Structural Analysis of the DNA-binding Domain of the *Erwinia amylovora* RcsB Protein and Its Interaction with the RcsAB Box*

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The transcriptional regulator RcsB interacts with other coactivators to control the expression of biosynthetic operons in enterobacteria. While in a heterodimer complex with the regulator RcsA the RcsB box consensus is recognized, DNA binding sites for RcsB without RcsA have also been identified. The conformation of RcsB might therefore be modulated upon interaction with various coactivators, resulting in the recognition of different DNA targets. We report the solution structure of the C-terminal DNA-binding domain of the RcsB protein from *Erwinia amylovora* spanning amino acid residues 129–215 solved by heteronuclear magnetic resonance (NMR) spectroscopy. The C-terminal domain is composed of four α-helices where two central helices form a helix-turn-helix motif similar to the structures of the regulatory proteins GerE, NarL, and TraR. Amino acid residues involved in the RcsA independent DNA binding of RcsB were identified by titration studies with a RcsA box consensus fragment. Data obtained from NMR spectroscopy together with surface plasmon resonance measurements demonstrate that the RcsAB box is specifically recognized by the RcsAB heterodimer as well as by RcsB alone. However, the binding constant of RcsB alone at target promoters from *Escherichia coli*, *Erwinia amylovora*, and *Pantoea stewartii* was approximately 1 order of magnitude higher compared with that of the RcsAB heterodimer. We present evidence that the obvious role of RcsA is not to alter the DNA binding specificity of RcsB but to stabilize RcsB-DNA complexes.

The RcsB protein is a key regulator in enteric and plant pathogenic bacteria and represents the transcriptional effector of a modified two-component system. RcsB is essential for the induction of exopolysaccharide (EPS) biosynthesis (1), an important factor for the virulence of bacterial pathogens. It is further involved in rcsA autoregulation (2, 3), in the regulation of cell division (4, 5), in the expression of the osmoregulated gene *osmc* (6) and probably in regulation of motility and chemotaxis (7–9). The Rcs regulation system is different from homologous systems as the phosphorylation of RcsB involves two other proteins, the membrane sensor RcsC (1) and the histidine-containing phosphotransmitter RcsD (YqJ) (8). A third protein, RcsF, might be additionally involved in the activation of RcsB (10). Whereas specific signals recognized by the sensor protein RcsC still remain unknown, the RcsB regulation pathway can be activated by osmotic shock (11) and desiccation, by mutations affecting the integrity and composition of the cell envelope (12, 13), by treatment with the cationic amphoteric substance chlorpromazine (9), and by overproduction of the chaperone-like transmembrane protein DjlA (14, 15).

A further modification of the RcsB regulation mechanism if compared with conventional two-component systems might be the ability of RcsB to recognize different nucleic acid structures in combination with other proteins. RcsB does interact with various coinducers like RcsA (12) and probably TviA (16), and the formation of alternative protein complexes might direct the regulator to different targets. It has been shown that the EPS production is induced by the binding of a heterodimer of RcsB and RcsA at the RcsAB box (17, 18). This 14-bp consensus sequence is present in the promoters of all analyzed Rcs-regulated operons for capsule synthesis, as well as in rcsA promoters. However, several other genes in *Escherichia coli*, like the osmoregulated gene *osmc* (6) as well as cell division genes controlled by the *fts* promoter (5), and the genes responsible for Vi antigen synthesis in *Salmonella typhi* (16) were reported to be controlled by RcsB in a completely RcsA independent mechanism. Still it remains unclear whether the RcsA independent regulation mechanisms are mediated by RcsA alone or by the interaction of RcsB with further, yet unidentified coinducers.

The requirement for RcsA in EPS regulation can be bypassed by increasing the copy number of RcsB (19), indicating that RcsA modulates the RcsB-mediated transcriptional activation, but that it seems not to be essential for the general regulation mechanism. RcsA might help to keep RcsB in an active conformation by stabilizing its phosphorylation. This assumption might be supported by the observation that a mutant in the putative RcsB phosphorylation motif results in the RcsA independent overproduction of EPS in *E. coli* (6). In any case, regulation of different operons by RcsB alone or in combinations with certain coactivators would imply different modes of DNA binding.

DNA binding activity of RcsB alone could not be clearly demonstrated so far. An interaction with specific DNA-binding sites by electrophoretic mobility shift assays could be shown only in combination with RcsA (17) or RNA polymerase (6). Two

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The atomic coordinates and structure factors (code 1NRL) 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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§ The abbreviations used are: EPS, exopolysaccharide; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; HSQC, heteronuclear single-quantum coherence; NOE, nuclear Overhauser effect; r.m.s.d., root mean square deviation; HTH, helix turn helix, SPR, surface plasmon resonance.
well conserved sequence motifs can be found in RcsB, a N-terminal phosphorylation motif involving three aspartic acid residues at positions 10, 11, and 56, and a C-terminal helix-turn-helix (HTH) DNA binding motif spanning amino acid positions 151–194 and sharing sequence homology with the autoinducer homoserine lactone-dependent LuxR-type regulators (20) and the phosphorylation controlled FixJ/UhpA family of activators (21). The 24-kDa protein RcsB can therefore be divided into a N-terminal “receiver” and probably protein interacting domain, and into a C-terminal “effector” domain interacting with DNA. To analyze the DNA binding properties of RcsB, we have solved the solution structure of the RcsB effector domain by high resolution 1H, 15N, and 13C NMR spectroscopy and we have further analyzed the interaction of RcsB with one of its DNA targets. Specific amino acid residues of RcsB interacting with the RcsAB box have been identified and we could show that RcsA considerably stabilizes the RcsAB-DNA complex. However, we also could demonstrate that RcsA is not essential for the specific recognition of the RcsAB box by RcsB.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Oligonucleotides, and DNA Techniques—Strains and plasmids used for DNA cloning and expression studies are listed in Table I. Standard DNA techniques were used as described elsewhere (24). The coding sequence for the Erwinia amylovora C-RcsB protein spanning residues 129 to 215 was amplified by standard PCR with Vent polymerase using the plasmid pQ-RcsBEA as a template and primers RcsBEA C-up (GGGAGATCTTACCGCGGCGCT) and RcsBEA C-low (ACCTGGCAATTTATTCACGGCGCT). The DNA was cloned with enzymes BamHI and PstI into the plasmid pQE30 resulting in plasmid pQ-CrsBEA, which was used as expression vector.

Expression and Purification of Proteins —The RcsA protein was produced with the plasmid pQ-RcsBEA (17) in strain BL21 as C-terminal fusion to the maltose-binding protein. The RcsB protein was produced with plasmid pQ-RcsBEA (17) with an N-terminal poly(His) tag in strain BL21 as C-terminal fusion to the maltose-binding protein. The RcsB protein was eluted with a NaCl gradient up to 1 M in 60 min at a flow rate of 2 ml/min. The C-RcsB protein eluted at approximately 500 mM NaCl and the combined fractions were concentrated by ultrafiltration up to a final concentration of 1 M in 50 mM phosphate buffer, pH 6.4.

Surface Plasmon Resonance (SPR) Technique—SPR measurements were performed with a BIAcore X instrument (BIAcore, Uppsala, Sweden) using immobilized DNA (about 60 resonance units) coupled to the streptavidin-coated sensor chip SA as recommended by the manufacturer. The experiments were carried out at a flow rate of 50 µl/min. The DNA fragments and proteins were diluted in running buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM dithiothreitol, 0.1 mM EDTA). Bovine serum albumin and λ-DNA were added to the protein solutions to a final concentration of 1 mg/ml, respectively. SPR mixtures of various concentrations ranging from approximately 47 ng to 7.5 µg were injected allowing an association time of 120 s and a dissociation time of 300 s. A reference flow cell loaded with a random DNA target of the same size as the probe DNA target was used to subtract unspecific DNA/protein interactions. Regeneration of the chip surface was achieved by removing all bound proteins with a pulse of 5 µl of 0.05% SDS in running buffer.

Kinetic analyses were done using the BIAevaluation 3.0 program. To determine the binding properties of the proteins, 1:1 Langmuir kinetics provided by the software were used.

Biochemical Assays —The enzymatic activity of the β-galactosidase was determined with the o-nitrophenyl-β-D-galactopyranoside assay after Miller (25). Electrophoretic mobility shift assays were done as described (17). The band intensities were visualized using a phosphoimaging plate. Intensities of the bands were quantified with Image-Quant version 4.1 from Amersham Biosciences.

NMR Data Collection and Processing—NMR data collection was carried out at 289 K on Bruker DMX and DRX spectrometers operating at 1H-resonance frequencies of 495, 600, and 800 MHz using 5-mm triple-resonance (1H/13C/15N) probes with Z- or XYZ-gradient capability. The reduced temperature was chosen because of instability of the protein at room temperature and millimolar concentrations needed for NMR measurements. All three-dimensional experiments made use of pulsed field gradients for coherence selection and artifact suppression, and utilized gradient sensitivity enhancement schemes wherever appropriate (26, 27). Homonuclear as well as 13C- and/or 15N-NMR TOCSY and NOESY experiments (28, 29) were acquired and processed as described elsewhere (30, 31). DNA titration experiments were performed at 600 MHz with an initial C-RcsB concentration of 0.4 mM at 15°C with a reconstituted 14-bp RcsAB box of the sequence TGAAGTACTCT in running buffer.

Restraints Generation and Structure Calculation —The NOE-derived distance restraints were determined from two-dimensional homonuclear NOESY and three-dimensional 1H- and 15N-edited NOESY-HSQC spectra, including a constant-time 1H-edited NOESY-HSQC for the aliphatic side chain region providing positive peaks for protons bound to a carbon atom with 1 or 2 aliphatic carbon neighbors, and negative peaks for protons bound to a carbon atom with 2 carbon atom neighbors (32). The sequential assignment was confirmed using the self-written program st2nmr (33). Semi-automated assignments of the NOE cross-peaks based on chemical shifts and a preliminary structure derived from manual homology building with NarL (Protein Data Bank code INRL) were obtained with the self-written program nmr2st (34). Stereoselective assignments of the prochiral methylene and isopropyl methyl groups were obtained using the program GLOMSA (35). Pseudo-atom correction for unassigned stereo partners and magnetically equivalent protons was applied as proposed (36).

All structure calculations were performed as described previously.
RESULTS

Specific Recognition of the RcsAB Box by the RcsB Protein—The RcsA independent activation of EPS biosynthesis in *E. coli* caused by multicopy *rcsB* strongly indicates a specific interaction of RcsB alone with Rcs-regulated promoters. We therefore analyzed the interaction of the *E. amylovora* RcsB protein in the absence of the coinducer RcsA with the promoters of EPS biosynthetic operons from three different species by the highly sensitive SPR technology. The selected promoters were Pwza from the *E. coli* operon for colanic acid biosynthesis, PamsG from the *E. amylovora* operon for amylovoran biosynthesis, and PcpsA from the *P. stewartii* operon for stewartan biosynthesis. PCR generated DNA fragments of the three promoters with approximately 110 bp in length and containing the previously described RcsAB boxes were analyzed by incubating with a 750 nM solution of purified RcsB. We could clearly demonstrate a specific interaction of RcsB alone with all three DNA fragments (Fig. 1A). In steady state kinetics using RcsB concentrations from 0.4 nM to 3 μM, the *Kd* values of the RcsB/DNA interaction were calculated to 4.6 ± 3 × 10^{-6} with Pwza, 2.2 ± 10^{-6} with PamsG, and 5.4 ± 10^{-6} with PcpsA. Characteristic for these interactions was a high instability and an immediate dissociation of the protein-DNA complexes. The half-lives of all three complexes was less than a second and too short to become estimated with our technique. This rapid dissociation of RcsB from its DNA target might contribute to the failure to receive a clear retared band by analyzing the RcsB/DNA interaction by other techniques like the electrophoretic mobility shift assay.

A 14-bp consensus sequence called the RcsAB box was previously described as the RcsB-binding region in the *wza*, *amsG*, and *cpsA* promoters (3). We next determined whether the RcsAB box is also important for the DNA binding of RcsB alone at these promoters. For this purpose, the 14-bp RcsAB box in fragment Pwza was deleted and the binding of RcsB to the modified 110-bp fragment was analyzed by SPR (Fig. 1C). We could not detect any binding, indicating that the RcsAB box is also essential for an interaction of RcsB alone with DNA.

RcsA Stabilizes the DNA Interaction of RcsB—As heterodimer formation with RcsA is obviously not a prerequisite for RcsB to recognize the RcsAB box, we now analyzed the influence of RcsA on the DNA binding characteristics of RcsB. Addition of RcsA protein equimolar to RcsB considerably decreased the dissociation of the proteins from the three DNA fragments and stabilized the protein-DNA complexes (Fig. 1B). We obtained *Kd* values of 3.8 ± 0.1 × 10^{-7} with Pwza, 2.8 ± 0.8 × 10^{-8} with PamsG, and 1.5 ± 0.3 × 10^{-7} with PcpsA. The half-lives of all three RcsAB-DNA complexes were extended to several minutes and the most stable complex was formed with PamsG, followed by Pwza and PcpsA. The *Kd* of the RcsB-Pwza complex using protein concentrations of 94 nM to 1.5 μM was calculated as 3.1 ± 1.7 × 10^{-3}. These results indicate that a major role of RcsA in the activation of EPS biosynthesis is the stabilization of the RcsB-DNA complex.

Characterization of the C-terminal DNA-binding Domain of RcsB—A further detailed characterization of the different binding modes of RcsB requires its structural analysis by solution NMR spectroscopy. Unfortunately, the full-length RcsB protein is sparingly stable in the concentrations required for NMR spectroscopy. In addition, only a few signals were obtained from the N-terminal domain indicating an open solvent accessible structure. These findings presently preclude the study of the full-length RcsB protein by NMR spectroscopy. In contrast, the C-terminal part spanning amino acids 129–215 (C-RcsB, Fig. 2) and including the complete effector domain was reasonably stable. The C-RcsB protein was overproduced from the expression plasmid pQ-CrcsB_EA in *E. coli*, generating a modified protein with an addition of 12 amino acid residues at the N-terminal end including a poly(His)_6 tag. The protein stayed soluble and could be purified in two steps using Ni^{2+}-chelate and heparin chromatography. To analyze whether the truncated RcsB protein is produced with a functional conformation, we transformed the plasmid in strain JB3034 x pEA101, containing a chromosomal *cpsB::lacZ* insertion as a reporter gene, and the *E. amylovora* *rcsA* gene on the compatible multicopy plasmid pEA101. The *lacZ* expression in this strain is activated, as the *cps* operon is strongly induced by binding of a RcsAB dimer and because of the increased copy number of...

Fig. 1. Interaction of the RcsB protein with DNA fragments containing the RcsB boxes of the promoters of *amsG*, *wza*, and *cpsA*. The protein/DNA interaction was analyzed by SPR technology using DNA fragments of approximately 110 bp. The concentration of each protein was 750 nM. Solid line, PamsG; dotted line, PcpsA; broken line, Pwza. A, DNA binding of RcsB alone. B, DNA binding of RcsB with RcsA. C, interaction of RcsB alone with Pwza after deletion of the 14-bp RcsAB box. The protein concentration was 1.5 μM.
C-RcsB Structure

**RcsB**

The solution structure of C-RcsB in the native state (Fig. 5). The DNA binding was analyzed in an electrophoretic mobility shift assay with a labeled 183-bp fragment from the *E. amylovora amsG* promoter containing the RcsAB box. The RcsB and RcsA proteins were added in concentrations of 2 and 15 μM, respectively. The formation of protein-DNA complexes was analyzed by electrophoresis in a 5% acrylamide gel and the separated bands were quantified with PhosphorImager.

**Short- and medium-range NOE patterns** were observed for the backbone protons in the NOEY spectra (Fig. 4). Five helical regions involving residues 133–142, 153–164, 168–177, 179–193, and 198–210 are indicated by the strong sequential NOE connectivities, as well as consensus chemical shift index data calculated from chemical shift index data calculated from $^1$H, $^{12}$C, $^{13}$Cβ, and $^{13}$CO chemical shift values (Ref. 39; data not shown). The three prolines in positions 131, 153, and 212 are all present in the trans configuration, as indicated by the observation of strong NOE connectivities between their H$^n$ protons and the H$^i$ of the corresponding preceding residues.

The experimental NOEY peaks from two- and three-dimensiona spectra were assigned, integrated, and transformed into upper distance restraints. A total of 1618 were found to be meaningful and therefore taken into account by the program DYANA (38) in the structure calculations. 22 stereospecific assignments of diastereotopic groups were obtained with the program GLOMSA (35). Diastereotopic methyl groups with non-degenerate proton resonances could be stereospecifically assigned for 5 of 6 valine and 4 of 9 leucine residues. One-hundred structures were finally calculated. An ensemble of 20 final energy-minimized structures was selected to represent the solution structure of C-RcsB in the native state (Fig. 5). The statistical information for this family of structures is summarized in Table II. This analysis does not include residues 129–130, 144–151, and 213–215 that display trivial NOE connectivities only and are most probably located in highly mobile regions of the protein. The residues 144–152 represent the loop region connecting the first and the second helical region, displaying local r.m.s.d. values of 1.26–3.57 Å. The vast majority of the residues in the NMR ensemble are located in the core (allowed) region of the Ramachandran plot (Table II). Only
0.1% of the total number of residues fall into disallowed regions.

The solution structure of C-RcsB contains five helices (Fig. 6), designated α6 to α10 according to the corresponding helices in the homologous proteins NarL (43) and GerE (44) (Fig. 2). The central DNA binding HTH motif is formed by helices α8 and α9 and is supported by α7. Helix α10 completes a hydrophobic core of the domain. The N-terminal helix α6 has modest hydrophobic interactions with the C-terminal helix α10. The three helices α7 to α9 are fixed to their proper positions by a hydrophobic cluster formed by the side chains of Val-158, Ile-171, and Ile-182. Leu-175 anchors the loop between α8 and α9 to the cluster. A conserved glycine residue is located at position 165 with \((\psi, \chi)\) values of \((145^\circ, -45^\circ)\) to assure a proper angle between helices α7 and α8. Other highly conserved residues contributing to the stability of the fold are Glu-155 at the amino end of α7 and Lys-192 at the carboxyl end of α9.

The overall molecular structure of the protein is homologous to the C-terminal DNA-binding domains of the transcriptional regulators NarL (Ref. 43, Protein Data Bank code 1RNL) and TraR (45, 46). Helices α6 and α7 are connected by a flexible tether that is not visible in the x-ray structure analysis of NarL, and it is presumably disordered. This region corresponds to the region 143–149 of C-RcsB that is highly mobile in the solution structure and was excluded from the structure statistics (see above). The proper angle between α7 and α8 is assured by Gly-170 that adopts \((\psi, \chi)\) values of \((85^\circ, 15^\circ)\). A further related structure is reported from the small transcriptional regulator GerE (Ref. 43, Protein Data Bank code 1FSE); here a helix corresponding to helix α6 of C-RcsB is missing while the four C-terminal helices including the HTH motif are well conserved. The residue corresponding to Gly-165 of C-RcsB and Gly-170 of NarL is Asp-26; it adopts \((\psi, \chi)\) values of \((70^\circ, 37^\circ)\). Both x-ray structures therefore place the corresponding residue in the \(\alpha_L\) region of the Ramachandran plot. It remains unclear whether the discrepancy with the NMR solu-
Identification of DNA Interacting Residues in the C-terminal Domain of RcsB—NMR chemical shift perturbation mapping was performed to study interactions of C-RcsB with the RcsAB box. A 0.4 mM solution of the 15N-labeled C-RcsB protein was titrated with a 4 mM solution of the reconstituted PamsG14 fragment representing the 14-bp RcsAB box from the E. amylovora PamsG promoter. The protein:DNA ratios used for the titration were 8:1, 2.7:1, 1.5:1, and 1:1. Several residues showing significant chemical shift differences upon addition of the DNA could be identified (Fig. 8), giving evidence for a specific interaction of C-RcsB with the RcsAB box. The detection of only a few specific chemical shift differences after an interaction of C-RcsB with the RcsAB box by NMR spectroscopy is in accordance with our results from SPR experiments, showing specific but weak interactions of RcsB alone with DNA fragments containing the RcsAB box.

DISCUSSION

Proteins with structures homologous to RcsB are the regulator for nitrate uptake NarL (43) of E. coli, belonging to the FixJ family of response regulators, the sporulation regulator GerE (44) of Bacillus subtilis, representing an autonomous effector domain, and the autoinducer dependent regulator TraR of Agrobacterium tumefaciens (45, 46), a member of the LuxR family of regulators. C-RcsB could best be aligned with NarL and GerE, showing that the effector domain starts with...
helix α7 and that the HTH motif is composed of helices α8, the scaffold helix, and α9, the recognition helix. The complete structural unit is composed of a four-helix bundle including helices α7 and α10. Amino acid residues 143 to 152 represent a flexible tether between the RcsB receiver and effector domain, and helix α6 might function as a linker helix between the two domains as suggested for the homologous helices α6 of NarL and TraR. The analyzed C-RcsB protein therefore comprises the complete effector domain including the interdomain region. The C-RcsB protein was found to be correctly folded as indicated by competition experiments in DNA interaction assays. The inhibition of RcsAB-DNA complex formation by C-RcsB is most likely because of an interaction of its HTH motif with the DNA target, but an additional interaction with either the RcsA or RcsB protein cannot be excluded. For GerE as well as for TraR, an involvement of helix α10 in protein dimerization was reported (44, 45). Inactive protein complexes might therefore be formed by oligomerization of C-RcsB with RcsA or RcsB. However, it is not known whether RcsB forms dimers or oligomers in solution or upon binding to DNA.

The DNA binding surface of C-RcsB at the 14-bp RcsAB box was analyzed by heteronuclear NMR spectroscopy in DNA titration experiments. In general, the amide protons in the majority of residues exhibit none or only very small chemical shift changes indicating that the overall fold of the protein is not altered upon binding to DNA. The data gave no evidence for a very strong interaction, which is in agreement with our results obtained by SPR measurements of the RcsB/RcsAB box interactions, where the DNA binding affinity and the half-time of the RcsB-DNA complex was considerably decreased in the absence of RcsA. However, a specific recognition of the RcsAB box by RcsB alone was evident. The highest accumulation of chemical shift changes of C-RcsB was found in the recognition helix of the HTH motif and in the linker between scaffold and recognition helix, the turn of the HTH motif. The sequence RSIKTIS of the C-RcsB HTH motif is most likely responsible for DNA recognition and we could identify five of these residues being involved in the interaction with the RcsAB box. Accordingly, the N-terminal ends of recognition helices in homologous HTH containing proteins are supposed to contact specific bases in the major groove of the DNA binding motifs (43, 45). Chemical shift variations of C-RcsB were detected at positions of the highly conserved residues Val-157 and Ile-182 that participate in a hydrophobic cluster fixing helices α7 to α9 in their proper position. In addition, residue Glu-154 showing also a considerable chemical shift change upon complexation is involved in a salt bridge stabilizing the HTH motif. A less clear explanation can be given for the chemical shift perturbation in Ser-206 and Val-208. Both residues are located at the C terminus of helix α10 and are not in the proximity of the DNA-binding site. A possible explanation would be the formation of a dimer upon DNA binding. In a model of C-RcsB bound to DNA obtained by backbone superposition of two identical C-RcsB structures (residues 153–215) on subunits A and C (residues 176–228; r.m.s.d. 1.2 and 1.1 Å) of TraR bound to DNA (Protein Data Bank code 1L3L (46)), the residues Ser-206 and Val-208 are located in the α10 helix that determines the contact between the two monomers. However, the NMR chemical shift perturbation data do not indicate a significant change in the chemical environment of other residues in α10 that should come in close contact upon dimerization. Additionally, to obtain the dimer of C-RcsB in a position suitable for DNA binding the linker helix α6 had to be deleted because of overlap with α10 of the other dimer unit. We may conclude that binding to DNA as dimer in a fashion equivalent to TraR would require a significant change in the position of the linker helix α6. Interestingly, a similar observation that a change in position of α6 is necessary to allow the entry of α9 into the major groove was made in the case of NarL (43).

The RcsAB box has been identified as the binding site for the RcsAB heterodimer and might represent only a suboptimal binding site for RcsB alone. This assumption is supported by our observation that the affinity of RcsB to the RcsAB box is 1 order of magnitude lower in the absence of RcsA. The weaker binding specificity of the RcsB HTH recognition helix might be compensated in combination with corresponding helices from coactivators like RcsA. Interaction with a coinducer could, furthermore, allosterically affect the binding specificity of the HTH motif. Unfortunately, the effect of RcsA on the structural conformation and on the DNA interaction of RcsB could not be analyzed by NMR spectroscopy so far. The RcsA protein can only be produced in an active conformation when fused to the large maltose-binding protein (17) and tends to aggregate at higher concentrations.

Potential DNA targets recognized by RcsB alone or at least independently from RcsA have been proposed for the ftsAIp promoter regulating ftsAZ and for omcC expression (5, 6). The proposed 18-bp RcsB box differs from the RcsAB box mostly in nucleotide positions located at the 3′ end of the consensus (6), and therefore positioning of RcsB at the left site of the RcsAB box in the RcsAB-DNA complex was suggested. However, it was not possible to demonstrate the interaction of RcsB alone with the RcsB box by electrophoretical mobility shift assays, indicating also a somehow instable complex. An α-helix of a HTH motif can access only one side of the DNA and it is therefore able to bind no more than 5 base pairs because of the curvature of the DNA major groove. The length of the suggested RcsB box gives therefore evidence for the interaction of more than one RcsB monomer with the DNA, or for the involvement of other yet unidentified coactivators. If the formation of RcsB homodimers or homo-oligomers enables or supports DNA binding, then the protein interface should involve at least parts of the C-terminal domain as the C-RcsB protein was still able to interact with DNA.

Our results show that heterodimerization with RcsA is not required for a RcsB/DNA interaction but that it considerably enhances its efficiency. The formation of relatively unstable complexes of RcsB alone with promoters containing RcsAB or RcsB boxes might therefore be important to maintain an essential basal level of expression of the corresponding genes. Modulation of the DNA recognition specificity or the stabilization of RcsB-DNA complexes by heterodimerization with specific coinducers as a response to distinct external signals might represent an additional option to rapidly increase the expression of selected genes or operons. The results presented in this report indicate that in the case of the RcsA/RcsB heterodimer formation, the stabilization of the protein-DNA complex is the predominant function.

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REFERENCES
1. Stout, V., and Gottesman, S. (1990) J. Bacteriol. 172, 659–669
2. Ebel, W., and Trempy, J. E. (1999) J. Bacteriol. 181, 577–584
3. Wehland, M., and Bernhard, F. (2000) J. Biol. Chem. 275, 7013–7020
4. Ghaisas, F. G., Phoenix, P., and Drapeau, G. R. (1992) J. Bacteriol. 174, 3964–3971
5. Carballes, F., Bertrand, C., Bouche, J. P., and Cam, K. (1999) Mol. Microbiol. 32, 443–450
6. Davalos-Garcia, M., Contrer, A., Toscas, I., Gutierrez, C., and Cam, K. (2001) J. Bacteriol. 183, 5870–5876
7. Arricau, N., Hermant, D., Wazir, H., Echchichon, C., Dufay, P. S., and Popoff, M. Y. (1998) Mol. Microbiol. 29, 825–830
8. Takeda, S. I., Fuijsawa, Y., Matusbara, M., Aiba, H., and Mizuno, T. (2001) Mol. Microbiol. 40, 440–450
9. Conter, A., Sturny, R., Gutierrez, C., and Cam, K. (2002) J. Bacteriol. 184, 2850–2853
10. Gervais, F. G., and Drapeau, G. R. (1992) J. Bacteriol. 174, 8016–8022
11. Sledjeski, D. D., and Gottesman, S. (1996) J. Bacteriol. 178, 1294–1296
12. Gottesman, S. (1995) in Two-component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) p. 253–262, ASM Press, Washington, D. C.
13. Clavel, T., Lazaroni, J. C., Vianney, A., and Portalier, R. (1996) Mol. Microbiol. 19, 19–25
14. Clarke, D. J., Holland, I. B., and Jacq, A. (1997) Mol. Microbiol. 25, 933–944
15. Kelley, W. L., and Georgopoulos, C. (1997) Mol. Microbiol. 25, 913–931
16. Virlogeux, I., Waxin, H., Ecobichon, C., Lee, J. O., and Popoff, M. Y. (1996) J. Bacteriol. 178, 1691–1698
17. Kelm, O., Kiecker, C., Geider, K., and Bernhard, F. (1997) Mol. Gen. Genet. 256, 72–83
18. Wehland, M., Kiecker, C., Coplin, D. L., Kelm, O., Saenger, W., and Bernhard, F. (1997) J. Bacteriol. 179, 3300–3307
19. Brill, J. A., Quinlan-Walshe, C., and Gottesman, S. (1988) J. Bacteriol. 170, 111–112
20. Henikoff, S., Wallace, J. C., and Brown, J. P. (1990) Methods Enzymol. 183, 111–132
21. Kahn, D., and Ditta, G. (1991) Mol. Microbiol. 5, 987–997
22. Bullock, W. O., Fernandez, J. M., and Stuart, J. M. (1987) BioTechniques 5, 376–379
23. Bernhard, F., Poetter, K., Geider, K., and Coplin, D. L. (1990) Mol. Plant-Microbe Interact. 3, 429–437
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual (Ford, N., Nolan, C., and Ferguson, M., eds) 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Kay, L. E., Reifer, P., and Saarinen, T. (1992) J. Am. Chem. Soc. 114, 10663–10665
27. Schleucher, J., Sattler, M., and Griesinger, C. (1993) Angew. Chem. Int. Ed. Engl. 32, 1489–1491
28. Kay, L. E., Marion, D., and Bax, A. (1989) J. Magn. Reson. 84, 72–84
29. Zuiderweg, E. R. P., and Fesik, S. W. (1989) Biochemistry 28, 2387–2391
30. Pristovicic, P., Lueke, C., Reinecke, B., Lohr, F., Ludwig, B., and Ruterjans, H. (2000) J. Biomol. NMR 16, 353–354
31. Lueke, C., Reinecke, B., Lohr, F., Pristovicic, P., Ludwig, B., and Ruterjans, H. (2000) J. Biomol. NMR 18, 365–366
32. Vuister, G. W., and Bax, A. (1992) J. Magn. Reson. 98, 428–435
33. Pristovicic, P., Ruterjans, H., and Jerala, R. (2002) J. Comput. Chem. 23, 335–340
34. Pristovicic, P., Lueke, C., Reinecke, B., Ludwig, B., and Ruterjans, H. (2000) Eur. J. Biochem. 267, 4205–4212
35. Guntiert, P., Braun, W., and Wuthrich, K. (1991) J. Mol. Biol. 217, 517–530
36. Wuthrich, K. (1986) in NMR of Proteins and Nucleic Acids, Wiley, New York
37. Reincke, B., Perez, C., Pristovicic, P., Lueke, C., Ludwig, B., Lohr, F., Rogov, V., Ludwig, B., and Ruterjans, H. (2001) Biochemistry 40, 12312–12320
38. Guntiert, P., Mumenthaler, C., and Wuthrich, K. (1997) J. Mol. Biol. 273, 283–298
39. Wishart, D. S., and Sykes, B. D. (1994) J. Biomol. NMR 4, 171–181
40. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291