Structural dynamics of the two-component response regulator RstA in recognition of promoter DNA element

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ABSTRACT

The RstA/RstB system is a bacterial two-component regulatory system consisting of the membrane sensor, RstB and its cognate response regulator (RR) RstA. The RstA of Klebsiella pneumoniae (kpRstA) consists of an N-terminal receiver domain (RD, residues 1–119) and a C-terminal DNA-binding domain (DBD, residues 130–236). Phosphorylation of kpRstA induces dimerization, which allows two kpRstA DBDs to bind to a tandem repeat, called the RstA box, and regulate the expression of downstream genes. Here we report the solution and crystal structures of the free kpRstA RD, DBD and DBD/RstA box DNA complex. The structure of the kpRstA DBD/RstA box complex suggests that the two protomers interact with the RstA box in an asymmetric fashion. Equilibrium binding studies further reveal that the two protomers within the kpRstA dimer bind to the RstA box in a sequential manner. Taken together, our results suggest a binding model where dimerization of the kpRstA RDs provides the platform to allow the first kpRstA DBD protomer to anchor protein–DNA interaction, whereas the second protomer plays a key role in ensuring correct recognition of the RstA box.

INTRODUCTION

Bacteria and plants frequently use two-component signal transduction systems (TCSs) to adapt to environmental changes and to survive under stress conditions (1–4). TCSs are especially abundant in bacteria and absent in metazoans, making them attractive targets for antibacterial drug discovery (5). Typical TCSs couple a transmembrane histidine protein kinase (HK), which detects changes in the environment, to a cytosolic response regulator (RR), which often alters gene expression. External stimulus leads to autophosphorylation of the HK, which is then able to form a complex with the cognate RR and allow the transfer of the phosphate group to a conserved Asp residue of the RR. Phosphorylation alters intra-molecular interactions within the RR, which allows it to bind to DNA, resulting in up- or down-regulation of downstream genes (6).

The RstB/RstA system is a ubiquitous TCS composed of the membrane-associated histidine kinase RstB and its cognate response regulator RstA. In Escherichia coli and Salmonella, RstA is under the control of the PhoP/PhoQ TCS, which monitors extracellular Mg²⁺ levels, and up-regulates the acid-induced asr (acid shock RNA) gene and the biofilm regulator csgD gene under acidic conditions (2,7,8). Overexpression of RstA in Salmonella enterica serovar Typhimurium induced degradation of RpoS and altered biofilm formation (9). Moreover, RstA directly binds to the promoter of the fep operon in Salmonella and promotes the expression of the iron transporter FeoB under iron-replete conditions (10). Since both biofilm formation and iron acquisition are often associated with the ability of the organism to establish infection (11,12), RstA may play a regulatory role in virulence. Preliminary experiments on Yersinia pseudotuberculosis have also shown that mutations on the rstA gene attenuated virulence in mouse models (13).

Klebsiella pneumoniae is an important pathogen associated with nosocomial infections (14). Emergence of K. pneumoniae variants with multiple antimicrobial resistances has
become a serious problem in hospitals worldwide. Association of RstB/RstA with multiple virulence factors highlights its potential as a drug target, and emphasizes the importance of investigating its mechanism of action. *K. pneumoniae* RstA (kpRstA) contains an N-terminal receiver domain (RD, residues 1–119) and a C-terminal DNA-binding domain (DBD, residues 131–236), with an overall sequence identity of 79% compared to RstA from *E. coli* and *S. enterica* serovar Typhimurium (see Supplemental Figure S1). As a member of the OmpR/PhoB subfamily, phosphorylation of the conserved Asp in the RD is believed to result in dimerization of the domain through a conserved α4–β5–α5 region, accompanied by the binding of the DBD to cognate sequences in the promoter region of RstA-regulated genes. Genomic systematic evolution of ligands by exponential enrichment (SELEX) experiments in *E. coli* have shown that RstA binds to the consensus sequence TACATNTNGT-TACA with tandem TACA recognition sites (8). Like other members of the OmpR/PhoB subfamily, it is implied that dimerization of RstA is necessary for binding to this tandem sequence, with each DBD binding to a single recognition site (15,16). The consensus sequence, called the RstA box, can also accommodate imperfections in the TACA sequence. *Klebsiella* RstA is capable of activating the RstA box of the *asr* gene in *K. pneumoniae*, which contains a RstA box with a TACA/TACT imperfect repeat instead of the canonical TACA repeat found in the *E. coli* *asr* gene (17).

Although proteins of the OmpR/PhoB subfamily share a high degree of structural similarity, there is little consensus on how they recognize the tandem DNA sequences in the context of a dimer. For example, the two DBDs of the OmpR dimer can bind to different DNA sequences in either head-to-tail or head-to-head orientations (18–20), whereas the DBDs of PhoB and PmrA bind to DNA exclusively in a head-to-tail orientation (21,22). Moreover, binding of PhoB to DNA induces bending in the DNA, which is not observed for PmrA. These observations imply divergent structural bases for tandem sequence recognition within the same protein subfamily. Here we report the characterization of kpRstA binding to the RstA box sequence located in the *asr* promoter of *K. pneumoniae* by isothermal titration calorimetry (ITC). The structures of kpRstA RD activated with BeF3−, free kpRstA DBD and kpRstA DBD in complex with DNA are also disclosed through a combination of X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Furthermore, we compare the backbone dynamics of the DBD in the bound and free forms. Surprisingly, the kpRstA dimer binds to the RstA box DNA sequence in a sequential fashion which has never been observed in other RRs. The combination of multifaceted approaches allows us to formulate a sequence of events behind the activation and unique DNA recognition mechanism of kpRstA.

**MATERIALS AND METHODS**

**Chemicals and reagents**

BeSO4 was purchased from Mitsuwas Pure Chemicals (Japan). Other chemical reagents were purchased from Sigma (USA) and Merck (USA). DNA sequences were synthesized by Genomics Ltd (Taiwan). Double-stranded DNA was obtained by mixing together complementary strands in the buffer for the current experiment, followed by denaturation at 95°C for 5 min and slowly cooling down to room temperature.

**Cloning, protein expression and purification**

Full-length kpRstA was cloned into a modified pET32a(+) vector which lacked the region coding for the enterokinase cleavage site. kpRstA RD and kpRstA DBD were cloned into pET6H vector (a gift from Prof. J.-J. Lin, National Yang Ming University, Taiwan). The proteins were expressed in *E. coli* BL21(DE3) strain by growing the cells in 1.0 liter LB medium at 37°C until reaching an OD600 of 0.6–0.8 followed by induction with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were then allowed to grow overnight at 18°C before harvest. Isotope-labeled proteins for NMR experiments were produced by centrifuging and re-suspending the cells in 250 ml 2xM9 medium supplemented with 1.0 g/l of 15NH4Cl and/or 1.0 g/l u-13C-glucose prior to induction. Deuterated samples were obtained by substitution of water and u-13C-glucose with 2H2O and u-13C2H-glucose in this step. To solve the crystallographic phase problem, selenomethionine (SeMet)-labeled samples of kpRstA RD and kpRstA DBD were obtained by growing *E. coli* B834(DE3) strain in LeMaster medium supplemented with 50 µg/ml SeMet, 1% glucose, 1 mM MgSO4, 0.25 mg/ml MgSO4 and 7.68 µg/ml FeSO4 during the induction phase. To achieve sufficient labeling, we constructed the kpRstA DBD (L153M/L168M) mutant where both Leu153 and Leu168 were changed to Met. Another loss-of-function mutant, R207A, was constructed for ITC experiments. The mutations were introduced into pET6H-kpRstA-DBD through site-directed mutagenesis by polymerase chain reaction (PCR) with the primers listed in Supplemental Table S1. All expressed proteins contained an N-terminal His-tag that was later removed in the case of full-length kpRstA.

The cells were lysed with a microfluidizer in loading buffer (20 mM Tris, 100 mM NaCl, pH 7.5) and the lysate spun down at 30 000 g on an Avanti J-26XP centrifuge (Beckman-Coulter, USA). The supernatant was loaded onto Ni-NTA resin (Qiagen, USA) pre-equilibrated with the loading buffer and eluted with the same buffer containing 300 mM imidazole. The eluted proteins were further purified by size-exclusion chromatography using an Akta fast-performance chromatography system (FPLC) equipped with a HiLoad Superdex-75 16/60 column (GE Healthcare, USA) equilibrated with the elution buffer solution (20 mM sodium phosphate, 50 mM NaCl, pH 6.0). The His-tag of full-length kpRstA was removed by digestion with 75 U of thrombin (GE Healthcare, USA) for 2 days at 4°C and the cleaved protein was further purified following manufacturer’s instructions. The resulting full-length kpRstA contained a GSAMA sequence at the N-terminus, whereas both kpRstA-RD and kpRstA-DBD contained an extraneous N-terminal M-6H-AMG sequence.

**Isothermal titration calorimetry**

ITC was performed with a Microcal ITC200 calorimeter (GE Healthcare, USA) at 25°C. Protein and DNA solution
were dialyzed overnight against the same reaction buffer (20 mM Tris, pH 7.5, 100 mM NaCl in the case of kpRstA DBD and 20 mM Tris, pH 7.5, 100 mM NaCl, 7 mM MgCl₂ with or without 5.3 mM BeSO₄ and 35 mM NaF for full-length kpRstA). For kpRstA DBD, the titration was conducted by injecting 1 μl (first injection) or 2 μl (second to 19th injections) of 2 mM protein solution into 200 μl of 80 μM DNA (16- or 22-bp) solution. For full-length kpRstA, the titration was conducted by injecting 1.2 μl (first to 17th injections) or 1.6 μl (18th to 29th injections) of 1.0 mM protein solution into 200 μl of 57 μM 22-bp DNA solution. An initial delay of 180 s was applied before the first injection, with a 120 s interval between two successive injections. The binding isotherms of kpRstA DBD against 16-bp DNA were fitted to a single-site binding model while those of kpRstA DBD and full-length kpRstA against the 22-bp DNA were fitted to a sequential binding model under the Microlab Origin package (GE Healthcare, USA).

NMR experiments

The NMR spectra were acquired on Bruker AVANCE 500, 600 or 800 MHz spectrometers (Bruker, Germany) equipped with 5-mm triple resonance cryoprobes at 25 or 37°C. NMR data were acquired in Shigemi tubes on 0.1–1.0 mM protein samples in NMR buffer (20 mM sodium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) D₂O) at pH 6 for free kpRstA DBD and at pH 6.5 for the kpRstA DBD/DNA complex. The ¹⁵N,¹³C- and ¹³C,²H-labeled protein/DNA complex samples were prepared at a protein-to-DNA molar ratio of 1:2. As the 16-bp DNA substrate gave the best NMR spectra, they were used for all NMR studies. Protein backbone resonance assignments of kpRstA DBD and its complex with DNA were achieved by standard triple resonance experiments, including HNCA, HN(CO)CA, CBCA(CO)NH, HNCO and HN(CA)CO (23–27). Ambiguities in the backbone assignment were resolved by specific amino labeling of the protein. Complete assignment of free kpRstA DBD has been described elsewhere (28). ¹H chemical shifts were externally referenced to 0 ppm through the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), whereas ¹³C and ¹⁵N chemical shifts were indirectly referenced according to the recommendations of the International Union of Pure and Applied Chemistry (29). The weighted chemical shift perturbations (CSPs) of kpRstA DBD backbone ¹⁵N and ¹H resonances upon DNA binding were calculated with the following equation: \( \Delta \delta = \left( \frac{\Delta \delta_{\text{HN}}}{2} + 0.154 \Delta \delta_{\text{N}} \right)^{1/2} \).

Both ¹³C- and ¹⁵N-edited NOESY-HSQC experiments were conducted with a mixing time of 120 ms to obtain distance restraints for the structure calculation of free kpRstA DBD. The NMR spectra were processed using Bruker TOPSPIN 3.0 and analyzed with Sparky (Goddard, T.D. and Kneller, D.G., University of California San Francisco) and CARA (available from: http://www.nmr.ch). To measure one-bond ¹H-¹⁵N residual dipolar couplings (RDC), free kpRstA DBD was partially aligned in liquid crystalline phase containing 15 mg/ml P1β1 bacteriophage (Asla Biotech Ltd, Latvia). Changes in splitting relative to the isotropic ¹JHN values were measured using DSSE-HSQC experiments to obtain one bond ¹H-¹⁵N RDCs (30). The measured RDCs were analyzed using the program PALES (31).

The ¹⁵N spin-lattice/longitudinal relaxation rate, ¹⁵N-R₁, spin-spin/transverse relaxation rate, ¹⁵N-R₂, and [¹H-¹⁵N]NOE were determined by using standard pulse sequences (32). Each ¹⁵N-R₁ was determined with ten randomly ordered delays of 0.8, 118.8, 238.8, 358.8, 478.8, 598.8, 718.8, 898.8, 1018.8 and 1288.8 ms. Similarly, each ¹⁵N-R₂ was determined from 10 delays, in random order, of 0, 34.3, 51.5, 68.6, 102.9, 120.1, 154.4, 188.7, 205.8 and 223.0 ms for free kpRstA DBD; 0, 17.2, 34.3, 51.5, 68.6, 85.8 and 102.9 ms for the kpRstA DBD/DNA complex. Both rate constants were determined using the program Protein Dynamics Center (Bruker, Germany), assuming monoeponential decay of the peak intensities. The errors in peak intensities were calculated from two duplicate experiments. The steady-state heteronuclear [¹H-¹⁵N] NOE experiment was carried out in duplicate in an interleaved manner, with and without proton saturation. The NOE was calculated as the error-weighed average ratio of peak intensities, with error estimated by standard deviation of three pairs of repeated experiments. The reduced spectral density analysis was performed as previously described (33–37).

NMR structure determination

The solution structure of kpRstA DBD was calculated with CYANA 3.0 based on experimental restraints (38,39). Distance restraints were generated by picking and quantifying NOE peaks using the automatic peak picking routine of AURELIA (Bruker, Germany) followed by automated NOE cross-peak assignment with CYANA 3.0. The restraints were grouped into short- (≤ 1 Å), middle- (1–5 Å) and long-range (≥ 5 Å) distances. Backbone torsion angle restraints were predicted from backbone chemical shifts with TALOS+ (40), and only those identified as ‘good’ by the program were used in the structure calculation. Hydro-
gen bond restraints were generated for secondary structure regions by assigning the following distance constraints: 1.8–2.6 Å for HN–O and 2.7–3.5 Å for N–O. A list of restraint statistics is provided in Supplemental Table S2. Measured RDCs (71 out of 119) were also used in the calculation. Out of 150 calculated conformers, the 20 conformers with the lowest target functions were deposited to the Protein Data Bank (PDB) with accession code: 2MLK. All structural figures were prepared with MOLMOL (41) and PyMOL (42).

Crystallization and data collection

For crystallization trials, kpRstA DBD was mixed with 23-bp DNA substrate (Figure 2C) at a molar ratio of 2:1 (DNA:protein) and concentrated to 20 mg/ml in preparation buffer (20 mM Tris-HCl, pH 7.5 and 100 mM NaCl). kpRstA RD was similarly prepared except that the preparation buffer contained additional 5.3 mM BeSO4, 35 mM NaF and 7 mM MgCl2. Initial crystallization trials were performed with commercially available kits (Hampton Research, USA) using the hanging drop vapor diffusion method (43). Specifically, 1 μl of concentrated complex was mixed with an equal amount of reservoir solution and equilibrated against 500 μl of reservoir solution at 26°C. After optimization of crystallization conditions, rhombus-shaped kpRstA RD crystals appeared in 100 mM Bis–Tris, pH 6.5 and 25% (v/v) polyethylene glycol 3350, whereas kpRstA DBD/DNA formed plate-shaped crystals in 100 mM Bis–Tris, pH 5.5, 28% (v/v) polyethylene glycol 400 and 1.32 M sodium formate. The phase of kpRstA RD crystals were determined by single-wavelength anomalous diffraction (SAD) of SeMet-labeled crystals at 0.97874 Å (Se-peak). The phase of kpRstA DBD/DNA crystals were determined by collecting multi-wavelength anomalous diffraction (MAD) data on a single SeMet-labeled crystal of kpRstA DBD(L153M/L168M)–DNA complex at three different wavelengths: 0.97879 Å (Se-edge), 0.96357 Å (Se-remote) and 0.97862 Å (Se-peak). All data were collected on an ADSC Quantum-315 CCD area detector at beamline BL13B1 of the National Synchrotron Radiation Research Center, Hsinchu, Taiwan. X-ray diffraction data integration and scaling were performed using the HKL2000 package (44). For the MAD experiment, data from each wavelength were indexed according to the same crystal orientation matrix, but integrated and scaled independently. Diffraction data statistics are listed in Supplemental Table S2.
Crystal structure determination and refinement

Crystals of kpRstA RD and kpRstA DBD/DNA complex diffraction to 3.1 and 2.7 Å, respectively. Experimental phases of kpRstA DBD/DNA complex were obtained at 2.7 Å using the MAD method implemented in the program AutoSol from the PHENIX suite (45). The programs COOT (46) and PHENIX were then used for rounds of manual model rebuilding and refinement, respectively. The final model for kpRstA DBD/DNA complex contained two proteins (102 residues in chain A and 101 residues in chain B), one double-stranded DNA molecule (22 nucleotides in chain C and 23 nucleotides in chain D) and 63 water molecules in the asymmetric unit. The structure was refined to a final $R_{	ext{work}}$ and $R_{	ext{free}}$ of 21.6 and 27.1%, respectively, with good stereochemistry. The structure of kpRstA RD was solved by employing the MR-SAD protocol as implemented in PHENIX (47) using a poly-alanine monomer of the PhoB RD structure (PDB code: 1ZES) as the search model. Molecular replacement was performed with the AutoMR program within PHENIX using the data set collected at the absorption edge (0.9789 Å). The resulting model contained four kpRstA RD molecules (corresponding to two dimers, with 117 residues in each chain), four BeF$_3^-$, four Mg$^{2+}$ atoms and 38 water molecules in the asymmetric unit. The structure was refined to a final $R_{	ext{work}}$ and $R_{	ext{free}}$ of 18.7 and 22.3%, respectively. The Ramachandran plot outliers of SeMet-RstA RD are mostly located on the C-terminus (Arg117 of chain A and Leu116 of chain B) and in loop regions (Asp9, Asp10 on chain B and Ile53 on chain C). Detailed refinement parameters for both structures are available in Supplemental Table S2. Coordinates of kpRstA DBD/DNA and kpRstA RD have been deposited to the PDB with accession codes: 4NHJ and 4NIC, respectively.

RESULTS

Promoter binding affinity and thermodynamics of kpRstA–RstA box interaction

The binding affinities of kpRstA DBD to the upstream half-site of the RstA box (DNA-16a), downstream half-site of the RstA box (DNA-16b) and the full-length RstA box (DNA-22) were studied by isothermal titration calorimetry (Figure 1). As shown on Figure 1B and Table 1, the titration curve of kpRstA DBD to DNA-16a could be fitted well with a single binding site of $K_D = 8.17 \pm 0.54 \mu M$ with $\Delta H = -10.12 \pm 0.12 \text{ kcal/mol}$ and $\Delta S = -10.7 \text{ cal/mol/deg}$, indicating that the binding is enthalpically driven. In contrast, the binding of kpRstA DBD to DNA-16b did not generate enough heat to obtain reliable thermodynamic parameters, suggesting extremely weak protein–DNA interactions (Figure 1C). The titration curve of kpRstA DBD to the full length RstA box (DNA-22) was fitted with a sequential binding model of $K_{D1} = 10.3 \pm 1.13 \mu M$, $\Delta H_1 = -9.48 \pm 0.23 \text{ kcal/mol}$, $\Delta S_1 = -8.98 \text{ cal/mol/deg}$ and $K_{D2} = 5.15 \pm 0.26 \mu M$, $\Delta H_2 = -6.89 \pm 0.25 \text{ kcal/mol}$, $\Delta S_2 = 1.07 \text{ cal/mol/deg}$ (Figure 1D). Although the data can be fit equally well with two independent equivalent sites of $K_D = 3.40 \pm 0.33 \mu M$, $\Delta H = -7.95 \pm 0.08 \text{ kcal/mol}$, $\Delta S = -1.63 \text{ cal/mol/deg}$, the results would not agree with the half-site binding results which showed that the downstream half-site binds to DNA poorly. The results suggest that binding of kpRstA DBD to the first site enhances the binding of the second kpRstA DBD to the second site. Mutation of Arg207, one of the key residues responsible for DNA binding (see Figure 4C), to Ala completely abolished the DNA binding of kpRstA DBD (Figure 1E).

The titration curve of full-length kpRstA protein to DNA-22 was characterized by a biphasic behavior. The binding isotherms fit poorly to either a one-site model or multiple-site model and only the sequential binding model gave a satisfactory fit with $K_{D1} = 45.45 \pm 8.41 \mu M$, $\Delta H_1 = -9.79 \pm 1.07 \text{ kcal/mol}$, $\Delta S_1 = -13.0 \text{ cal/mol/deg}$ and $K_{D2} = 5.05 \pm 0.72 \mu M$, $\Delta H_2 = -0.48 \pm 1.11 \text{ kcal/mol}$, $\Delta S_2 = 22.6 \text{ cal/mol/deg}$ (Figure 1F). The lower affinity of the first site compared to that of kpRstA DBD is consistent with the inhibitory effect of the RD on DNA binding to DNA reported for other TCS response regulators. The RD of PrrA, e.g. sterically blocks the DNA-binding site of its DBD in the absence of BeF$_3^-$, which results in lower affinity of full-length PrrA towards DNA when compared to the DBD by itself (48). PhoB exemplifies another possibility, where the RD is capable of adopting an alternative dimer conformation in the absence of BeF$_3^-$ that has been proposed to interfere with the correct alignment of the DBDs for binding (49,50). Interestingly, binding to the second site was entropy-driven, which suggests changes in conformation and/or formation of hydrophobic contacts. The binding affinity of the full-length kpRstA towards DNA-22 was significantly enhanced in the presence of BeF$_3^-$ ($K_D = 9.14 \pm 1.84 \mu M$ and $K_{D2} = 0.59 \pm 0.09 \mu M$), primarily through increases in binding enthalpy, although strong entropic contributions persisted for the second site (see Table 1). Size exclusion chromatography showed that the presence of BeF$_3^-$ induced dimerization of full-length kpRstA (data not shown), which suggests that the enhanced binding is caused by the dimerization of the protein.

Structural overview of kpRstA RD, DBD and DBD/DNA complex

The structure of kpRstA RD in the presence of BeF$_3^-$ was solved to a resolution of 3.2 Å (Figure 2A). Similar to the structure reported for TorR RD (51), four kpRstA RD molecules, which correspond to two dimers, were found in the asymmetric unit (see Supplemental Figure S2). The two dimers within the asymmetric unit had similar structures as revealed by structural alignment, with an rmsd of 0.47 Å over 117 Cα. Structural analyses revealed that the overall structure of kpRstA RD has the traditional (βα)$_5$ fold of RR regulatory domains that consists of a central five-stranded parallel β-sheet surrounded by five-helices.

The structural topology of kpRstA DBD in solution consists of the N-terminal three-strand antiparallel β-sheet (β1, Thr137–Ser139; β2, Leu143–Asp146; β3, Arg149–Leu153) followed by three helices assuming the helix-turn-helix DNA binding motif topology (α1, Thr63–Thr74; α2, Arg77–Arg81; α3, Leu117–Leu126). The structure of the β-hairpin (β2, β3) of the DBD was not fully defined, with the following residues in the β3 strand: Ser139–Asp146 and the following residues in the α1 helix: Thr63–Asp72. The DBD contains a loop insertion that is characteristic of the RstA box binding domain. The loop fold of the DBD consists of a 310 helix (Thr74–Thr77) followed by an extended loop (Thr78–Asn82) and a β-hairpin (β3, β4). The β3 strand of the DBD is positioned at the bottom of the binding pocket, while the β4 strand is located at the top of the binding pocket. The DBD binds to the RstA box (DNA-22) in a partially buried manner, with a buried surface area of 2650 Å$^2$.
Arg182–Arg190; α3, Asp198–Leu212) and ending with a β-hairpin (β4, Ile222–Val225; β5, Lys228–Phe232) (Figure 2B). The N-terminal β-sheet interacts with helix α1 primarily through hydrophobic interactions while stacking interaction between the aromatic rings of Phe140 and Phe232 brings the N-terminal β-sheet to the vicinity of the C-terminus. The overall structure of kpRstA DBD shares a winged helix-turn-helix (wHTH) fold typical for the OmpR/PhoB RR superfamily, such as OmpR (PDB ID: 1OPC), PhoB (PDB ID: 1GXQ/1GXP) and KdpE (PDB ID: 3ZQ7), with Cα rmsd ranging from 1.1 to 1.4 Å.

The crystal structure of kpRstA DBD/DNA-23 consists of two protein molecules and one 23-bp DNA per asymmetric unit (Figure 2C). In the crystal packing, the DNA duplexes formed a pseudo-continuous DNA helix stabilized by base-stacking interactions between symmetry-related DNA. Superimposition of the two kpRstA DBDs in the asymmetric unit showed that their structures are very similar, with an rmsd of 0.67 Å over 93 Cα atoms (see Suplemental Figure S2). The two DBDs bind on the same side of the DNA in a head-to-tail fashion and the electrostatic potential surface of the protein dimer forms a long and continuous positively charged region for DNA binding. The α3 recognition helix is docked into the major groove of the DNA and forms numerous interactions with the RstA box. The C-terminal β-hairpin also makes contact with the DNA, mostly close to the phosphate backbone. Unlike the bent DNA conformation observed for the PhoB DBD/DNA complex (21), the DNA-23 in kpRstA DBD/DNA-23 complex only displayed slight bending (∼10°).

**Dimerization of kpRstA RD**

All structures of the OmpR/PhoB superfamily RRs solved to date adopt a similar dimeric structure in the phosphorylated (active) state, which can be mimicked by the BeF$_3^-$-bound form (6). In kpRstA RD, the BeF$_3^-$ molecule is non-covalently bound to Asp52 within the phosphorylation site, and forms a hydrogen bond with the side-chain of Thr79, a salt bridge to Lys101, and van der Waals contacts with the backbone nitrogen atoms of Ile53, Met54 and Ser80 (Figure 3A). One of the fluorine atoms is coordinated to an Mg$^{2+}$ atom, which in turn coordinates with the main chain carboxyl oxygen of Met54 and the side-chain carboxyl oxygen atoms of Glu8, Asp9 and Asp52 to form a 6-fold coordination of octahedral geometry. The interactions found in the phosphorylation site of kpRstA RD are analogous to those of other structures of active RRs from different subfamilies, underscoring the conserved nature of the phosphorylation site environment (6).

Previous studies on receiver domains of RRs have demonstrated that a molecular switch comprised of a highly conserved residue pair (Thr/Ser and Tyr/Phe) was able to exchange between inward (active) and outward (inactive) conformations in response to phosphorylation (51–54). Superimposing the structure of activated kpRstA RD onto the inactive PhoP RD (PDB ID: 2PKX) suggests that the signature switch residues are likely Thr79 and Tyr98, which are located in the β4-α4 loop and β5, respectively (Figure 3B) (53). This molecular switch could propagate the structural changes at the phosphorylation site to the dimer interface, which spans the α4–β5–α5 region. Residues in this region are highly conserved in the OmpR/PhoB superfamily of RRs. The dimer interface of kpRstA RD has a buried surface area of 689.5 Å$^2$ as calculated with PISA (55). Two conserved intermolecular salt bridges are formed between Asp97 (β5)–Arg111 (α5) at the center and Glu92 (α4)–Arg113 (α5) at the outer sides of the interface (Figure 3C). Additionally, six hydrophobic residues (Ile88, Leu91, Ala106, Val107, Ala110 and Leu114) cluster together to form an extensive hydrophobic patch that holds together

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Table 1. Binding parameters of full-length (FL) kpRstA and kpRstA DBD towards RstA box DNA sequences

| Sample                  | Model                  | N       | $K_D$ (μM) | $\Delta H$ (cal/mol) | $\Delta S$ (cal/mol/deg) |
|-------------------------|------------------------|---------|------------|----------------------|--------------------------|
| DBD/DNA-16a             | One-site               | 1.05    | 8.17 ± 0.53| −10100 ± 120         | −10.7                   |
| DBD/DNA-16b             | -                      | -       | -          | -                    | -                        |
| DBD/DNA-22              | Sequential             | Set as 2| 10.31 ± 1.14| −9480 ± 233          | −8.98                    |
| DBD-R207A/DNA-22        | -                      | -       | -          | -                    | -                        |
| FL/DNA-22 (without BeF$_3^-$) | Sequential             | Set as 2| 45.45 ± 8.41| −9792 ± 1070         | −13.0                    |
| FL/DNA-22 (with BeF$_3^-$) | Sequential             | Set as 2| 5.05 ± 0.72 | −478.7 ± 1110       | 22.6                     |
|                         |                        |         | 9.14 ± 1.84 | −17400 ± 1380       | −35.3                    |
|                         |                        |         | 0.59 ± 0.09 | −3028 ± 1400        | 18.4                     |
the α4 and α5 helices between adjacent molecules (Figure 3D).

Interaction between kpRstA DBD and DNA

The two DNA-bound DBDs interact with each other with a buried surface area of 362 Å² per monomer at the protein–protein interface. The interface mostly consists of B4 and B5 of the upstream DBD (bound to the 5′ half-site of the coding strand) on one end and the two loops between β2–β3 and β3–α1 of the downstream DBD (bound to the 3′ half-site of the coding strand) on the other end (Figure 4A). Intermolecular van der Waals contacts are formed between Gly177 (α1–α2 loop)–Val148 (β2–β3 loop), Glu218 (α3–B4 loop)–Ala160 (β3–α1 loop), Val225 (B4–Arg150 (β3), Val225 (B4)–Thr163 (α1), Leu231 (B4)–Arg150 (β3), Pro234 (C-terminal tail)–Val148 (β2–β3 loop). Moreover, Lys223 (B4) and His235 (C-terminal tail) form hydrogen bonds with Asn149 (β2–β3 loop) and Arg226 (B4–β5 loop) forms a hydrogen bond with Thr163 (α1). Lys223 (B4) is also within hydrogen bonding distance to Arg150 (β3). The extensive interactions observed between the upstream and downstream DBDs suggest that the two binding events are not independent of each other, and formed the basis for the choice of a sequential binding model when fitting the ITC data. Similar DBD–DBD interfaces have been observed for DNA-bound PhoB and KdpE (21,56), even though the residues involved share little homology amongst the different proteins (see Supplemental Figure S1).

The two kpRstA DBDs within an asymmetric unit bind to the DNA with similar contacts (Figure 4B and C), with contact surfaces of 882 Å² between DNA and protein and 899 Å² for the upstream and downstream protomers, respectively. The upstream DBD binds the RstA box from T4 (coding strand) to A15′ (the prime sign indicates the template strand), and the downstream DBD binds from C13 to G5′ (Figure 4B). Specific interactions between the α3 recognition helix residues and DNA, which include Val203-T6, Arg207-G5, Arg199-G17′ and Arg199-T16′, provide the basis for sequence specificity. It should be noted that while the upstream Arg199 forms two specific hydrogen bonds with the template strand of the RstA box TACA, G17′ and T16′, the downstream Arg199 contacts the DNA duplex by forming three hydrogen bonds: Arg199-A17′, Arg199-G17′ and Arg199-A6′ (Figure 4C). This observation implies that the side chain of Arg199 is capable of recognizing nucleotide sequences that deviate from the canonical RstA box. Similarly, there are numerous interactions between the second kpRstA DBD and the second RstA box. Base specificity is established mainly through the residues Arg200, Ser201, Val204 and Arg208 (Figure 4C).

The binding interface of the kpRstA DBD/DNA complex in solution was also examined through NMR CSP studies (Figure 5A and B). Residues perturbed by the binding of DNA-16a localize primarily at the C-terminal region including helices α2, α3, B4, B5 and the α2–α3 loop, with the largest perturbations occurring at or near the α3 recognition helix. Slight perturbation of the N-terminal region of α1 was also observed. Noticeably, the N-terminal β-sheet showed very little perturbation. The observed CSPs are in full agreement with the DBD/DNA interactions identified in the crystal structure of the complex.

Structural and backbone dynamics changes of kpRstA DBD upon DNA binding

Superposition of the DNA-free NMR structure and DNA-bound crystal structure of the DBD revealed that their overall structures are similar with an rmsd of 1.43 Å for 101 Ca atoms (Figure 6A). However, superimposing the N-terminal halves (res. 135–190) of the two structures resulted in an rmsd of 1.12 Å for 52 Ca atoms (Figure 6B), whereas superposition of the C-terminal halves (res. 191–235) resulted in an rmsd of 1.70 Å for 36 Ca atoms (Figure 6C), indicating that the region spanning the transactivation loop to β4 and β5 undergoes large conformational changes upon DNA-binding. Noticeably, the α3–β4 loop swings ∼90° towards the template strand of the DNA in the complex, which results in Ala216 interacting with the ribose part of G17′. Moreover, insertion of Arg226 into the minor groove brings the β4–β5 loop into contact with the DNA and clamps down the template strand alongside the α3 recognition helix, further stabilizing the interaction between the DBD and DNA.

To gain further insights into the dynamic behavior of kpRstA DBD, we investigated the backbone dynamics
of DNA-free kpRstA DBD and kpRstA DBD/DNA-16a complex by measuring $^{15}$N-R1, $^{15}$N-R2 and $[^1H, ^{15}N]$-NOE at 800 MHz (Figure 7A) and calculating the reduced spectral density functions from these relaxation parameters (33,34,57,58). Reduced spectral density functions reflect the degree of motion in particular frequency regions. Thus, higher value of $J(\omega)$ indicates the presence of motion at the frequency $\omega$ MHz region. The results are shown in Figure 7B. $J(0)$, $J(N)$ and $J(0.87H)$ represent reduced spectral density function at 0, 80 and 696 MHz, respectively. Overall, the DNA-free kpRstA DBD (filled circles) is rigid, except two loop regions. The loop connecting $\alpha$2 and $\alpha$3 (Lys187–Ser200) is characterized by large $J(0.87H)$ indicative of the presence of high frequency fast motion. Another loop region showing flexibility is that connecting $\alpha$3 and $\beta$4 (Ala216–Glu218). This region also showed elevated $J(0.87H)$ suggesting the presence of fast motion. Moreover, this loop region also showed considerable slow motion, as indicated by large $J(0)$. Interestingly, these two loop regions juxtapose helix $\alpha$3. In the DNA-bound form (open circles), the DBD showed a similar dynamic behavior and the two flexible loops between Ala216–Glu218 and Lys187–Ser200 also exhibit higher flexibility as observed in the free form. However, reliable relaxation measurements cannot be obtained for many resonances between $\alpha$2 and $\alpha$3, indicating that this region becomes more flexible upon DNA-binding. One also observes higher and more scattered $J(0)$ for the $\alpha$3–$\beta$4 region, suggesting that the entire C-terminal region beyond $\alpha$2 exhibit slow motion, possibly some conformational exchange process. The functional significance of the enhanced dynamics of the two regions is discussed in the following section. In agreement with the monomeric protein sizes, the overall rotational correlation times deduced from the relaxation data are 6.75 and 12.5 ns for the DNA-free and DNA-bound form, respectively.

**DISCUSSION**

**kpRstA DBD binds DNA in sequential mode**

Modulation of gene expression by RRs in bacteria has been well studied, and much of the structural changes resulting from the activation of the RD are relatively well understood (51,52,54,59). On the other hand, the DNA-bound structure of PhoB DBD has for a long time been the only source of information for structural changes of the DBD upon binding to DNA (21). Recently, the structures of *K. pneumoniae* PmrA DBD and *E. coli* KdpE bound to their respective cognate DNA sequences have been solved (22,56). Although PhoB, PmrA and KdpE belong to the same OmpR/PhoB subfamily, their DBDs have shown very different structural and functional characteristics when bound to DNA. For example, binding of PhoB to its cognate sequence introduces a ~40° bend in the DNA, whereas binding of KdpE or PmrA to DNA only introduces slight bending. However, binding of KdpE to DNA results in a much larger dimer surface than in PmrA. This lack of a consensus binding mechanism is also observed for kpRstA, which belongs to the same subfamily. kpRstA DBD binds to the RstA box without inducing substantial bending of the DNA and forms a dimer interface comparable to that of KdpE. Yet unlike KdpE, binding of kpRstA DBD to the two half-sites of the RstA box have very different affinities, whereas KdpE DBD reportedly binds to its two cognate half-sites with similar affinity (60). Moreover, binding of full-length kpRstA to the full-length RstA box is clearly biphasic in sequential mode (Figure 1D and E), which has never been reported for other RRs from the OmpR/PhoB subfamily. These observations suggest that the DNA-binding properties of RRs in the same family need to be examined on an individual basis, even though they might share a similar structural fold.

The affinities of kpRstA DBD and full-length kpRstA toward cognate DNA measured in this study are gener-
ally weaker than those reported for other RRs. PmrA and KdpE DBDs all bind to their respective high-affinity half-sites at sub-micromolar concentrations (22,60), which are one to two orders of magnitude stronger than the affinity of kpRstA DBD towards DNA-16a (Figure 2A). Full-length kpRstA also binds to the full-length RstA box (Figure 2E and F) at lower affinity than binding of PhoB towards a consensus pho box (61). Although all response regulators with data available show no difference in either structure or function between the BeF$_3^-$ bound and phosphorylated forms (62), we cannot exclude the possibility that BeF$_3^-$ is not able to activate kpRstA to the same extent as phosphorylation. Interestingly, the promoter region of the asr operon harbors binding sites to both RstA and PhoB, although either protein is capable of activating the operon by itself (7). We speculate that the arrangement of a strong- and weak-binding element would enhance the dynamic range of gene expression and allow for fine-tuning of gene regulation. The csgD promoter region also contains RstA and OmpR binding sites, but in this case the two sites overlap extensively (7). Since the RstB/RstA and EnvZ/OmpR TCS respond to different types of environmental stress, the large difference in DNA-binding affinity between RstA and OmpR should have little relevance on the regulation of CsgD by RstA.

The lack of affinity for the downstream half-site displayed by the DBD and the biphasic binding behavior of full-length kpRstA indicate that the two DBDs in the kpRstA dimer bind to the full-length RstA box in a sequential fashion (Figure 2). Our structural studies suggest that binding of the first DBD to the upstream half-site bring the $\alpha3$–$\beta4$ loop close to the DNA, which would then place Glu218 in a position to form inter-DBD contacts with Ala160 (Figure 4B). Backbone dynamics further suggest that Arg226 becomes more rigid when interacting with DNA, as observed from the increase in J(0) and concomitant decrease in J(N) and J(0.87H) in Figure 7, which would help stabilize the Arg226–Thr163 inter-DBD hydrogen bond. Thus, binding of one kpRstA DBD promotes the binding of the second DBD to the second site on RstA box in a cooperative manner. Whether this sequential binding behavior is dependent on the sequence of the DNA, particularly of the downstream half-site is currently unknown. It is worth noting that, unlike in E. coli, neither asr nor csgD of K. pneumoniae contain the classic RstA box (TACAGNNNNNTACA) sequences in their promoter region. In fact, K. pneumoniae homologs of the putative E. coli genes that may be targets of RstA as proposed by Osagawara et al. (8) did not harbor the classic RstA box either. The unexpected absence of classic RstA boxes in homologous genes hints at divergent RstA–DNA recognition between K. pneumoniae and E. coli.

**RD dimerization is entropy driven**

Binding of the second DBD to the second half-site features a strong entropic component (see Table 1) contingent on the presence of the RD. In protein–DNA complexes, favorable binding entropy primarily originates from desolvation of nonpolar surfaces (63). Incidentally, the RD of kpRstA contains an extensive hydrophobic patch at the $\alpha4$–$\beta5$–$\alpha5$ region, which forms the dimer interface in the active form (Figure 3D). Inactive kpRstA RD spontaneously forms dimers at high concentrations as observed from the line-widths in NMR spectra (data not shown), suggesting that the dimer interface is exposed to the solvent. The $\alpha4$–$\beta5$–$\alpha5$ region also contains a number of charged residues, which may harbor non-specific binding sites for anions and cations. Based on these premises, we would argue that the entropic contribution arises from the release of water and ions at the $\alpha4$–$\beta5$–$\alpha5$ region upon RD dimerization, which is induced by the binding of the second DBD to the downstream half-site. However, in the presence of BeF$_3^-$, one would expect the dimerization to occur before titrating the protein into the DNA solution, hence the entropic energy should noticeably decrease. This is not observed in our ITC experiments. One possibility is that the RD dimer interface within the full-length kpRstA can access different conformations depending on whether the RD is bound to DNA and the RD binds BeF$_3^-$, and only when both types of interactions are present will the interface adopt its final form, similar to the multiple states observed for the CheY response regulator under different experimental conditions (64,65). Effort is currently under way to test this possibility.

**The 'prime-and-lock' model for the activation of kpRstA**

Taken together, we propose that binding between kpRstA and its cognate DNA sequences utilizes a ‘prime-and-lock’ mechanism, shown in Figure 8. The process is divided into the following steps: (i) phosphorylation of the RD results in formation of RstA dimers with an intermediate RD–RD interface conformation (state I). Due to the presence of two DBDs close in space, the DNA-binding affinity is increased compared to the unphosphorylated form. (ii) Binding of the first DBD to the upstream half-site of the RstA box causes conformational changes in the first DBD, ‘priming’ it for interaction with the second DBD. (iii) The second DBD binds to the downstream half-site and ‘locks’ the complex through DBD–DBD and RD–RD interactions. At the same time, the RD–RD dimer assumes its final conformation (state F), providing the entropic energy required for the second binding event. The flexible transactivation loop is now in position to recruit the $\sigma$ subunit of RNA polymerase for downstream gene activation (66).
The enhanced dynamics allows the transactivation loop to sample the space around the promoter in a short time, increasing the chance of encountering the σ subunit. Thus, our model presents the following highlights: (a) Phosphorylation of the RD is a pre-requisite for kpRstA to bind to DNA, since otherwise the binding affinity would be too weak. As such, tight control of RstA-activated genes can be achieved simply by controlling the population of phosphorylated kpRstA. (b) Binding of both DBDs within a kpRstA dimer to the DNA is necessary for the formation of a stable activation complex. The first binding event anchors the dimer to potential binding sites, allowing the second binding event to occur provided that a downstream half-site is available. In the absence of the downstream half-site (i.e. wrong binding site), the binding affinity of the first DBD towards the upstream half-site alone would not be enough to maintain the activation complex. (c) Binding to DNA instigates functionally relevant changes in backbone dynamics of the DBD. The current paradigm for regulation in RRs emphasizes the importance of interfaces created between domains rather than conformational changes (56,67). Also, the importance of interfaces in promoting the strong binding of RstA to DNA is clearly the most important factor in cooperative binding of the second DBD to DNA. We observed extensive interactions between the upstream and downstream DBDs indicating that the two binding events are not independent of each other, and formed the basis for the choice of a sequential binding for our model. However, significant structural changes were also observed between the DNA-free and the DNA-bound RstA DBDs. Noticeably, the α3–β4 transactivation loop swings ∼90° toward the template strand of the DNA in forming the complex. In conclusion, our model provides a framework for RstA–DNA interaction that can be experimentally validated in future studies.

ACCESSION NUMBERS
PDB IDs: 2MLK, 1ZES, 4NHJ, 4NIC, 1OPC, 1GXQ, 1GXP, 3ZQ7 and 2PKX.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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