Crystal Structures of SlyA Protein, a Master Virulence Regulator of Salmonella, in Free and DNA-bound States*§

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SlyA is a master virulence regulator that controls the transcription of numerous genes in Salmonella enterica. We present here crystal structures of SlyA by itself and bound to a high-affinity DNA operator sequence in the slyA gene. SlyA interacts with DNA through direct recognition of a guanine base by Arg-65, as well as interactions between conserved Arg-86 and the minor groove and a large network of non-base-specific contacts with the sugar phosphate backbone. Our structures, together with an unpublished structure of SlyA bound to the small molecule effector salicylate (Protein Data Bank code 3DEU), reveal that, unlike many other MarR family proteins, SlyA dissociates from DNA without large conformational changes when bound to this effector. We propose that SlyA and other MarR global regulators rely more on indirect readout of DNA sequence to exert control over many genes, in contrast to proteins (such as OhrR) that recognize a single operator.

The transcription factor SlyA is a master regulator of virulence genes in the human bacterial pathogen Salmonella enterica. Genes regulated by SlyA allow the bacterium to proliferate in macrophages, withstand oxidative stress, and resist antimicrobial peptides (1–4). SlyA recognizes short (12–17 bp) AT-rich pseudo-palindromic sequences in the promoters of slyA, ugtL, pagC, and mig-14, as well as that of srrA in SPI2 (Salmonella pathogenicity island 2) (3–6). In vitro, SlyA forms a dimer that is the active form of the protein (5, 6). It is thought that the mechanism of SlyA-based regulation involves counteracting the silencing effect of the bacterial nucleoid protein H-NS, which associates with the AT-rich Salmonella virulence genes (reviewed in Ref. 7). This mechanism may involve competition between SlyA and H-NS for DNA binding or remodeling of DNA-H-NS complexes to permit transcription (8, 9).

SlyA belongs to the MarR superfamily of bacterial transcription factors (reviewed in Ref. 10). Extensive crystal structure data exist for MarR proteins, which reveal the conserved architecture of the family: a helix-turn-helix motif connected to a euKaryote-like β-wing for DNA binding and an α-helical dimerization domain (11–16). One MarR family member, OhrR, has been crystallized in the presence of its DNA target, the ohrA operator (14). Crystal structures exist for two MarR-type global regulators in Gram-positive Staphylococcus aureus, MgrA and SarZ, but neither crystal structure includes a bound DNA, and no other structural work has been reported for global regulators from Gram-negative bacteria. There is one unpublished structure in the Protein Data Bank (code 3DEU) of SlyA bound to the drug salicylate, a proposed effector of some MarR-type proteins (11).

The biochemical basis of SlyA function remains largely uncharacterized. Based on alignments between SlyA and other MarR proteins with known structures, targeted mutagenesis of SlyA identified a number of residues whose loss impaired DNA binding and dimerization (6, 17). However, in the absence of any crystal structure, the molecular details of the SlyA-DNA interaction and the mechanism of SlyA-mediated regulation have remained unclear. We undertook the structural characterization of the apo-SlyA and DNA-bound SlyA using x-ray crystallography and report these structures here. We confirmed the identity of key residues for DNA binding and dimerization and propose a general mechanism for DNA recognition by MarR family members that regulate multiple genes. The structures described herein also open new pathways for designing small molecules that target SlyA and related proteins as potential alternative anti-infection approaches.

**EXPERIMENTAL PROCEDURES**

**Cloning of the slyA Gene for Recombinant Expression**—The slyA ORF was amplified from the purified genomic DNA of S. enterica serovar typhimurium strain LT2 (American Type Culture Collection) using the following PCR primers: TACCTCCATATCTGCAAGTAGTCTGATC (forward) and TTATCCATTCCATGTTAAATCAGAGGTGG (reverse). Ligation-independent cloning of the amplified slyA ORF into plasmid pMCSG19 was carried out essentially as described by Donnelly et al. (18). Briefly, the purified PCR product was treated with ligation-independent cloning-qualified T4 DNA polymerase (Novagen) in the presence of 5 mM DTT and 2.5 mM dCTP to generate single-stranded ends. The insert was then annealed to linearized pMCSG19 and transformed into Escherichia coli BL21(DE3) cells carrying pRK1037. The resulting recombinant construct encodes the SlyA protein fused to maltose-binding protein with a His6 tag.
at its N terminus. Maltose-binding protein was cleaved from the protein in vivo by tobacco vein mottling virus protease expressed from pRK1037. The SlyA coding sequence in our construct contains the dipeptide Glu-Pro at residues 97 and 98; the UniProt Database sequence (P40676) contains Asp-Ala at these positions as a result of a two-nucleotide inversion. Because the original sequence of SlyA contains Glu-Pro at these positions (1), as well as many other SlyA proteins from different Salmonella strains in the NCBI Entrez Gene Database, we did not attempt to mutate the SlyA coding sequence. The identity of these residues as Glu-Pro was supported by independent cloning and sequencing experiments.

Overexpression and Purification of SlyA—E. coli cultures were grown in LB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37 °C with shaking at 250 rpm until A₆₀₀ = 0.5–0.6 was reached. To induce protein expression, isopropyl β-D-thiogalactopyranoside was added to the cultures to a final concentration of 1 mM, and cultures were further incubated for 3 h at 30 °C. Cell pellets were washed with a 0.9% saline solution, flash-frozen in liquid nitrogen, and stored at −80 °C until needed for protein purification.

Cell pellets were resuspended in buffer A (10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM DTT, and 20 mM imidazole). To lyse cells, spermine and lysozyme were added to the suspension to 20 mM and 0.3 mg/ml, respectively. The suspension was incubated on ice for 20 min and sonicated. Clarified cell lysates were applied to a 5-ml HisTrap nickel-nitrilotriacetic acid affinity column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A, and His-tagged SlyA was eluted on a 20–500 mM imidazole gradient. Fractions containing 0.25 mM hexatantalum tetrabromide and allowed to soak overnight. After rinsing in crystallization solution supplemented with 20% glycerol, crystals were flash-frozen in liquid nitrogen for data collection.

Intensity data for native and hexatantalum tetrabromide-soaked crystals were collected at the Advanced Photon Source, Argonne National Laboratory, using General Medicine and Cancer Institutes Collaborative Access Team beamline 23-ID-B. Data for multi-wavelength anomalous dispersion phasing were collected from one hexatantalum tetrabromide-soaked crystal at three wavelengths near the tantalum LIII-edge. All data were processed in HKL2000 (20). Phase calculation and density modification using the multi-wavelength anomalous dispersion data were performed by the AutoSol module of the PHENIX suite (21), with a resolution cutoff of 5.0 Å. Two hexatantalum tetrabromide clusters were located in the low-resolution electron density map. Phases from the multi-wavelength anomalous dispersion data set were then combined with amplitudes from the native data set in AutoBuild to generate an initial model and electron density maps for the native complex. Model building was completed using Coot, and the model was refined in PHENIX. Figures were prepared with PyMOL (25).

Electrophoretic Mobility Shift Assay—Binding reactions (10 μl) containing 100 nM purified tag-free SlyA and 25 nM high-affinity DNA were incubated in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.2% Triton X-100, and 50 μg/ml bovine serum albumin. Where indicated, reactions were supplemented with sodium salicylate from 0.5 M stock solution. After 20 min, loading buffer containing 20% glycerol was added to each reaction. The 8% native polyacrylamide gel was prerun for 30 min in 0.5 X Tris borate/EDTA before sample loading. Free and bound DNAs were resolved by electrophoresis at 85 V for 60 min at room temperature. The gel was stained with SYBR Gold (Invitrogen), and the DNA was visualized in a UV cabinet.

2 The abbreviations used are: SeMet, selenomethionine; HTH, helix-turn-helix.
**RESULTS**

**Structure of the Apo-SlyA Protein**—The SlyA protein structure was solved by single-wavelength anomalous dispersion from a SeMet-substituted crystal form. Apo-SlyA crystallized in space group P6\(_2\),2 and was refined to 2.4 Å (Table 1). The final structure contains three selenium atoms, including two in the dimerization domain, Leu-12 from one monomer interacts hydrophobically with Ile-130 and Leu-133 from the second subunit. The two Leu-126 residues, one from each subunit, interact with each other to complete the hydrophobic dimer interface. The bottom half of the dimer is in a “closed” position, with the N termini of the two α4 helices separated by ~15 Å. Glu-59 and Arg-65 from each subunit form intermolecular salt bridges to hold these helices in place.

**Structure of SlyA Bound to a High-affinity DNA Site**—To investigate the mode of DNA binding by SlyA, SlyA was crystallized in the presence of a 22-bp duplex derived from a sequence just downstream of the slyA transcriptional start site. This sequence contains a 12-bp high-affinity binding site, TTAGCAAGCTAA, which was discovered by DNase footprinting of the slyA promoter region and confirmed by SELEX (5). The complex of SlyA and this DNA fragment crystallized in space group P4\(_2\),2\(_2\),2. Complex crystals were soaked in hexatan-

| TABLE 1 |
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| **Data collection, phasing, and refinement statistics** |
| TaBr, hexatantalum tetradecabromide; r.m.s.d., root mean square deviation. |
| **Data collection** | **SeMet-SlyA** |
| Space group | P6\(_2\),2 |
| Cell dimensions | 0.97938 |
| Resolution (Å) | 50–2.20 (2.24–2.20) |
| R\(_{merge}\) (%) | 9.6 (71.3) |
| Completeness (%) | 94.0 (58.2) |
| Redundancy | 10.8 (3.3) |
| **Native SlyA-DNA complex** | **TaBr-SlyA-DNA** |
| Space group | P4\(_2\),2 |
| Cell dimensions | 0.97934 |
| Resolution (Å) | 50–3.08 (3.09–2.97) |
| R\(_{merge}\) (%) | 5–4.00 (4.14–4.00) |
| Completeness (%) | 98.8 (99.0) |
| Redundancy | 25.9 (26.2) |
| **Phasing** | **Figure of merit (before/after density modification)** |
| Phasing resolution (Å) | 42.6–2.25 |
| 50–5.00 |
| **Refinement** | **SeMet-SlyA** |
| Resolution (Å) | 39–2.40 (2.46–2.40) |
| R\(_{free}\)/R\(_{work}\) (%) | 46–2.96 (3.09–2.96) |
| 21.3/26.6 (31.4/49.1) |
| Mean B factor (Å\(^2\)) | 48.85 |
| 67.23 |
| Ramachandran plot (%/#) | 93.8/105 |
| 91.4/233 |
| Additional allowed | 6.2/7 |
| 8.6/22 |
| Generously allowed | 0/0 |
| 0/0 |
| Disallowed | 0/0 |
| 0/0 |

\(a\) Values in parentheses are for the highest resolution shell.

\(b\) R\(_{merge}\) = Σ|I\(_{hkI}\)| – (I) / Σ|I\(_{hkI}\)| where I\(_{hkI}\) is the observed intensity for reflection hkl, and (I) is the mean intensity.

\(c\) R\(_{free}\) = Σ|F\(_{obs}\)| – |F\(_{calc}\)| / Σ|F\(_{obs}\)|. R\(_{free}\) is calculated identically for a test set of random reflections totaling 5% for the SeMet-SlyA data set and 10% for the SlyA-DNA data set.

\(d\) Ramachandran statistics are from PROCHECK (31).
dimeric axis of symmetry. The base of the triangular dimer is oriented at an angle of \(\sim 19^\circ\) with respect to the DNA and measures 72 Å from the tip of one wing (defined as Ca of Ser-83) to the other.

The SlyA dimer in the complex looks very different from that in the apo crystal (Fig. 2B). Superposition of 258 Ca atoms from the two dimers results in a root mean square deviation of 5.4 Å. Notably, binding to DNA causes a large motion in the \(\alpha_4\) helices of both subunits, so these helices can fit into consecutive major grooves \(\sim 32\) Å apart (Fig. 2, B and C).

**Interactions between SlyA and the High-affinity DNA Site**—In the SlyA-DNA complex, the DNA bends upward toward the bottom of the SlyA dimer at the pseudo-palindromic center, generating a kink of \(\sim 16^\circ\), and the DNA is undertwisted by 1.2°. Near the center of the duplex, the major grooves widen by up to 2.3 Å to accommodate the \(\alpha_4\) recognition helices, whereas near the ends of the binding site, the minor grooves narrow slightly. At the very end of the duplex, the minor groove actually grows wider, perhaps on account of contacts between DNA molecules in the crystal.

Each SlyA subunit makes a nearly identical set of contacts with the two halves of the high-affinity sequence (Fig. 3A). The \(\alpha_4\) recognition helices emerge from the major grooves at the pseudo-palindromic center, with their N termini pointed into each groove. The sole direct contact with the DNA bases in this region is provided by Arg-65, which forms bidentate hydrogen bonds between the guanidinium NH groups and O6 and N7 of guanine 14 (Fig. 3B and supplemental Fig. S1A). Two residues

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**FIGURE 1. Structure of apo-SlyA.** A, schematic of the SeMet-substituted SlyA monomer with secondary structures labeled. The approximate position of the disordered wing region is indicated by the dashed black lines. B, SlyA dimer. C, ClustalW sequence alignment of SlyA with other MarR family regulators. SlyA secondary structures are depicted as green rectangles (\(\alpha\)-helices) and arrows (\(\beta\)-strands). Asterisks, conserved residues; colons, highly similar residues; periods, less similar residues.
from this helix participate in van der Waals contacts with the DNA bases: Cβ of Pro-61 contacts O4 of thymine 8 of one strand and thymine 16 on the opposite strand, whereas Oγ of Ser-62 approaches C5 of cytosine 15. Oγ of Ser-62 also contacts the phosphate group of cytosine 15, along with the backbone NH of Glu-59. In one subunit, the side chain of Glu-59 also contacts the phosphate group of thymine 16, whereas in the other subunit, Oγ of Ser-62 contributes a third van der Waals contact at the sugar ring of guanine 14. The side chain of Thr-32 on one subunit, a residue found in the linker between helices 1 and 2, also approaches the adenine 13 phosphate group.

The outer portions of the high-affinity sequence are contacted by residues from the β-wing and helix 3. In our structure, several of these contacts are unique to each subunit because the natural slyA sequence bordering the 12-bp pseudo-palindrome is not symmetrical; the sequence reads AATAAC in the sense direction and TTATAA in the opposite strand. The most pronounced effect of this asymmetry is seen in the wing-minor groove interaction network. Here, the side chain of Arg-86 extends into the minor groove and is sandwiched between two sugar rings belonging to specifically positioned nucleotides, one being three positions ahead of the 5′-end of the pseudo-palindromic sequence (i.e. adenine 4/thymine 4′) and the other being five positions after the 3′-position of the same element on the antiparallel strand (i.e. adenine 23/thymine 23′). Arg-86 is also held in place by an electrostatic interaction with the side chain of Asp-84 and a contact between the backbone NH of Arg-85 and the O2 atoms of thymine 22 and thymine 4′ (Fig. 3C and supplemental Fig. S1B), whereas N3 of adenine 22 makes a van der Waals contact with Cβ of Arg-86 on the “upstream” subunit. Also, Arg-85 of the downstream subunit curves around to form an interaction between its side chain and the adenine 23 phosphate, and Cβ of upstream Asp-84 contacts C5′ of thymine 23′.

Besides the interactions described above, there exists a large collection of contacts between the sugar phosphate DNA backbone, the SlyA wing, and helix 3. O3′ of the adenine 5 sugar engages with Cβ of Ala-87. The phosphate group on nucleotide 6 is an epicenter of the interaction network; here, there are contacts made with backbone NH groups from Gln-49, Ile-50, and Lys-88, with Ile-50 and Ala-87 also contributing van der Waals contacts from their aliphatic side chains. Ser-48 Cβ makes a van der Waals contact with this group in the upstream subunit. The positive dipole of helix 3 also points directly toward this phosphate. The phosphate group of thymine 7 can form hydrogen bonds with the side chain nitrogen atoms of Gln-49, Arg-78, or Lys-88. Finally, an extra van der Waals con-
tact is found between Oε of downstream Gln-49 and C3’ of the sugar ring of adenine 6’. Disruption of the SlyA-DNA Complex by Salicylate—At the beginning of this study, we found an unpublished structure of SlyA bound to the drug salicylate in the Protein Data Bank (code 3DEU). Salicylate is known to modulate the interaction between DNA and E. coli MarR and was co-crystallized in the structure of MarR (11). We hypothesized that salicylate may cause SlyA to dissociate from DNA. We performed a gel shift assay using purified native SlyA and DNA representing the high-affinity SlyA-binding sequence. The addition of salicylate in quantities of 50 mM (the concentration used to co-crystallize SlyA for the 3DEU structure) or 25 mM led to complete dissociation of SlyA from the high-affinity target DNA, suggesting that salicylate could be an effector for SlyA at high concentrations (Fig. 4A).

DISCUSSION

We have solved the structures of the Salmonella virulence factor SlyA, including its structure in complex with a high-affinity DNA sequence, which, to our knowledge, represents the first such crystal structure of this master virulence regulator. Previous biochemical and genetic studies revealed the importance of several residues, including Leu-12, Leu-63, Arg-65,
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FIGURE 4. Salicylate disrupts the SlyA-DNA interaction. A, salicylate (SAL) disrupts the SlyA-DNA interaction in a gel shift assay. Lane 1, 22-mer DNA (25 nM) containing the high-affinity SlyA-binding site (5); lane 2, DNA plus 25 mM sodium salicylate; lane 3, DNA plus 100 nM SlyA; lane 4, DNA plus SlyA and 25 mM sodium salicylate; lane 5, DNA plus SlyA and 50 mM sodium salicylate. Free and bound DNAs were resolved on a nondenaturing 8% polyacrylamide gel. B, alignment of DNA-bound SlyA (cyan) with the SlyA monomer complexed to salicylate (Protein Data Bank code 3DEU; pink). DNA and the protein are shown as ribbons, and salicylate molecules are shown as sticks. C, stereo view showing steric clash between salicylate and guanine 14 and displacement of Arg-65. Cyan helix and sticks, SlyA-DNA complex; pink helix and sticks, SlyA-salicylate complex.

Leu-67, Leu-70, and Leu-126, for SlyA function based on targeted mutagenesis that abolished transcriptional activation and/or DNA binding when these residues were changed to alanine (6, 17). Our structures permit us to unequivocally assign functions to many of these residues, whose roles include maintenance of structural integrity, dimerization, and DNA binding.

The SlyA wing shares many features with that of OhrR, the first MarR family protein crystallized with DNA (14). SlyA Arg-86 mirrors OhrR Arg-94, which enters the minor groove, and both are held in place by a conserved aspartic acid (Asp-84 and Asp-92, respectively). Both half-sites of the ohrA operator contain a conserved thymine that is hydrogen-bonded to OhrR Arg-94 in the crystal structure. The proposed role of OhrR Arg-94 is to select pyrimidines over purines in the ohrA minor groove. In contrast, our structure suggests a role for indirect readout of DNA by SlyA Arg-86 because both Arg-86 residues can be said to “measure” the width of the minor groove by interacting with a pair of sugars at specific positions, whereas only one of these residues also forms hydrogen bonds with the minor groove base surfaces (Fig. 3). The large number of contacts with the DNA backbone versus the bases of the slyA operator provides further evidence that SlyA uses an indirect contact mechanism to bind DNA. As a regulator of multiple genes, SlyA must recognize several sequences in the Salmonella genome. These sequences need not be identical in length or sequence, as has been shown by DNase footprinting assays of several SlyA-responsive promoters (3–6). We predict that indirect readout and reliance on DNA shape rather than sequence for high-affinity recognition will be a hallmark of the MarR family proteins that are global virulence regulators. Instead of reading one promoter sequence, this property enables the global regulatory function.

The binding of MarR family proteins to DNA is often modulated by small molecule ligands, thereby linking environmental signals with gene expression responses (10). In the presence of the high-affinity binding site from slyA, purified SlyA required no cofactor to bind DNA, but the interaction could be disrupted by salicylate (Fig. 4A). Superposition of 122 Cα atoms from a SlyA monomer bound to salicylate onto one monomer from our SlyA-DNA complex showed few large conformational changes, with an overall root mean square deviation of 1.6 Å (Fig. 4B). Close inspection of the superposed molecules revealed that one of three salicylate molecules in the Protein Data Bank 3DEU structure occupies the same place as the DNA backbone at guanine 14; furthermore, helix α4 of salicylate-bound SlyA undergoes a rotation of ~35° around its axis, which would lead to disruption of protein-DNA contacts in this region, including the interaction between Arg-65 and guanine 14 (Fig. 4C). Translation of this motion to the neighboring wing appears to pull it out and away from the minor groove, although many residues in this region are not modeled in the 3DEU structure. Conformational changes to the winged HTH domain are a common mechanism of DNA derepression across the MarR family, linking ligand binding to gene response. Oxidation of a cysteine in Xanthomonas campestris OhrR by organic hydroperoxide disrupts a critical hydrogen-bonding network and leads to formation of an intersubunit disulfide bond, causing a 28° rotation in the winged HTH domain (26). Similar oxidation of a homologous cysteine to a mixed disulfide produces large motions in the S. aureus global regulator SarZ. Compared with its reduced and sulfenic acid forms, the mixed-disulfide SarZ appears to be incompatible with DNA binding (16). Binding of the antirepressor peptide ArmR to MexR causes helix α4 in that protein to shift up to 13 Å and the wing to move up to 18 Å, leading to dissociation from DNA (27). In contrast with these large motions, the conformational change in SlyA upon salicylate binding is much more subtle. Interestingly, MexR can also use an oxidation-sensing mechanism involving intersubunit disulfide bond formation to release from DNA (28). The crystal structure of the oxidized MexR dimer also reveals little overall structural change compared with the DNA-binding form; instead, the disulfide bonds would inhibit a MexR-DNA complex via steric clashes with the DNA backbone (29).

There is growing interest in the concept of antivirulence as an approach to creating new antibiotics that evade the ever-present challenge of drug resistance. Such therapies would inhibit the activity of important virulence factors, which leave...
the bacterium viable but in a latent state, thereby eliminating the evolutionary pressure to acquire antibiotic resistance genes (30). The flexible HTH domains of SlyA and closely related proteins, such as RovA in Yersinia, could be attractive targets for rational drug design efforts in this vein. Given the major roles played by SlyA in the pathogenesis of Salmonella, the structures presented here provide a platform for this work.

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