; β4, residues 61–65) (Fig. 1). Comparison of the CcpA-Ser46-P structure in our ternary complex to that of HPr-Ser46-P in the CcpA-(HPr-Ser46-P)-cre structure results in a r.m.s. deviation of 1.2 Å for 80 corresponding Ca atoms, showing that CcpA-Ser46-P and HPr-Ser46-P adopt essentially the same structure when bound to CcpA. Only two significant differences are found between the HPr-Ser46-P and CcpA-Ser46-P structures bound to CcpA. The first is the conformation of helix α3, which is tilted slightly differently in the two proteins and the second is the absence of HPr-Ser46-P β strand β3 (residues 86–88) from Crh-Ser46-P. These secondary elements are located distal to the regions that interact with CcpA and likely have little impact on CcpA-Ser46-P and HPr-Ser46-P binding to CcpA.

Two structures of Cpr, but not CcpA-Ser46-P, have been reported; one determined by NMR and the other by crystallography. The NMR structure shows a monomer with the same overall fold as Crh in our structure (36). However, the NMR analysis suggested that Cpr dimersize at high concentrations and a subsequent crystal structure revealed a domain-swapped dimer in which the N-terminal β strand, β1, exchanges subunits (35). Superimposition of the Ca atoms of our CcpA-Ser46-P crystal structure onto the corresponding Ca atoms of a Crh subunit in the domain-swapped structure, excluding residues 1–12, which are involved in domain exchange, results in r.m.s. deviation of 1.1 Å. Intriguingly, the crystal structure of CcpA-Ser46-P is also identically domain swapped. What role, if any, Cpr dimerization may play in vivo is unclear. However, our structure, obtained under high protein concentrations (200 μM) clearly reveals that the monomer of Cpr-Ser46-P functions as a corepressor for Cpr, a finding consistent with recent studies examining CcpA binding to Crh-Ser46-P and HPr-Ser46-P by surface plasmon resonance (34).

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usually to accommodate to the precise major groove sequence of the DNA as well as the conformation of the DNA, the latter of which may be influenced by the adjacent DNA or adjacent bound proteins. Similar plasticity has been observed in structures of the Lac repressor (41, 42). However, there appears to be a thermodynamic cost associated with such binding flexibility as CcpA binds the amyE cre nearly 12-fold more tightly than the xyl cre, both of which are responsive to CCR (43). This plasticity allows CcpA to bind half-sites with altered sequences and provides an explanation for how CcpA can bind such a large number of degenerate cre elements that are found in different contexts throughout bacilli genomes (43, 44).

CcpA-Crh-Ser46-P Interactions—Each Crh-Ser46-P monomer docks onto a CcpA subunit in a manner similar to that utilized by HPr-Ser46-P. Indeed, the primary interaction interface is forged between Crh-Ser46-P residues located on helices H9251 and H9252 and the turn between strand H9252 and helix H9251 and the surface exposed region of CcpA that includes helices I and IX from the N-subdomain of CcpA. Thus, CcpA interaction interfaces of HPr-like proteins can be broken into two regions: contact regions I (CRI), encompassing residues 15–28 (helices H9251 and the preceding loop) and contact region II (CRII), comprised primarily of H9252 and phosphorylated residue Ser46 (Fig. 3).

Contact Region II: Conserved CcpA-HPr/Crh Interactions—Residues from CRII of Crh-Ser46-P include the phosphoresidue Ser46-P, which is located one residue N-terminal to a2, and the remainder of a2. Residues from this region are completely conserved between HPr and Crh and in addition to Ser46-P include Ile47, Met48, and Met51 (Fig. 3, A and B). As in the CcpA-(HPr-Ser46-P)-cre structure, Crh-Ser46-P residue Ser46-P is contacted specifically by two CcpA basic residues, Arg303 and Lys307, which are located near the center of CcpA helix IX (Fig. 4A). This interaction is buttressed by Tyr89. CcpA cannot bind HPr-like proteins at physiologically relevant concentrations unless they are phosphorylated on residue Ser46 (6, 26, 34). Such phosphorylation of Ser46 increases the binding affinity of CcpA for cre DNA, indicating the critical nature of this interaction in both binding specificity and affinity (26, 34). In addition, several hydrophobic residues on H9252 interact with hydrophobic residues on CcpA helix IX to form a water-free interface. These Crh-Ser46-P residues, which are conserved in HPr, include Ile47, Met48, and Met51 and interact with CcpA residues Ala299, Val300, and Leu304 (Fig. 3B).

Contact Region I: Discrimination in the Binding of HPr-like Proteins to CcpA—CRI, consisting of the loop-a1 motif, contains loop residues Gln15 and Ala16 and a1 residues Arg17, Ala20, Val23, and Gln24 (Fig. 3). Crh-Ser46-P residues Val23 and Gln24 make similar contacts with CcpA in the CcpA-(HPr-Ser46-P)-cre and CcpA-(Crh-Ser46-P)-cre structures. However, unlike CRII, in which all CcpA interacting residues are conserved between HPr and Crh, CRI contains two positions, 15 and 20, that differ between HPr and Crh. In all Crh proteins sequenced thus far these residues are glutamine and alanine, whereas in all HPr proteins they are histidine and threonine (Fig. 3A). In the CcpA-(HPr-Ser46-P)-cre structure, His15 of HPr hydrogen bonds to the side chain of CcpA residue Asp296 (N/H9254-O/H9254, 2.5 Å), whereas the O/H9254 of Asp296 engages in a hydrogen bond with the amide nitrogen of HPr residue Ala16. Just as the interactions between HPr CRII residue Ser46-P and CcpA basic residues Arg303 and Lys307 serve as key tethering points for HPr-Ser46-P CRII, the
interactions between HPr-Ser46-P residues His15 and Ala16 and CcpA residue Asp296, serve as the anchor for HPr-Ser46-P contact region I to CcpA. In addition, these interactions function to partition the high energy CCR and low energy phosphoenolpyruvate:sugar phosphotransferase pathways because CcpA cannot bind HPr-His15-P and HPr-Ser46-P cannot bind the phosphoenolpyruvate:sugar phosphotransferase enzyme, E1 (45).

We predicted that the conservative H15Q substitution should retain the key hydrogen bond with CcpA residue Asp296 (27). Surprisingly, only two of the three crystallographically independent CcpA-(Crh-Ser46-P)-cre complexes show an interaction between Crh-Ser46-P residue Gln15 and CcpA residue Asp296. Moreover, these contacts are weak (between 3.5 and 4.5 Å). Indeed, the structure shows that the primary contact to Crh-Ser46-P residue Gln15 is from the side chain of CcpA residue Arg324 (Figs. 3, B and C, and 4A). By contrast, in the CcpA-(HPr-Ser46-P)-cre complex, Arg234 is greater than 5.0 Å from His15 (Fig. 3C). In the CcpA-(Crh-Ser46-P)-cre structure the Arg234 side chain rotates down into the CcpA-Crh interface to hydrogen bond with Gln15 (aver-
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The T20A substitution in Crh is the only other difference between the CcpA interacting residues of HPr-Ser46-P and Crh-Ser46-P (Fig. 3A). In the CcpA-(HPr-Ser46-P)-cre structure Thr20 hydroxyl bonds to the phenolic side chain of CcpA residue Tyr295. Obviously, this contact is not possible in the CcpA-(Crh-Ser46-P)-cre complex. The last key CcpA interacting residue in CRI is Arg17. In the CcpA-(HPr-Ser46-P)-cre structure, the guanidinium side chain of Arg17 makes two contacts to residues Asp69 and Asp99, which are located on the other subunit of the CcpA dimer. These “cross-contacts” have been postulated to be important in the allosteric DNA-binding mechanism of CcpA. In the CcpA-(Crh-Ser46-P)-cre structure, the cross-contacts are weakened as Arg17 interacts with only side chain of Asp99 (Fig. 3B). Thus, the interactions between CcpA and CRII of both HPr-Ser46-P and Crh-Ser46-P are highly conserved, but the contacts between CcpA and the CRI of these CCR coregulators are different.

Basis for Differential Binding of Crh-Ser46-P and HPr-Ser46-P to CcpA—The CcpA-(Crh-Ser46-P)-cre structure demonstrates that although Crh-Ser46-P and HPr-Ser46-P bind CcpA in the same manner globally, there are several differences, specifically in the CRI-CcpA interface, that may explain the reduced affinity of Crh-Ser46-P for CcpA compared with HPr-Ser46-P (Fig. 4A). Underscoring these differences, superimpositions of the Ca atoms of one CcpA subunit of the CcpA-(Crh-Ser46-P)-cre structure onto those of a CcpA subunit of the CcpA-(HPr-Ser46-P)-cre structure reveals that whereas the CRII of HPr and Crh dock nearly identically onto CcpA, the CRIIs do not. Specifically, the CRI of Crh-Ser46-P is shifted away from the CcpA molecule as compared with the corresponding region of HPr-Ser46-P (Fig. 4B). This relocation results from the loosening of interactions between loop α1 (CRI) residues of Crh-Ser46-P to CcpA, primarily the weaker Gln15–Arg324 contact, and the loss of the Thr20–Tyr295 hydrogen bond. The shift of CRI away from CcpA also explains the loss of the cross-contact from Arg17 to CcpA residue Asp99, which in this conformation is too far (>4 Å) to form a meaningful interaction. The different docking mode of Crh-Ser46-P is highlighted quantitatively by the buried surface area between CcpA and Crh-Ser46-P, which, whereas still sizeable, is 100 Å2 less than that observed between HPr-Ser46-P and CcpA (1300 Å2 compared with 1400 Å2). An additional contribution to the different binding affinities of Crh-Ser46-P and HPr-Ser46-P to CcpA may be the difference in the unliganded/liganded states of these phosphoproteins, if Crh actually exists in a monomer to dimer equilibrium in vivo. Indeed, we find that the domain-swapped Crh dimer would not be competent for CcpA binding. However, the physiological relevance, if any, of the domain-swapped Crh dimer remains to be determined.

Crp-Ser46-P Is a Functional Corepressor for CcpA—Binding of HPr-Ser46-P or Crh-Ser46-P to CcpA allows the regulator to bind cre sites with high affinity and specificity. The CcpA-(HPr-Ser46-P)-cre and apo-CcpA structures reveal that HPr-Ser46-P mediates a novel two-component allosteric DNA-binding activation mechanism that involves rotation of the CcpA subdomains as well as a direct, coregulator-induced relocation of a pivot-point residue, Thr61, located between the N-domain and the DNA-binding domain of CcpA (27). Key to the relocation of Thr61 is the interaction between Ser46-P and CcpA residue Arg303, which causes a rotation of Arg303 and a concomitant shift in the position of Tyr89. This movement is translated to the entire Tyr89 loop, which causes the hydrogen bond between Tyr295 and Thr306 to break. Tyr89 then rotates toward the CcpA dimer interface, dislodging Thr61 (27). The final result is the juxtaposition of the DNA-binding hinge regions, allowing formation of the hinge helices in the presence of cognate DNA. What is notable about this activation mechanism is the principal role played by residues of helix α2. Overlays of the CcpA-(Crh-Ser46-P)-cre and CcpA-(HPr-Ser46-P)-cre structures show that although α1 of the Crh complex is significantly shifted as compared with α1 in the HPr complex, α2 remains similarly docked against CcpA (Fig. 4B). Moreover, α2 residues of both Crh and HPr make essentially identical contacts to CcpA. Therefore, despite the weakening of the CRI interface, the interactions between the CRII of Crh-Ser46-P and CcpA remain adequately positioned to flip the DNA-binding switch to “on.” This finding provides an atomic explanation for data showing that although...
that of HPr-Ser\(^{46}\)-P is explained readily by weaker Crh-Ser\(^{46}\)-P interactions of its CRI to CcpA, which causes this region to shift away from CcpA. Nonetheless, the interface between CcpA and helix \(\alpha2\) (CRII) of Crh-Ser\(^{46}\)-P is maintained and thereby demonstrates this contact region is necessary and sufficient to throw the allosteric switch and activate cre binding by CcpA. Finally, this study, which reveals how small differences in protein-protein interfaces can have a significant impact on the structure of a complex, underscores the need to take great caution in modeling protein-protein interactions even when a highly homologous structure is available.

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