Quaternary Structural Transitions in the DeoR-Type Repressor UlaR Control Transcriptional Readout from the L-Ascorbate Utilization Regulon in *Escherichia coli*[†]

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ABSTRACT: UlaR is a DNA binding protein of the DeoR family of eubacterial transcriptional repressors which maintains the utilization of the L-ascorbate *ula* regulon in a repressed state. The availability of L-ascorbate in the growth medium releases UlaR-mediated repression on the *ula* regulon, thereby activating transcription. The molecular details of this induction by L-ascorbate have remained elusive to date. Here we have identified L-ascorbate 6-phosphate as a direct effector of UlaR; using a combination of site-directed mutagenesis, gel retardation, isothermal titration calorimetry, and analytical ultracentrifugation studies, we have identified the key amino acid residues that mediate L-ascorbate 6-phosphate binding and constructed the first model of regulation of a DeoR family member, establishing the basis of the *ula* regulon transcription control by UlaR. In this model, specific quaternary rearrangements of the DeoR-type repressor are the molecular underpinning of the activating and repressing forms. A DNA-bound UlaR tetramer establishes repression, whereas an L-ascorbate-6-phosphate-induced breakdown of the tetrameric configuration in favor of an UlaR dimeric state results in dissociation of UlaR from DNA and allows transcription of *ulaG* and *ulaABCDEF* structural genes. Despite the fact that similar changes have been described for other unrelated repressor factors, this is the first report to demonstrate that specific oligomerization changes are responsible for the activating and repressing forms of a DeoR-type eubacterial transcriptional repressor.

Transcriptional regulators of the DeoR[1] family are widespread among Gram-positive and Gram-negative eubacteria. In *Escherichia coli*, the repertoire of DeoR family regulators includes 14 members, of which eight have well-characterized functions, while the rest are annotated on the basis of sequence homology (*1, 2*). Members of the DeoR family typically act as repressors in sugar and nucleoside metabolism (*3, 4*). Examples of systems controlled by DeoR-type regulators are the glycerol-3-phosphate (GlpR), L-fucose (FucR), L-ascorbate (UlaR), and deoxyribonucleoside (DeoR) systems (*5*−*8*). For most of these systems, the inducer is a phosphorylated sugar. Proteins of the DeoR family share several common features. First, their length (between 240 and 260 amino acids) is highly conserved (*2*). Second, the domain organization is reminiscent of the two-component systems of signal transduction, with a sensor C-terminal domain, which binds the inducer and often acts as an oligomerization domain, and an N-terminal domain, which folds in a DNA-binding helix−turn−helix (HTH) motif (*9*−*11*). Helix 2 (or the recognition helix) of the HTH motif is well-conserved across the DeoR family, whereas helix 1

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[1] Abbreviations: ArgR, arginine repressor; L-ascorbate-6-P, L-ascorbate 6-phosphate (likewise, P stands for phosphate in all sugar phosphates mentioned); AUC, analytical ultracentrifugation; CRP, cAMP receptor protein; DBD, DNA binding domain; DeoR, deoxyribose nucleoside repressor; ds, double-stranded; EMSA, electrophoretic mobility shift assay; EthR, ethionamide resistance repressor; FNR, fumarate nitrate reductase regulator; FucR, fucose repressor; GalR, galactose repressor; GlpR, glycerol 3-phosphate repressor; HTH, helix−turn−helix; IHF, integration host factor; ITC, isothermal titration calorimetry; LacR, lactose repressor; LldR, L-lactose dehydrogenase repressor; MBP, maltose binding protein; PTS, phosphotransferase system; QacR, *qac*A gene repressor; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TetR, tetracycline repressor; Ula, utilization of L-ascorbic acid; UlaR, Ula regulon repressor protein.

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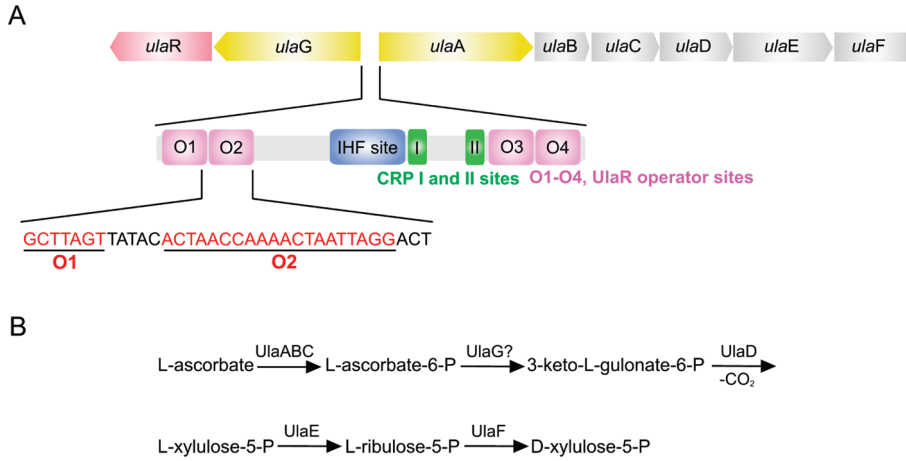

FIGURE 1: Scheme of the *ula* regulon. Directions of the two transcriptional units of the *ula* regulon are shown by arrows emanating from the first transcribed gene of each unit (yellow), *ulaG* and *ula ABCDEF*, respectively. The gene encoding the UlaR repressor is colored red. All other genes are colored gray. The interoperon region is amplified to show the position of the four operator sites (O1−O4), regulatory binding sites CRP I and CRP II, and the integration host factor (IHF) site. A region consisting of the end of O1 and all of O2, which was used throughout this study, is further magnified. UlaR recognition sites are colored pink. (B) Scheme of the L-ascorbate-6-P pathway. The question mark by UlaG means a putative functional assignment.

presents more sequence variation (with the exception of an absolutely conserved arginine residue, which presumably stabilizes helix 3) (*2*). Third, the repressors of the DeoR family bind to several operator sites within the promoter regions of the target genes, with widely varying configurations. Thus, in the *deo* system, DeoR binds distant multiple operators with a dyad symmetry (*12*); in contrast, in the *glp* system, GlpR binds adjacent operators in tandem (*13*−*15*). Oligomerization of DeoR proteins and cooperative binding to several operator sites have been shown to facilitate DNA looping and enhance regulation (*16*). This is the case for DeoR, which arranges in an octamer (*12*), or GlpR and AgaR, which form tetramers (*17*). In an unrelated system, the ArgR repressor, binding of its inducer (arginine) is required for negative feedback inhibition of arginine biosynthetic genes by stabilization of a trimer−trimer interface required for binding DNA (*18*). Other examples of transcription factor regulation by oligomerization are found in TetR family proteins QacR and EthR. QacR is a dimer of dimers on DNA, or a dimer upon induction by several drugs (*19, 20*); EthR is an octamer of dimers on DNA, and it becomes a dimer upon induction (*21*). One further example is that of the GalR repressosome, where GalR binds the two operators in the *gal* promoter and exerts full repression in a dimer of dimers configuration which, upon induction by D-galactose, dissociates into DNA-unbound dimers (*22*).

In *E. coli*, the *ula* regulon controls the anaerobic utilization of L-ascorbate as a carbon and energy source (*11, 23*). This system is formed by two transcriptional units (*24*), one formed by *ulaG*, proposed to encode an L-ascorbate-6-P lactonase, and the other formed by the *ulaABCDEF* genes, which encode the three components of the L-ascorbate phosphotransferase system (UlaABC) (*25*) and three catabolic enzymes (UlaDEF) (*23*) (Figure 1 and Figure S1 of the Supporting Information). The UlaABC transporter is involved in the uptake and phosphorylation of L-ascorbate (*25*), which becomes internalized as L-ascorbate-6-P. The latter is then channeled to the pentose phosphate pathway as D-xylulose-5-P through the sequential action of L-ascorbate-6-P lactonase, UlaD, UlaE, and UlaF. The *ula* regulon is regulated by a DeoR-like repressor protein termed UlaR (YjfQ; Uniprot accession number P0A9W0 or ULAR_ECOLI) (*24*) and by the general regulators cyclic AMP (cAMP) receptor protein (CRP) and fumarate nitrate reductase regulator (FNR) (*26*). UlaR is a 251-amino acid protein that represses the two divergently transcribed units *ulaG* and *ulaABCDEF* via simultaneous binding at the two operator sites present in each promoter (Figure 1A) (*6, 24*). Consistent with the known DeoR domain organization, UlaR comprises two domains, an N-terminal HTH domain (residues 1−62) and a C-terminal sugar−phosphate binding domain (residues 69−251) (*24*) (Figure 2). The sugar−phosphate binding domain belongs to the DeoR-C family of the ISOCOT superfamily and is structurally related to *E. coli* D-ribose-5-P isomerase (*27*).

Here we show that L-ascorbate-6-P is a small-molecule effector of UlaR and that binding of L-ascorbate-6-P to UlaR severely impairs the capacity of UlaR to bind to its cognate operator sites. Further, we provide experimental evidence that demonstrates that UlaR activity is controlled by homotypic tetramer−dimer transitions regulated by L-ascorbate-6-P. On the basis of these results, we propose a working model for transcription regulation of the *ula* regulon by UlaR, whereby under normal conditions, UlaR binds to the four operator sites of the *ula* operons in a homotetrameric arrangement thus eliciting total repression (*6*). In contrast, the presence of L-ascorbate-6-P breaks this configuration down into DNA-free UlaR homodimers and allows transcription to proceed. Although changes in the oligomerization state of DeoR-type regulators have been shown to facilitate binding to the promoter regions (*12, 17*), this is, to the best of our knowledge, the first study to show that these changes underlie the regulatory switch between the DNA-bound and the DNA-free states of a DeoR-type transcriptional repressor.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The genotypes and sources of the *E. coli* strains used in this study are as follows. The wild-type strain was *E. coli* K-12, here termed ECL1 (*28*), with genotype HfrC *phoA8 relA1 tonA22* $T2^r$. Strain XL1 Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*

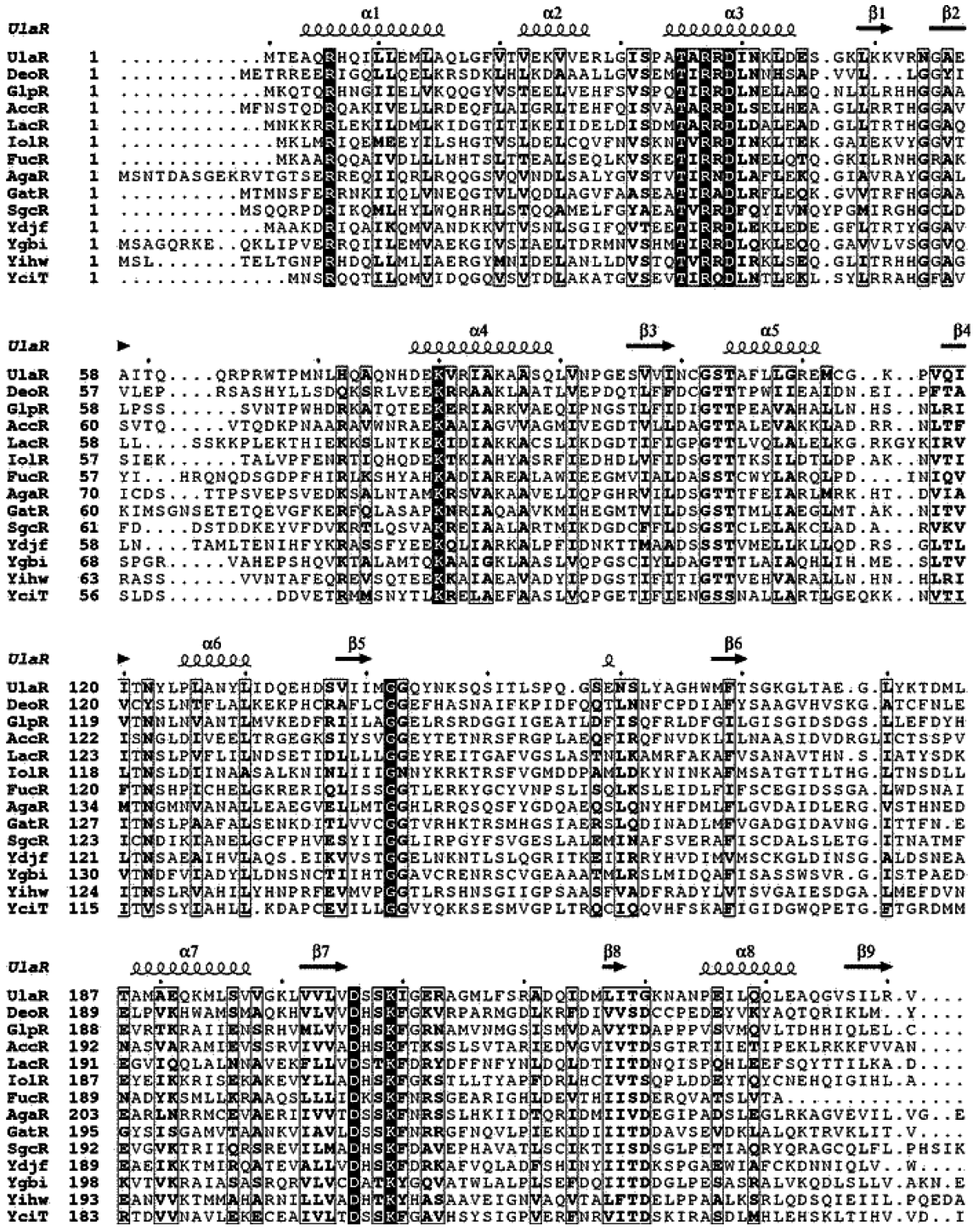

FIGURE 2: Multiple-sequence alignment of DeoR-type transcriptional regulators in *E. coli*. The alignment was prepared with ClustalX (*39*) and rendered using the ESPript version 2.2 server with default parameters (*40*). Secondary structure prediction was conducted with the PredictProtein server (*41*). Strictly conserved residues are shown as white letters on a black background; similar residues are boxed.

[F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (*tet*^{R})]} (Stratagene) was used to clone and express the UlaR fusion proteins. Plasmid pMAL-c2X (Ap^{R}) encoding an N-terminal maltose binding protein (MBP) fusion upstream the multicloning site was obtained from New England Biolabs.

DNA Manipulations, Cloning, and Site-Directed Mutagenesis. Standard molecular biology techniques were used throughout this study unless otherwise stated (*29*). Bacterial genomic DNA was prepared from the ECL1 strain as described by Silhavy et al. (*30*). For routine work, plasmid DNA was purified with the Wizard Plus Midiprep DNA purification kit (Promega). DNA sequencing was carried out with an automated ABI 377 DNA sequencer and the fluorescent dye termination method. A DNA fragment encoding the full-length *ulaR* gene was amplified by polymerase chain reaction (PCR) using ECL1 strain chromosomal DNA as template and primers UlaR-pMalfwd (5′-CT**GAATTC**ATGACTGAAGCACAAAGACATC-3′) and UlaR-pMalrev (5′-TAA**GGATCC**TTAAACACGCAGAATGCTGAC-3′) (Table 1), which introduced *Eco*RI and *Bam*HI restriction sites (in bold) for ligation into pMAL-c2X, downstream and in frame with MBP, respectively, thereby yielding the

Table 1: Sequences of DNA Oligonucleotides Used in This Study[a]

cloning[b]	
UlaR-pMALfwd	CT**GAATTC**ATGACTGAAGCACAAAGACATC
UlaR-pMALrev	TAA**GGATCC**TTAAACACGCAGAATGCTGAC
UlaR-pMAL-C-fwd	CTGAATTCAATCTGCATCAGGCGCAGAATCAC
UlaR-pMAL-C-rev	TAAGGATCCTTAGCCGGTGATAAGCATATCGAT
D8YJF	GCGAATTCATTAAATCTTTCCATACTC
YJFD1	TCGGATCCTCAATATGTGATTGGTT
annealing[b]	
Op2 + 15fwd	TCA**GGATTAATCAAAACCAATCA**CATATTGATTCG
Op2 + 15rev	ACGAATCAATATG**TGATTGGTTTTGATTAATCC**TG
mutagenesis[c]	
UlaR-H72A fwd	CCGATGAATCTG**GCT**CAGGCGCAGAAT
UlaR-H72A rev	ATTCTGGCGCTG**AGC**CAGATTCATCGG
UlaR-K80A fwd	AATCACGATGAA**GCA**GTACGTATC
UlaR-K80A rev	GATACGTAC**TGC**TTCATCGTGATT
UlaR-D206G fwd	CTGGTGGTACTGGTT**GGT**AGCAGTAAG
UlaR-D206G rev	CTTACTGCT**ACC**AACCAGTACCACCAG
UlaR-K209A fwd	GTTGATAGCAGT**GCG**ATTGGCGAACGC
UlaR-K209A rev	GCGTTCGCCAAT**CGC**ACTGCTATCAAC

[a] Restriction sites are shown in bold; start and stop codons are underlined. [b] Primers for Probe 115 amplification. Operator regions are shown in bold. [c] Mutagenic primers with substituted bases are shown in bold.

pMALP6 plasmid. Two C-terminal truncations of UlaR, 70−251 and 70−231, were generated by PCR using primers UlaR-pMAL-C-fwd (5′-CT**GAATTC**AATCTGCATCAGGCGCAGAATCAC-3′), UlaR-pMALrev, UlaR-pMAL-C-fwd, and UlaR-pMALrev, respectively, and pMALP6 as the template and were subcloned into pMAL-c2X, yielding pMALP6CF and pMALP6C. PCRs were performed with Pfu DNA polymerase (Stratagene) under standard conditions. Site-directed mutagenesis of the wild-type *ulaR* gene was done following the QuickChange mutagenesis protocol (Stratagene). Successful incorporation of the appropriate substitutions and the absence of unwanted mutations were confirmed by sequencing all constructs. All the primers used in this study are listed in Table 1.

β-Galactosidase Activity. β-Galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl β-D-galactopyranoside and expressed in Miller units (*31*). The data reported are a representative set from at least four separate experiments performed in duplicate.

Expression and Purification of Recombinant UlaR Proteins. MBP−UlaR fusion proteins were overexpressed in XL1 Blue using the following protocol. One freshly transformed colony was used to inoculate 2 mL of starter culture of Luria-Bertani (LB) medium, 100 μg/mL ampicillin, and 0.2% (w/v) glucose, which was grown overnight at 37 °C until saturation; 200 μL of the first starter culture was used to inoculate 10 mL of a second starter culture (same medium and growth conditions). The next day, 1 L of LB medium, 100 mg/mL ampicillin, and 0.2% (w/v) glucose was inoculated with the entire second starter culture and grown at 37 °C until the OD_{600} reached ∼0.5−0.7. At this point, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.3 mM, and expression was allowed to proceed for 12 h. Cells were harvested by centrifugation at 4000*g* for 15 min at 4 °C, resuspended in 60 mL of column buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA], and lysed by sonication. The lysate was centrifuged at 10000*g* for 30 min at 4 °C, and the supernatant was diluted four times in column buffer prior to being loaded on an amylose column, previously equilibrated with 8 column volumes (CV) of column buffer. After sample application, 12 CV of column buffer was used to wash loosely bound impurities, and the pure fusion protein was eluted with 10 mM maltose in column buffer. Digestion of the purified fusion proteins with Factor Xa was conducted at 1 unit/μL following the manufacturer's protocols (New England Biolabs).

DNA Binding Assays. For electrophoretic mobility shift assays (EMSAs), a double-stranded (ds) DNA fragment containing the UlaR operator O2 site (Probe 115) was obtained by PCR with D8Y-JF and YJF-D1 primers (Table 1). EMSAs were performed with purified recombinant UlaR protein. Native polyacrylamide gels (5%) containing 10% (v/v) glycerol in 1× TBE buffer [0.089 M Tris-borate (pH 8) and 0.002 M EDTA] were used to electrophorese gel shift reaction mixtures at 4 °C after a 30 min prerun without protein samples. Protein samples were mixed with DNA (ca. 2.5 nM final concentration) in a 20 μL reaction mixture containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10% (v/v) glycerol, and 2 mM dithiothreitol (DTT). Poly(dI-dC) was used as a nonspecific competitor at a 500-fold molar excess. After incubation for 15 min at 30 °C, 4 μL of a 6× gel loading buffer was added and the reaction mixtures were loaded directly onto the gel. Gel-shifted complexes with unmodified dsDNA oligonucleotides were visualized by ethidium bromide staining and UV transillumination.

Formation of the UlaR−DNA Complex. Protein−DNA complexes for analytical ultracentrifugation (AUC) were prepared as follows. We created a 35 bp dsDNA oligonucleotide encompassing the full O2 operator (Op2 + 15) by mixing two complementary oligonucleotides, Op2 + 15fwd and Op2 + 15rev (Table 1) (MWG), heating them to 80 °C for 30 min in a water bath, and then allowing them to cool slowly overnight. Op2 + 15 was added to purified MBP-UlaR (6 mg/mL) in a slight excess DNA:UlaR molar ratio (1.1:1), and binding was ensured by incubating the mixture for 60 min at 30 °C. The MBP fusion protein was cleaved by overnight incubation with Factor Xa. The UlaR−Op2 + 15 complex was further purified by size exclusion chromatography on a HiLoad Superdex200 column (GE Healthcare). Protein concentrations were determined following the method described by Lowry, using bovine serum albumin (BSA) as the standard (*32*). Further information regarding the purity and quality of the UlaR−DNA complex appears in Figure S2A of the Supporting Information.

UlaR−L-Ascorbate-6-P Complex. L-Ascorbate-6-P was chemically synthesized by reacting 6-bromo-6-deoxy-L-ascorbic acid with hydrogen phosphate ion at 25 °C, essentially as described by Liao et al. (*33*). To produce the UlaR−L-ascorbate-6-P complex, equimolar amounts of purified MBP−UlaR fusion protein and L-ascorbate-6-P were mixed and incubated at 30 °C for 1 h. After overnight cleavage with Factor Xa, the UlaR−L-ascorbate-6-P complex was purified from the remaining MBP and Factor Xa on a CM (carboxymethyl) Sepharose FF column (GE Healthcare). Under our experimental conditions, MBP flowed through CM Sepharose, while UlaR was retained. The UlaR−L-ascorbate-6-P complex was eluted in 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, and 1 mM DTT. Further information regarding the purity and quality of the UlaR−L-ascorbate-6-P complex appears in Figure S2B of the Supporting Information.

Isothermal Titration Calorimetry. Experiments were performed using a VP-ITC microcalorimeter with VPViewer2000 for instrument control and data acquisition (MicroCal). Protein samples including MBP fusions of wild-type UlaR and D206G, K209A, H72A, and K80A mutants, or UlaR after cleavage with Factor Xa, at 28−212 μM were prepared in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA. Lyophilized L-ascorbate-6-P was dissolved in the same buffer to a final concentration of 100 mM to make a stock solution. Further dilutions to 0.056−4.24 mM were made from the same buffer for the experiments. Protein concentrations were determined by the absorbance at 280 nm in 6 M guanidinium hydrochloride, using theoretical extinction coefficients. All samples were degassed at 15 °C for 8 min beforehand. Each UlaR protein was thermostated to 20 °C in a stirred (300 rpm) sample cell (1.41 mL), and the titrations were carried out as follows. (i) For MBP and UlaR control samples, a 28 μM protein sample was titrated with 0.56 mM L-ascorbate-6-P using 20 injections (15 μL for 30 s each). (ii) For the MPB−UlaR fusion protein (both full-length or C-terminal domain), a 56 μM protein sample was titrated with 1.12 mM L-ascorbate-6-P using 28 injections (4 μL for 8 s each) followed by 28 injections (8 μL for 16 s each). (iii) For the MBP−UlaR D206G mutant fusion protein, a 114 μM protein sample was titrated with 2.24 mM L-ascorbate-6-phosphate using 15 injections (30 μL for 60 s each). (iv) For the MBP−UlaR K209A mutant fusion protein, a 212 μM protein sample was titrated with 4.24 mM L-ascorbate-6-P using 15 injections (30 μL for 60 s each). The calorimetric raw data were analyzed using MicroCal Origin using manual integration, and binding isotherms were fitted by a nonlinear iterative least-squares algorithm to a model consisting of a single set of binding sites. Binding parameters were calculated from the fits.

Analytical Ultracentrifugation. Sedimentation velocity experiments were conducted in a Beckmann Optima XL-1 ultracentrifuge at initial loading concentrations of 0.1−0.4 mg/mL UlaR. Two complexes were prepared for AUC−SV analysis. The first complex was a UlaR−DNA complex with a short DNA sequence containing operator O2 with 15 flanking base pairs [O2 + 15 (Table 1)]; the second complex was UlaR in the presence of 7 mM L-ascorbate-6-P. Aliquots (100 μL) at varying concentrations of each UlaR complex in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA (UlaR−DNA complex) or 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, and 1 mM DTT (UlaR−L-ascorbate-6-P complex) were loaded into the three top channels of a six-channel centerpiece, and the three down channels were filled with 90 μL of buffer as a reference. Centrifugation was conducted at 20 °C in an AnTi-50 six-hole rotor at 20000, 30000, 45000, and 55000 rpm, during which visible sedimentation occurred. Radial absorbance scans were taken in continuous scan mode at 260 nm every 1 min with two replicates and a step size of 0.005 cm. The fringe displacement profiles were acquired at 1 min intervals.

Data analysis was performed by direct boundary modeling by solutions of the Lamm equation, available with SEDFIT (*34*). Data were best fitted by a model describing two independently sedimenting species, and the program returned values of the sedimentation and diffusion coefficients (*s* and *D*, respectively) for each species along with their standard errors. The final fits were obtained using size distributions $c^{(P)}(S)$ implementing prior knowledge for the determination of macromolecular size distributions (*34, 35*).

RESULTS

L-Ascorbate-6-P Is an Inducer Molecule of UlaR. Since members of the DeoR family typically have a phosphorylated sugar as their inducer, and often this sugar is the first intermediate of the pathway, we hypothesized that the most probable candidate inducer of UlaR was L-ascorbate-6-P. To test this hypothesis rigorously, we also examined all phosphorylated intermediates of the L-ascorbate pathway (Figure 1B) with respect to their capacity to act as inducers of the *ula* regulon.

Previous work from our laboratory has shown that addition of L-ascorbate in the growth medium induces expression of the *ula* regulon, suggesting that the metabolism of L-ascorbate (Figure S1 of the Supporting Information) could give rise to the inducer of the regulon (*24*). Furthermore, we have previously reported that constitutive expression of the *yiaK-S* operon in strain JA134 allows the metabolism of the rare sugar L-lyxose through a metabolic pathway in which L-xylulose-5-P is endogenously generated (*36*) (Figure S1 of the Supporting Information). Subsequently, L-xylulose-5-P is metabolized to L-ribulose-5-P and D-xylulose-5-P, the latter of which enters the pentose phosphate pathway by the sequential action of *yiaR* and *yiaS* (*37*). Since these phosphorylated compounds (L-xylulose-5-P, L-ribulose-5-P, and D-xylulose-5-P) are also generated endogenously in the L-ascorbate metabolism, we analyzed whether these metabolites could act as possible inducers of the *ulaA-F* regulon. This was approached by analyzing the expression of a *ulaA−lacZ* reporter fusion (*24*) upon growth on L-ascorbate or L-lyxose in the genetic background of strain JA134. No induction was observed during growth on L-lyxose since β-galactosidase values were similar to those obtained in the absence of L-lyxose (55 Miller units), thereby discarding L-xylulose-5-P, L-ribulose-5-P, and D-xylulose-5-P as potential inducers of *ulaA-F* expression. In contrast, the same reporter strain grown in L-ascorbate produced a β-galactosidase value of 11750 Miller units. Hence, the two phosphorylated intermediates not shared between the L-ascorbate and L-lyxose catabolic pathways remained as candidate inducers (3-keto-gulonate-6-P and L-ascorbate-6-P). Neither of them is commercially available.

To investigate whether L-ascorbate-6-P could act as a potential inducer of the *ula* regulon, we produced it by chemical synthesis (Experimental Procedures). As a specific inducer and negative allosteric regulator of UlaR, L-ascorbate-6-P had to feature two properties. (i) L-Ascorbate-6-P should reduce the binding affinity of the UlaR−DNA complex; (ii) the interaction between L-ascorbate-6-P and UlaR should have high affinity and high specificity.

To determine whether UlaR binds L-ascorbate-6-P in its DNA-bound state, we first prepared UlaR−P115 DNA complexes and used quantitative EMSAs to determine that the dissociation constant of such complex is 0.2 nM (data not shown). Next, we incubated preformed UlaR−P115 DNA complexes (1 μg of UlaR) with increasing concentrations of L-ascorbate-6-P (in the range of 0−14 mM) and analyzed the effect on the UlaR−DNA complex by gel retardation (Figure 3A). Under the experimental conditions, the amount

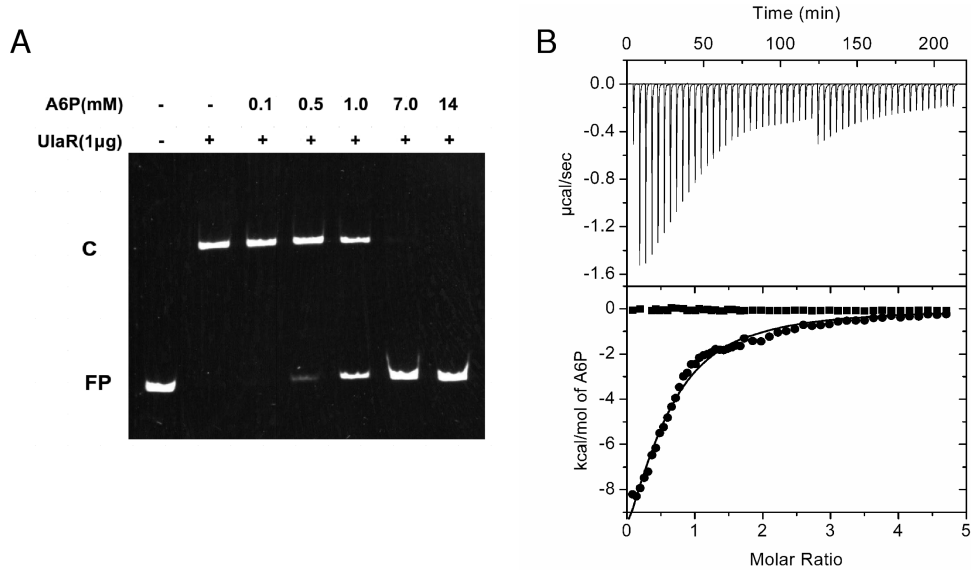

FIGURE 3: L-Ascorbate-6-P is the specific inducer of UlaR. (A) Disruption of the UlaR−DNA (a 115 bp DNA fragment containing operator O2, P115) complex by L-ascorbate-6-P. Prior to electrophoresis, 250 ng of P115 DNA was incubated with 3 μg of UlaR in the presence of increasing amounts of L-ascorbate-6-P (0.1−14 mM). The minimal amount of UlaR that results in the formation of a protein−DNA complex was determined in a separate experiment (data not shown) and was kept fixed throughout this experiment: lane 1, free DNA; lane 2, control UlaR; lanes 3−7, incubation of the UlaR−DNA complex with 0.1, 0.5, 1.0, 7.0, and 14 mM L-ascorbate-6-P, respectively; FP, free probe; and C, UlaR−DNA complex. (B) Binding isotherms and fitting of the calorimetric data of L-ascorbate-6-P titration of full-length UlaR (●) and control titration using the same injection protocol with buffer as the titrant (■).

of free probe (Figure 3A, FP) in the gel retardation increased as L-ascorbate-6-P was titrated in, indicating that significant inhibition of the UlaR−DNA interaction was achieved with increasing concentrations of L-ascorbate-6-P. Using EMSAs, we have determined that under our experimental conditions the half-dissociation concentration of L-ascorbate-6-P for the UlaR−P115 DNA complex is 1.4 mM, and indeed, the interaction between UlaR and DNA was almost totally abolished at L-ascorbate-6-P concentrations of >2 mM. This demonstrates that L-ascorbate-6-P has the capacity to dissociate UlaR−DNA complexes (Figure 3A). This critical concentration is similar to values determined in related systems, e.g., 3-fold lower than the value of 4 mM reported for tagatose 6-phosphate inhibition of the LacR repressor (*38*) and 3-fold higher than that for GalR (*22*). The specificity of the reported interaction was confirmed by the observation that other related sugars, such as L-xylulose-5-P, D-xylulose-5-P, L-ribulose-5-P, ribose-5-P, L-ascorbate, dehydroascorbate, and L-xylulose, were not able to dissociate the UlaR−DNA complex using the gel retardation assay (data not shown). Furthermore, L-ascorbate-6-P did not bind to the unrelated regulator LldR (L-lactate dehydrogenase regulator) in control gel retardation experiments, further supporting the specificity of the interaction between L-ascorbate-6-P and UlaR.

To provide a quantitative measure of UlaR affinity by L-ascorbate-6-P, we performed isothermal titration calorimetry (ITC) experiments in which L-ascorbate-6-P was titrated into a solution of purified full-length UlaR. The ITC experiments demonstrated that UlaR binds L-ascorbate-6-P with an association constant of $\sim 4 \times 10^4$ M^{-1} (dissociation constant of ~ 25 μM) and a stoichiometry of one molecule of L-ascorbate-6-P per UlaR dimer (Figure 3B and Table 2). The concentration of L-ascorbate-6-P necessary to dissociate 50% of the UlaR−DNA complex is 40-fold higher than the K_d determined by ITC, as a consequence of the higher affinity

Table 2: Isothermal Calorimetry Characterization of UlaR−L-Ascorbate-6-P Association

protein	N^a	K_a ($\times 10^4$ M^{-1})
UlaR	0.50 ± 0.00	2.3 ± 0.4
MBP−UlaR	0.51 ± 0.02	4.4 ± 0.3
MBP−UlaRΔN70	0.52 ± 0.02	2.0 ± 0.1
MBP−UlaR D206G	ND[b]	ND[b]
MBP−UlaR K209A	ND[b]	ND[b]
MBP control	ND[b]	ND[b]

[a] N is the number of binding sites for L-ascorbate-6-P per UlaR monomer. Best-fit parameters obtained by nonlinear least-squares analysis with a model assuming one set of single sites. [b] Not determined due to negligible binding of L-ascorbate-6-P to the protein sample.

of UlaR for DNA. Control experiments with MBP showed nondetectable binding to L-ascorbate-6-P, and the MBP−UlaR fusion protein, which was previously cleaved to remove MBP, exhibited identical thermodynamic parameters (Table 2).

The DeoR C-Terminal Domain Is the Effector Binding Domain of UlaR. As discussed earlier and in analogy with similar systems, the C-terminal domain of UlaR is probably responsible for recognizing and binding L-ascorbate-6-P and for bearing the oligomerization motif. This organization lends itself to the partitioning of residues in the DeoR-C domain into residues proximal to the tetramerization interface (and thus far from the HTH domain) and those distal to the interface (and so either belonging to or close to the HTH domain).

Indeed, the possible role of the C-terminal domain in inducer recognition had been suggested previously for LacR, where four amino acid residues were shown to be required for binding to the effector molecule tagatose-6-P (*38*). A multiple-sequence alignment of the DeoR family with UlaR reveals that the positions corresponding to the four residues in LacR are occupied by conserved positive amino acids, which are His72, Lys80, Asp206, and Lys209 in UlaR

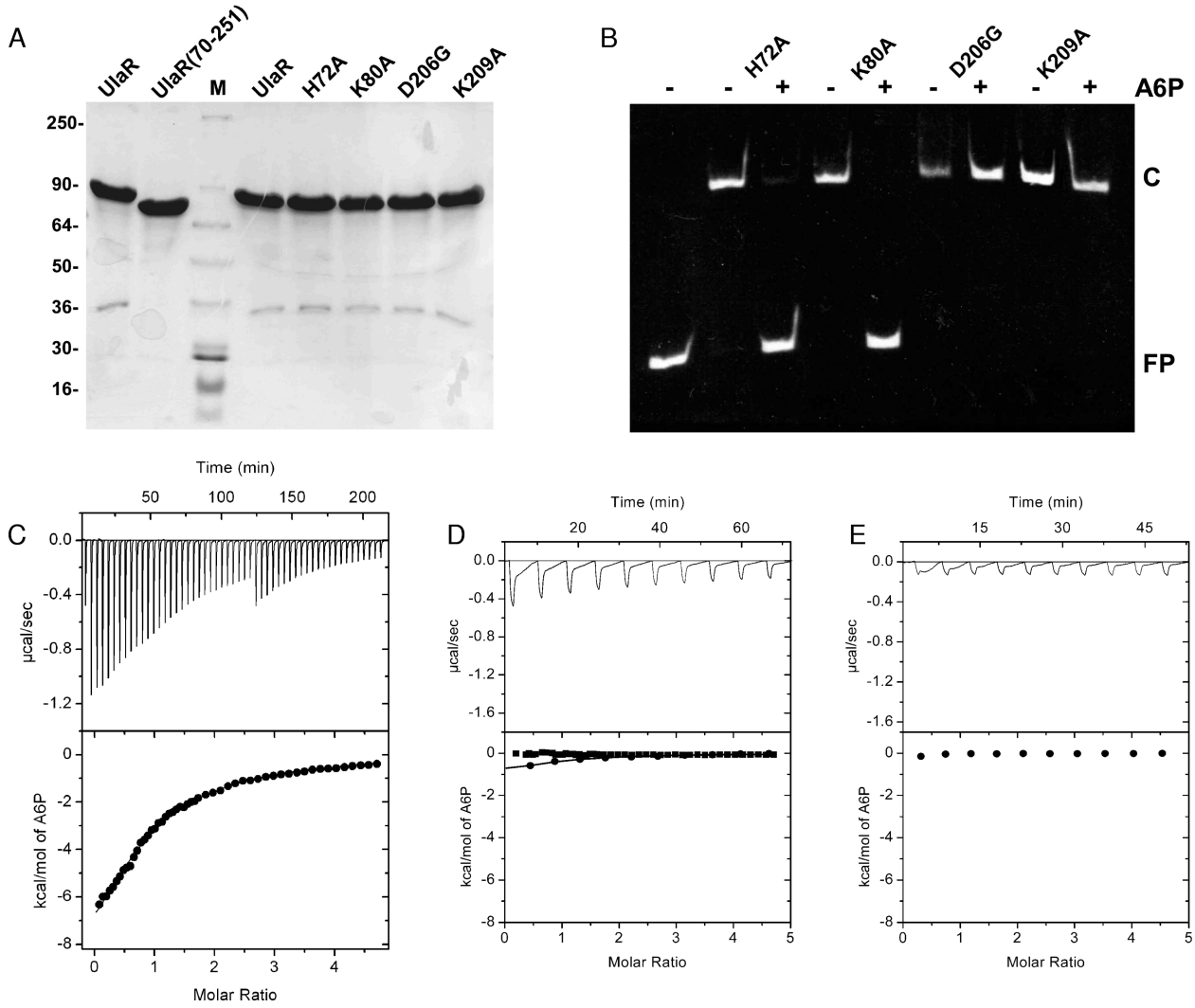

FIGURE 4: (A) Purified UlaR protein samples used in this study. Twenty micrograms of each pure MBP−UlaR variant fusion protein was loaded on a 12% SDS−PAGE gel, electrophoresed, and stained with Coomassie Brilliant Blue: lanes 1 and 4, full-length UlaR (residues 1−251); lane 2, C-terminal DeoR-C UlaR domain (70−251); lane 5, H72A mutant; lane 6, K80A mutant; lane 7, D206G mutant; lane 8, K209A mutant; and lane 3, molecular mass markers. (B) Gel mobility shift assays with C-terminal domain mutants of UlaR (cf. panel A) preincubated with or without 7 mM L-ascorbate-6-P (A6P; −, without A6P; +, with 7 mM A6P): FP, free probe; and C, mutant UlaR−DNA complex. Mutants H72A and K80A exhibited the same inducible phenotype as wild-type UlaR, and 7 mM L-ascorbate-6-P disrupted association with DNA. In contrast, mutants D206G and K209A were completely or only slightly sensitive to L-ascorbate-6-P, and consequently, their DNA complexes were not disrupted by 7 mM L-ascorbate-6-P. (C) Isothermal calorimetry (ITC) of the UlaR C-terminal domain (70−251) as L-ascorbate-6-P was titrated in (●). The binding isotherm was very similar to that of wild-type UlaR. (D and E) Titration of UlaR D206G and K209A mutants, respectively, with L-ascorbate-6-P followed by ITC showed no binding (●). Control titration using the same injection protocol with buffer as titrant (■).

(Figure 2). To investigate whether these residues were involved in L-ascorbate-6-P binding, we targeted each of them for mutagenesis into alanine or glycine. Of the four residues that were targeted, His72 and Lys80 are preceding or within the predicted α-helix connecting the HTH domain and the DeoR-C domain, and therefore named proximal mutations. The other two residues, Asp206 and Lys209 (distal mutations), are located within the DeoR-C domain and are remote from the HTH domain.

We first carried out ITC experiments with an N-terminal truncation of UlaR from residue 70 to 251 (termed UlaRΔN70) under the same conditions used with full-length UlaR, to assess whether UlaRΔN70 was sufficient to replicate the UlaR response to L-ascorbate-6-P (Figure 4C). Indeed, the binding isotherms and fitted calorimetric data for UlaRΔN70 when titrated with L-ascorbate-6-P were remarkably similar to those of full-length UlaR (Figure 3B), with a K_d of ∼50 μM (within a factor of 2 of that of full-length UlaR). Moreover, limited proteolysis experiments and N-terminal sequencing showed that a stable C-terminal truncation of UlaR started at residue 70 and, probably, ended in the native C-terminus at residue 251 (see Figure S3 of the Supporting Information). These results confirmed that UlaRΔN70 was the minimal module able to recognize L-ascorbate-6-P with near wild-type efficiency. Next, site-directed mutagenesis and ITC were performed to test the function of specific conserved residues of the UlaR C-terminal domain. The effect of H72A, K80A, D206G, and K209A mutations was analyzed by EMSA using purified protein preparations (Figure 4A,B). Gel mobility shift assays using probe P115 showed that, under our experimental conditions, D206G and K209A distal mutants exhibited impaired dissociation of protein−DNA complexes in the presence of 7 mM L-ascorbate-6-P (Figure 4B), thereby suggesting a prime role for these residues in

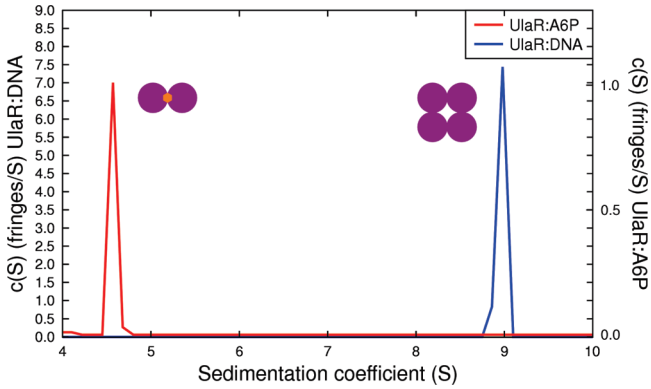

FIGURE 5: Analytical ultracentrifugation−sedimentation velocity analysis of UlaR bound to its cognate operator region O2 (blue) and to its inducer, L-ascorbate-6-P (red). Results of the two independent experiments are shown as sedimentation coefficient distributions $c(S)$ (fringes/S) vs sedimentation coefficient (S). For the DNA−UlaR complex, the major peak is at 8.9 S, corresponding to a DNA-bound UlaR tetramer (species 1). When inducer is preincubated with UlaR, the distribution of sedimentation coefficients peaks at ~4.6 S, the expected molecular size of a homodimer (species 2). Fitting residuals were 0.0072 fringe for the experiment with DNA and 0.0068 fringe with L-ascorbate-6-P. A schematic representation of dimeric and tetrameric UlaR (purple) is shown near the corresponding species.

L-ascorbate-6-P binding and/or in transducing the binding event to the DNA-binding domain. Accordingly, both UlaR D206G and K209A mutants were unresponsive to inducer by ITC (Figure 4D,E). In contrast, gel retardation experiments showed that proximal H72A and K80A mutants were at least as competent in binding L-ascorbate-6-P as wild-type UlaR (Figure 4B). These observations indicate that the proximal residues His72 and Lys80 have no apparent effect either on L-ascorbate-6-P binding or in the transmission of the allosteric signal from the inducer-binding domain to the DNA-binding motif.

Oligomerization Transition in UlaR. We used analytical ultracentrifugation (AUC) to probe for changes in the oligomerization states of UlaR under two conditions of biological relevance, the DNA-bound state and the L-ascorbate-6-P-bound state. The raw data of the AUC experiments, including quality indicators and root-mean-square distances, appear in Figure S4 of the Supporting Information. Sedimentation velocity (SV) experiments with UlaR were performed only in the presence of cognate ligands (DNA or L-ascorbate-6-P), since UlaR alone has limited solubility. We had preliminary evidence from native electrophoresis and dynamic light scattering that UlaR assumes a homotetrameric structure. In AUC−SV experiments where UlaR was preincubated with either DNA or L-ascorbate-6-P, two distinct species of UlaR were observed, species 1 and 2 (Figure 5). It is noteworthy that these two species were not observed to coexist or interconvert (over ~6−8 h); therefore, they represent distinct oligomerization states. Scanning data were best analyzed using sedimentation coefficient distributions $c(S)$ and sedimentation coefficient distributions with prior knowledge $c^{(P)}(S)$ models. In the presence of the cognate DNA sequence, UlaR sedimented with a sedimentation coefficient of ~8.9 S (species 1), corresponding to an apparent molecular mass of ~186 kDa (Figure 5, blue). This mass was bound by the predicted molecular mass of an UlaR homotetramer (4 × 27.6 kDa = 110.4 kDa) plus four DNA molecules (23.1 kDa each), which is remarkably close to the expected mass of 203.0 kDa. This deviation from ideality can be safely attributed to nonspherical mass distribution effects. For this $c(S)$ distribution, the minimal compatible molecular mass for a compact sphere is 120.5 kDa, which provides strong support for the presence of a tetrameric UlaR−DNA complex. In contrast, when UlaR was incubated with L-ascorbate-6-P and subjected to AUC−SV experiments under otherwise identical conditions and experimental parameters, a distinct single species was obtained from the $c(S)$ distribution, with an estimated sedimentation coefficient of ~4.5 S (species 2), equivalent to an apparent molecular mass of ~58.3 kDa (Figure 5, red line). This result is within a 5% error of the expected mass of an UlaR homodimer (2 × 27.6 kDa = 55.2 kDa) and allows unambiguous identification of the sedimenting species observed as an L-ascorbate-6-P−UlaR dimer complex.

DISCUSSION

In accordance with other model systems of repression by members of the DeoR family, we hypothesized that the phosphorylated sugar L-ascorbate-6-P could be the likely physiological inducer of UlaR. If so, the binding of the inducer to the UlaR repressor should bring about conformational changes sufficient to relieve transcription repression and hence allow transcription from the *ula* regulon to proceed. The complex mechanistic details of this activation process would depend on biochemical and biophysical features of the system, such as which distinct oligomerization states are accessible to UlaR, how those states interconvert upon various stimuli, and the roles of these states in activating or silencing transcription. Here we have started to unravel the sequence of quaternary structural transitions involved in the activation process.

Here we present the first direct evidence that L-ascorbate-6-P is indeed an inducer of UlaR. Besides, we have shown before that almost all the other phosphorylated intermediates of the L-ascorbate pathway (with the exception of 3-keto-L-gulonate-6-P) can be ruled out as inducers of the *ula* regulon, since growth on L-lyxose, which endogenously generates L-xylulose-5-P, L-ribulose-5-P, and D-xylulose-6-P, does not result in the induction of the *ula* regulon (*36, 37*). We demonstrate that UlaR and L-ascorbate-6-P form a stable complex and, using gel retardation assays, that this binding can occur both with purified UlaR and after UlaR is bound to DNA carrying one operator site for UlaR. Furthermore, at concentrations of >2 mM, L-ascorbate-6-P binding can displace UlaR from its operator site. The half-dissociation concentration (1.4 mM L-ascorbate-6-P) compares well with previously reported concentrations of physiological inducers (in the range of 0.5−4 mM), for example, GalR (*22*) or LacR (*38*). Together, these observations provide compelling evidence of the regulatory switch between UlaR in the presence of DNA (repression) or inducer (activation). These properties of the UlaR repressor protein are essential to understanding the repression−activation mechanism of the *ula* regulon.

From sequence homology, the UlaR C-terminal domain folds in a DeoR-C domain, for which there is a well-defined signature for ligand binding (*27*). To gain insight into the sugar−phosphate binding properties of UlaR, we characterized thermodynamically the association between UlaR and L-ascorbate-6-P, which yielded a K_d of ~25 μM and a

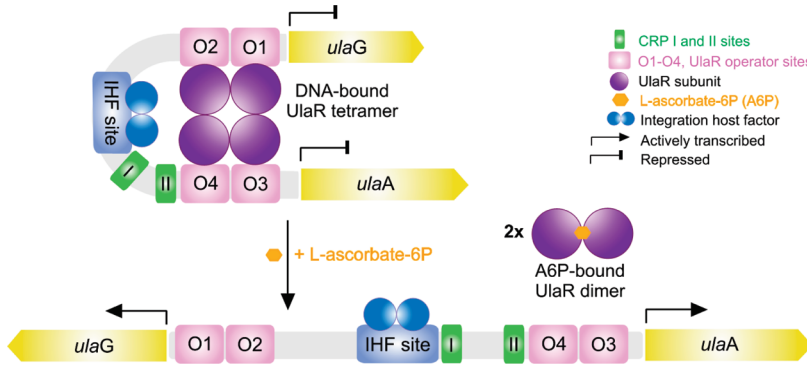

FIGURE 6: Proposed model for transcriptional regulation of the *ula* regulon by UlaR oligomerization transitions. UlaR is colored purple either as a tetramer (bound to its four operator sites, pink) or as a dimer (bound to L-ascorbate-6-P, orange). In the absence of inducer, transcription of the two divergently transcribed operons is effectively silenced by the UlaR tetramer. Conversely, when L-ascorbate-6-P is present, UlaR dissociates from DNA as an inducer-bound dimer, and hence, transcription repression is relieved. CRP I and CRP II sites, the integration host factor (IHF), and the IHF binding site are also shown.

stoichiometry of one sugar molecule per UlaR dimer (Figure 3B). This stoichiometry is in agreement with the evolutionary restraint acquired by members of the ISOCOT superfamily (*27*) and has been previously reported for other non-DeoR ISOCOT effector-binding domains, for example, QacR (*20*) and ribose-5-phosphate isomerase (*26*). Furthermore, a C-terminal truncation of UlaR spanning residues 70−251, UlaRΔN70 (the entire DeoR-C domain), behaved identically compared to full-length UlaR with respect to the binding constant and stoichiometry for L-ascorbate-6-P (Figure 4C), thereby indicating that the DeoR-C domain is sufficient for binding the inducer. Interestingly, the binding stoichiometry suggests that L-ascorbate-6-P binds in a pocket at the interface of an UlaR dimer. In fact, site-directed mutagenesis, gel retardation assays, and calorimetric analyses identified two distal residues, Asp206 and Lys209, directly involved in binding L-ascorbate-6-P. Mutations in these residues (D206G and K209A) caused either complete (K209A) or nearly complete (D206G) abrogation of binding. Since the K209A mutation had the net effect of removing one positive charge, it is tempting to propose that the side chain of Lys209 is directly involved in contacting the phosphate group of L-ascorbate-6-P. Since the binding stoichiometry determined by ITC was one molecule of L-ascorbate-6-P bound per UlaR dimer, it seems reasonable to propose a role for Asp206 and/or Lys209 in inducer recognition and, simultaneously, in modulating the oligomerization by affecting directly or indirectly the dimer interface. In contrast to these distal mutations, proximal mutations, such as H72A and K80A, have minimal effects on L-ascorbate-6-P binding as shown by EMSAs, thereby suggesting that they are not directly involved in the oligomerization surfaces (Figure 4B).

To address the quaternary configuration of the repressed state, we performed AUC sedimentation velocity experiments. The UlaR−DNA complexes (with either one operator sequence or two operator sequences present) migrated with an apparent molecular size corresponding to a DNA-bound tetrameric arrangement. This observation is consistent with postulated models in which a tetrameric UlaR enforces a loop in the DNA structure that enhances transcriptional repression. In contrast, addition of L-ascorbate-6-P to UlaR favored the formation of dimer complexes. Therefore, we demonstrate that the inducer, L-ascorbate-6-P, is a negative allosteric effector that weakens the affinity of UlaR for DNA and displaces the UlaR oligomerization state from a transcription-silencing tetrameric form to a transcription-activating dimeric form (Figure 6). As for UlaR, other transcription factors have been shown to adopt higher-order oligomeric structures when binding DNA. The formation of higher-order structures induced by ligand binding, rather than dissociation, has been observed in the distantly related ArgR transcription factor, in which arginine stabilizes a dimer of ArgR trimers, which then becomes competent for DNA binding (*18*). In the DeoR family, various quaternary structures have been described (e.g., GlpR and AgaR as tetramers and DeoR as an octamer) in association with DNA and are independent of the interoperon distance. However, a detailed analysis combining biophysical and biochemical methods of the transitions in the quaternary structure of a DeoR-type repressor, such as the one described here, had not been conducted to date. The evidence presented here suggests a mechanism for transcriptional regulation of the *ula* regulon in which (a) the repressor, UlaR, maintains repression by binding simultaneously to the two operators of the system adopting a tetramer structure and (b) the presence of the inducer, e.g., L-ascorbate-6-P, reduces the affinity of the UlaR−DNA complex and triggers a tetramer to dimer transition, with the concomitant lift of transcriptional repression. This mechanism provides a simple and effective means of regulating transcription from the *ula* regulon while avoiding undue transcription of the eight genes downstream from the *ula* operons. This paradigm, which has also been shown for other bacterial transcription factor families (e.g., TetR and GalR), is likely to be applicable to other repressors of the DeoR family.

Future research efforts must unravel the exact structural details of the homodimerization and homotetramerization processes, the surfaces involved, and their regulation by DNA binding and inducer binding, and how these structural transitions are orchestrated in vivo to produce a rapid metabolic switch as nutrient levels change in the extracellular milieu.

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SUPPORTING INFORMATION AVAILABLE

Scheme of the L-ascorbate and L-lyxose dissimilation pathways (Figure S1), further details of the purification and quality of UlaR−DNA and L-ascorbate-6-P complexes (Figure S2), descrip-

tion of the limited proteolysis protocol and results applied to UlaR to define the DeoR-C domain (Figure S3), and raw AUC data (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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