PROTEINS: Structure, Function, and Bioinformatics 64:814–816 (2006)

STRUCTURE NOTE

Structural Analysis of *B. subtilis* CcpA Effector Binding Site

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Introduction. In Gram-positive bacteria, the catabolite control protein A (CcpA) is the central regulator of a fundamental signal transduction pathway called carbon catabolite repression.[1] In response to the availability of glucose or other rapidly metabolizable carbohydrates in the growth medium, CcpA recognizes and binds *cis-acting* palindromic DNA sequences called catabolite response elements (*cre*)[2] and regulates the transcription of numerous carbon catabolic operons.

CcpA belongs to the LacI/GalR family of bacterial regulatory proteins characterized by an N-terminal helix–turn–helix type DNA-binding domain and a large C-terminal effector-binding domain.[3] Most of the family members bind to their cognate DNA depending upon the presence or absence of low-molecular-weight effectors. The effector-binding site is formed by a cleft between the two subdomains of the core protein that resembles periplasmic sugar-binding proteins.[4] Unlike other members of the family, CcpA is activated by a protein that acts as co-repressor, PserHPr, the Ser46-phosphorylated form of HPr, a phosphocarrier protein of the bacterial phosphotransferase system.[5] In bacilli, PserCrh, the Ser46-phosphorylated form of Crh, a protein homologous to HPr, is an alternative coregulator of CcpA.[6]

Small-molecule effectors have also been shown to regulate CcpA activity. Fructose-1, 6-bisphosphate (FBP) enhances the affinity of CcpA for PserHPr.[5] The ternary complex CcpA/PserHPr/FBP shows the highest affinity for DNA.[7] Glucose 6-phosphate (G6P) triggers cooperative binding of CcpA to *cre* sites at low pH.[8] Interestingly, the effects of FBP and G6P on the CcpA–PserHPr complex are not observed when PserCrh is used as co-repressor of CcpA instead of PserHPr.[9] It has also been proposed that NADP/NADPH could enhance interaction of CcpA with the transcription machinery.[7] Recent results suggesting direct interaction of CcpA with the RNA polymerase confirm this hypothesis.[10]

The X-ray structures of *Bacillus megaterium* apo CcpA and of ternary complex with a *cre* site and PserHPr or PserCrh have been reported.[11,12] The co-repressor molecule binds to the N-subdomain of CcpA's core, inducing a conformation change and suggesting an allosteric regulatory mechanism. The molecular mechanism of CcpA fine regulation by the small molecule effectors remains nevertheless unclear. We therefore decided to crystallize CcpA in presence of FBP. Here, we report the 2.45-Å resolution X-ray structure of the binary complex between *B. subtilis* PserHPr and ΔCcpA, a truncated form of CcpA lacking the N-terminal DNA-binding domain. We focus on the effector-binding mode.

X-ray Structure Determination. The *B. subtilis CcpA* gene deleted of the first 57 codons encoding the DNA binding domain was cloned into a pQE30 plasmid (Qiagen). The resulting ΔCcpA (residues Ala57 to Ser334) carrying a N-terminal histidine tag was expressed in the *Escherichia coli* strain M15 and purified by IMAC chromatography followed by gel filtration. *B. subtilis* HPr was produced in *E. coli* with a N-terminal histidine tag as described.[13] ATP-dependent seryl-phosphorylation of HPr was carried out as described using the *B. subtilis* HPr kinase/phosphorylase.[14]

Crystals were grown in sitting drops containing 70 μM ΔCcpA, 138.5 μM PserHPr, 4 mM FBP, 0.5% (v/v) MPD, 50 mM Mes (pH 6.5), 1.75 M ammonium sulfate over pits containing 1% (v/v) MPD, 100 mM Mes (pH 6.5), and 3.5 M ammonium sulfate. The asymmetric unit contains one ΔCcpA monomer bound to one PserHPr molecule. Diffraction data were collected at 100 K on an ADSC Quantum Q4 detector at the European Synchrotron Radiation Facility (Grenoble) on beamline ID14-H1 with a single crystal directly flash frozen in liquid nitrogen. Diffracted intensities, evaluated using MOSFLM,[15] and further processed with the CCP4 program suite,[16] were 99.9% complete at 2.45 Å resolution (Table I).

Grant sponsor: CNRS; Grant sponsor: ARC.

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Received 20 December 2005; Revised 21 February 2006; Accepted 2 March 2006

Published online 5 June 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.21001

TABLE I. Crystallographic Data and Refinement Statistics

Space group	$P4_12_12$
Unit cell parameters (Å)	a = b = 67.6, c = 167.2
Resolution range (Å)	20 − 2.45 (2.58–2.45)
Unique reflections	14990 (2132)
Completeness (%)	99.9 (99.9)
I/σ	6 (2)
R_{sym} (%)[a]	9.1 (36)
R_{cryst} (%)[b]	20.9
R_{free} (%)[c]	28.4
RMSD of bonds (Å)	0.006
RMSD of angles (degrees)	1.22
Average B-values CcpA (Å^2)	23
Average B-values PserHPr (Å^2)	38
Average B-values solvent (Å^2)	31.2

[a] $R_{sym} = \Sigma\,|I_i - \langle I\rangle|/\Sigma I_i$, where I_i is the intensity of a reflection, and $\langle I\rangle$ is the average intensity of that reflection.
[b] $R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma\,|F_{obs}|$
[c] Seven percent of the data were set aside for free R-factor calculation. The values for the last resolution shell are indicated in brackets.

The structure of the ΔCcpA–PserHPr complex was solved by molecular replacement with Phaser[17,18] using coordinates from the CcpA-PserHPr-DNA complex (PDB code 1RZR)[11] as a search model. Refinement by CNS[19] yielded a final model with R_{cryst} = 20.87 % and R_{free} = 28.4% and a good geometry (Table I). The electron density maps were examined using TURBO-FRODO.[20] The final model includes residues 61–333 of *B. subtilis* CcpA and residues 2–88 of *B. subtilis* PserHPr. The ΔCcpA/PserHPr dimer was generated by crystal symmetry [Fig. 1(A)]. The coordinates and structure factors have been deposited at the RCSB Protein Data Bank with the accession code 2FEP.

Structure Analysis. *B. subtilis* CcpA shares 75% sequence identity with *B. megaterium* CcpA and displays the same subunit fold.[11] The C-terminal PserHPr-binding core is in the active closed conformation described in the *B. megaterium* ternary complex[11] with a root-mean-square deviation (RMSD) of 0.7 Å for all equivalent CcpA Cα atoms. The binding mode of PserHPr is conserved with an RMSD of 0.97 Å between the two binary complexes. Thus the absence of DNA binding prevents neither the corepressor binding nor the CcpA conformation change.

Clear residual density was observed in the effector-binding cleft but it could not be accounted for by FBP present at 4 mM in the crystallization drop. It was interpreted as two sulfate ions stemming from the 3.5 M ammonium sulfate present in the crystallization solution. They both have partial occupancy (75%) in the final model. One sulfate ion (SO_4-1) interacts with the side chain of Tyr76 and through water molecules with the side chains of Ser73, Lys197, and Asp276. The other sulfate ion (SO_4-2) interacts directly with the side chains of Thr248, Asp276, and Thr278 and with Asp249 main-chain nitrogen. An additional interaction with the main-chain oxygen of Tyr222 is made through a bridging water molecule [Fig. 1(B)].

Small-Effector Binding. FBP increases the affinity of CcpA to PserHPr[5] and stimulates CcpA/PserHPr binding to *cre*.[9] In the crystal, we observe two sulfate ions instead of the expected co-crystallized FBP. However, their presence suggests a binding mode for FBP. Indeed, a FBP molecule can be modeled with its two phosphate groups superimposing on the two sulfate ions that are ~5 Å apart.

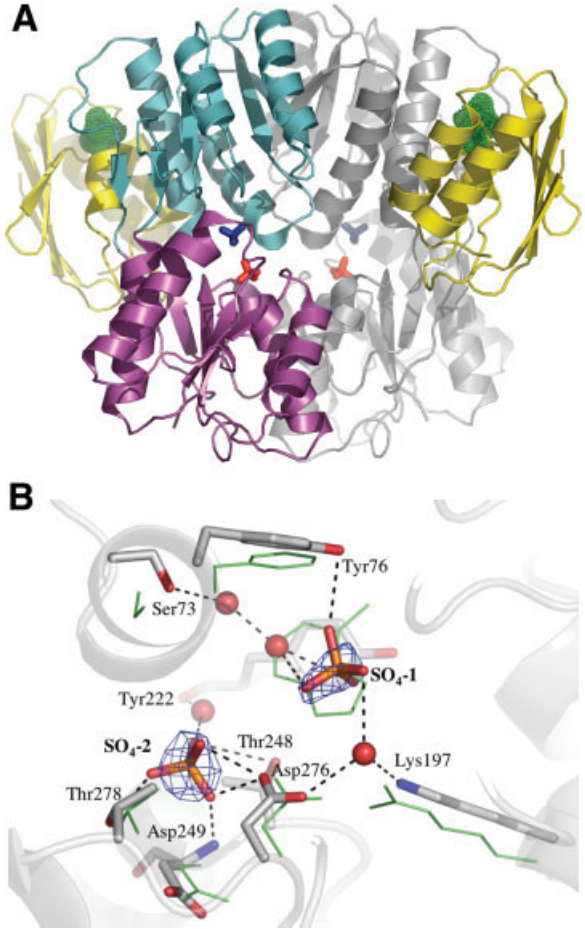

Fig. 1. *B. subtilis* ΔCcpA-PserHPr binary complex. **A:** Overall structure. The two PserHPr molecules are shown in yellow with their P-Ser46 highlighted in green dots. One subunit of the ΔCcpA core is shown in gray and the other in cyan (N-subdomain) and magenta (C-subdomain). The sulfate ions observed in the effector-binding clefts are shown in sticks (SO_4-1 in blue, SO_4-2 in red). **B:** Details of the effector-binding cleft. The two sulfate ions are shown as sticks in a Fo-Fc omit map contoured at 6σ. H-bonds with surrounding CcpA residues and water molecules are shown as black dashes. Superimposition of the *E. coli* PurR structure (PDB code 2PUA) bound to the effector analog 6-methylpurine is shown in green lines.

The residues of CcpA involved in the interaction with the two sulfate ions are mainly conserved in other members of the family. A superimposition with *E. coli* purine repressor PurR[21] bound to 6-methylpurine (PDB code 2PUR) shows that one sulfate ion (SO_4-1) occupies the position of the effector analog [Fig. 1(B)]. Superimposition with other members of the family yields the same result. This sulfate ion is likely to mimic the phosphate group common to G6P and FBP. The direct interaction between the other sulfate ion and Asp276 carboxylate implies that this residue is protonated at pH 6.5. Aspartate residues with pKa values >6.5 are mostly buried in active sites and implicated in ligand binding.[22] Asp276 is not strictly conserved among all members of the family but it has been

implicated in effector recognition. An Asp to Ala mutation at this position in the purine repressor protein PurR abolishes the interaction with small effector molecules.[23] In *B. megaterium* CcpA, an Asp to Gly mutation induces permanent, glucose-independent repression of gene expression and PserHPr-independent DNA binding.[24]

Structural comparison of CcpA with FBP-controlled enzymes shows that its C-terminal subdomain presents the same fold as the FBP-binding C-terminal domain of pyruvate kinase[25] with an RMS distance of 2.88 Å over 88 Cα atoms (PDB code 1A3W). Superimposition of pyruvate kinase onto CcpA reveals that the FBP molecule fits into CcpA's effector-binding cleft. The CcpA N-terminal subdomain presents a NADP-binding fold similar to the cofactor-binding domain of G6P dehydrogenase[26] with an RMSD of 3.8 Å over 122 Cα atoms (PDB code 1H94). Superimposition of the two structures suggests that NADP could bind in the effector-binding site of CcpA.

A complete understanding of the fine-tuning regulation of CcpA activity by these small molecule effectors requires additional structural and biochemical characterization.

Acknowledgments. We thank the Yeast Structural Genomics project (Orsay, France) for making their robotized crystallization platform available, the staff of beamline ID14-H1 at ESRF (Grenoble, France), Diep Le (LEBS, Gif-sur-Yvette, France) for EDMAN sequencing, Laurent Lariviére for help in data collection and Pr. Joël Janin for critical reading of the manuscript.

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