Cooperative Binding of DctD to the dctA Upstream Activation Sequence of Rhizobium meliloti Is Enhanced in a Constitutively Active Truncated Mutant

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DctD, a σ54-dependent, two-component regulator, binds to promoter distal (A) and promoter proximal (B) sites in an activation sequence located upstream of the dctA promoter. We report gel filtration and quantitative DNase I footprint experiments supporting a model in which DctD₂ binds to these sites cooperatively. The global analysis of upstream activation sequences containing sites A and B, A and B one-half helical turn out of phase, and only B yielded values for the intrinsic and cooperative binding free energies of ΔG₂ₐ = −9.5 ± 0.3, ΔG₂ₐ₋ₐ = −11.2 ± 0.2, and ΔG₂ₐ₋ₐ = −2.5 ± 0.5. A separate analysis of data from upstream activation sequences containing site A and a point mutant of site B, and site A and mutant site B one-half helical turn out of phase confirmed the estimate of cooperativity, yielding free energy values of ΔG₂ₐ = −9.4 ± 0.2, ΔG₂ₐ₋ₐ = −10.6 ± 0.2, and ΔG₂ₐ₋ₐ = −2.2 ± 0.4. We previously showed that removing the two-component receiver domain from DctD, making DctD₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-~

Rhizobium meliloti are capable of utilizing C₄-dicarboxylates as the sole carbon and energy source. These compounds are believed to be provided by the host plants to fuel nitrogen fixation during symbiosis (1–5). Transport of dicarboxylates into the bacteria requires a single genetic locus containing the three genes dctA, dctB, and dctD. The dctB and dctD genes are transcribed divergently from dctA, which encodes a transport protein whose expression is increased in the presence of C₄-dicarboxylates (6–9). Transcription from the dctA promoter requires the alternate sigma factor, σ54 (10). In free living cells dctB and dctD are required for this increased expression, but in symbiotic bacteroids, dctA expression occurs at only somewhat reduced levels in the absence of dctB and dctD (11–13).

The dctD gene product, DctD, belongs to the σ54-dependent activator sub-group of two-component response regulators. Two-component response regulators typically display homology in their NH₂-terminal 125 amino acids (6, 14, 15). These proteins are presumed to be phosphorylated at an aspartate located in the middle of this conserved region by histidine protein kinases, converting them from inactive forms to active ones (reviewed in Refs. 16 and 17). Like other σ54-dependent transcriptional activators, but not all two-component response regulators, DctD also has a central domain that contains an ATP binding motif (6). ATP hydrolysis is required for DctD and other σ54-dependent activators to catalyze the isomerization of closed complexes of the σ54-form of RNA polymerase and promoter DNA to open ones (18, 19). Although the phosphorylated form of DctD has recently been demonstrated (20), it has not been studied in detail. It is known, however, that destabilizing or removing the NH₂-terminal two-component receiver module from DctD yields proteins which constitutively hydrolyze ATP and stimulate transcription (19, 21, 22). Both the inactive, wild type form of DctD and DctD₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-~

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1 The abbreviations used are: UAS, upstream activation sequence; PCR, polymerase chain reaction; bp, base pair(s).
measure the intrinsic and cooperative free energy contributions to binding of DctD and DctD}_{1-142}, to the dctA UAS. Results show that the UAS is heterogeneous, with site A having less intrinsic affinity for DctD than site B, and that binding is cooperative. Moreover, the extent of cooperativity was significantly increased in the constitutively active mutant DctD}_{1-142} relative to that of the wild type protein. This increased cooperativity associated with the active state of truncated DctD is similar to the findings that phosphorylated NtrC has higher cooperativity than unphosphorylated NtrC, but it need not be caused by the same molecular mechanism.

**EXPERIMENTAL PROCEDURES**

**DNA Biochemistry**—Large scale DNA preparations were made by the alkaline lysis method (30). Small scale plasmid preparations were done by the boiling method (30). Transformations were done using calcium chloride to prepare competent cells. Preparation of single-strand M13 templates for sequencing was done according to the methods described in the Bio-Rad Mutagene kit. DNA sequencing was done using the Sequenase (U.S. Biochemical Corp.) kit. Restriction enzymes, DNA ligase, and Klenow DNA polymerase were purchased from Boehringer Mannheim and DuPont. Agarose was purchased from Life Technologies, Inc.; acrylamide and bisacrylamide were purchased from either Fisher or U. S. Biochemical Corp.

**Protein Assays**—Plasmid pTRH1 was used to express wild type DctD in JM109 cells under control of the lac promoter (23). In this plasmid, the dctD gene together with a small portion of the upstream dctB gene was cloned into pBS (Stratagene). One to 4 liters of culture were grown in Luria Broth (1% tryptone; 0.0.5% yeast extract; 0.5% NaCl) supplemented with 50 μg/ml ampicillin at 30°C until an optical density of 1.0 was reached, at which time isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1.0 mM and the culture was allowed to grow an additional 5–6 h. The cells were harvested by centrifugation and were resuspended in 20 mM Tris, 50 mM KCl, 5% glycerol, pH 7.9. The cells were then sonicated for 10 cycles of 30 s of disruption followed by 30 s of cooling using a Fisher model 300 Sonic Dismembrator. The solution was cleared by centrifugation at 12,000 rpm in a Sorvall SS-34 rotor for 40 min at 4°C. Dry ammonium sulfate was added to the supernatant to a final concentration of 35% saturation, which was incubated for 30 min at 0°C, and then centrifuged (12,000 rpm, 20 min, SS-34). The pellet was redissolved in the starting buffer, and dialyzed 1:1000 for 12 h at 4°C. A linear gradient was run from 50 mM to 300 mM KCl over a space between the wells to fully separate material in adjacent lanes. The gel was poured using a customized comb with 6-mm wells and 6 mm of gel. The running buffer was 0.5 M Tris-HCl/boric acid/EDTA electrophoresis buffer; Ref. 30). The gel was allowed to elute into TE by shaking overnight and ethanol-precipitated at −70°C. The radiolabeled DNA (−10 pm, 10,000 cpm) was allowed to bind to purified DctD or DctD}_{1-142} at 25°C in 30 μl of binding buffer (20 mM Tris, pH 7.9, 50 mM KCl, 5% glycerol). No excess carrier DNA was added. Protein, diluted in binding buffer, was titrated over 7–8 log units with 3 concentration points/log. After 20 min, DNase I (Life Technologies, Inc.) was diluted appropriately (in 20 mM Tris, pH 7.6, 5 mM MgCl₂, 50 mM NaCl) and 10 μl was added to each binding reaction. The digestion was stopped after 30 s by adding 25 μl of 1% SDS, 10 mM EDTA, and the mix was then brought to 100 μl, supplemented with ammonium acetate and 1 μg of tRNA, and precipitated with ethanol. The precipitated DNA was allowed to dry under vacuum for at least 60 min, after which the samples were dissolved in sequencing stop solution and loaded onto a 6% denaturing polyacrylamide gel (0.5 × Tris-HCl/boric acid/EDTA electrophoresis buffer; Ref. 30). The gel was poured using a customized comb with 6-mm wells and 6 mm of space between the wells to fully separate material in adjacent lanes. The gels were dried after electrophoreses and were exposed to film. After the gel was developed and visualized, dried gels were cut in half (typically between lanes 12 and 13) and exposed for 40 min on a Betascope 603 Blot Analyzer (Betagen). The regions encompassing the A and B sites were each blocked off along with unoccupied regions above and below the sites (to be used as standards to normalize the data), and raw counts were collected within the regions. In every gel, a lane was ran with no protein to be used as a control. Fractional protection for a given protein concentration was calculated using the formula given in Equation 1, where N is an experimental lane and R is the control lane with no protein added (31).

**DNase I Footprinting**—These experiments were conducted using the quantitative DNase I footprint titration method essentially as described by others (31–33). End-labeled templates were made by first digesting 2–3 μg of the pUC constructs just described with BamHI. The digested plasmid was then end-labeled by incubation with 1 μM dCTP, dATP, dGTP, and [α-32P]dATP and 1 unit of Klenow enzyme. It was then digested with EcoRI, and the mix was run on a 5% polyacrylamide gel. The appropriate band was cut out of the gel, and the DNA was allowed to elute into TE by shaking overnight and ethanol-precipitated at −70°C. The radiolabeled DNA (~10 pm, 10,000 cpm) was allowed to bind to purified DctD or DctD}_{1-142} at 25°C in 30 μl of binding buffer (20 mM Tris, pH 7.9, 50 mM KCl, 5% glycerol). No excess carrier DNA was added. Protein, diluted in binding buffer, was titrated over 7–8 log units with 3 concentration points/log. After 20 min, DNase I (Life Technologies, Inc.) was diluted appropriately (in 20 mM Tris, pH 7.6, 5 mM MgCl₂, 50 mM NaCl) and 10 μl was added to each binding reaction. The digestion was stopped after 30 s by adding 25 μl of 1% SDS, 10 mM EDTA, and the mix was then brought to 100 μl, supplemented with ammonium acetate and 1 μg of tRNA, and precipitated with ethanol. The precipitated DNA was allowed to dry under vacuum for at least 60 min, after which the samples were dissolved in sequencing stop solution and loaded onto a 6% denaturing polyacrylamide gel (0.5 × Tris-HCl/horic acid/EDTA electrophoresis buffer; Ref. 30). The gel was poured using a customized comb with 6-mm wells and 6 mm of space between the wells to fully separate material in adjacent lanes. The gels were dried after electrophoreses and were exposed to film. After the gel was developed and visualized, dried gels were cut in half (typically between lanes 12 and 13) and exposed for 40 min on a Betascope 603 Blot Analyzer (Betagen). The regions encompassing the A and B sites were each blocked off along with unoccupied regions above and below the sites (to be used as standards to normalize the data), and raw counts were collected within the regions. In every gel, a lane was ran with no protein to be used as a control. Fractional protection for a given protein concentration was calculated using the formula given in Equation 1, where N is an experimental lane and R is the control lane with no protein added (31).

**Numerical Analysis**—The binding data were analyzed as described (32, 33) with the appropriate binding functions using nonlinear least-squares parameter estimation. These analyses were performed on a personal computer (486i CPU) using Lahey Fortran F77L (Lahey Computer Systems, Inc., Incline Village, NV) and NONLIN (Michael John- son, University of Virginia, Charlottesville, VA), a fitting program that includes information about covariance in its error analysis (34). The reported 95% confidence limits of the parameters were obtained by examining the N-dimensional parameter space for the variance ratio predicted by an F statistic. The method approximates the worst case joint confidence intervals and does not assume that the confidence limits are symmetrical about the optimal values.²

² The software is provided together with an explicit tutorial at the Internet site http://www.bmb.psu.edu/DNaseIV.
Fractional protection from DNase I is directly proportional to fractional occupancy, but even saturating concentrations of protein do not completely protect from DNase I. For this reason, the final equation used in fitting the protection data (P) and the appropriate fractional occupancy function (Y) was as given in Equation 11.

\[
P = (U - L) \times Y + L
\]

where \(U\) is the upper limit of protection, and \(L\) is the lower limit of protection. A concentration range of at least 7 orders of magnitude was used in each titration to aid in estimating the transition end point parameters. For calculating protein concentration, DctD was considered to be a dimer (see results) present in excess of DNA, and protein preparations were assumed to be 100% active. Gel shift experiments showed that \(\pm 99%\) of each of the labeled DNA fragments was bindable.

**RESULTS**

*Purification of DctD—* Purification of *R. meliloti* DctD from an overexpression strain was accomplished in a three-step procedure starting with a sonication of cells to generate a crude extract, followed by a 35% ammonium sulfate precipitation, and finally elution from a phosphocellulose column using a KCl gradient. This purification procedure is similar to the procedure described for purification of DctD<sub>1-142</sub> (19), with the exception that wild type DctD seems to be more soluble than the truncated mutant and does not require potassium thiocyanate or deoxycholate to prevent it from precipitation. Fig. 2A shows material from one such purification separated on an SDS-polyacrylamide gel. For all preparations used in this study, 95–98% purity was obtained from the phosphocellulose chromatography.

*DctD Is a Homodimer—* Small zone gel filtration experiments indicated that at 2.3 \(\mu\)M concentrations, DctD emerged from the column in a single peak at 90-ml elution volume (Fig. 2B). For this column, a 90-ml elution volume was typical of proteins with a molecular mass of 120,000 daltons. Since the predicted monomer size for DctD is 56,000, we propose that the protein is a homodimer at this concentration. To determine if a shift in the equilibrium concentrations of monomer and dimer occurs over concentrations relevant to the DNA binding studies reported here, we ran samples on the gel filtration column at concentrations down to 2.3 \(\mu\)M monomer. To detect these low amounts of protein, fractions were collected and immunoblotted to find the elution volume of the protein. As can be seen in Fig. 2B, neither the elution volume nor the shape of the peak changed between \(-1\) \(\mu\)M and \(-1\) \(\mu\)M concentrations of DctD<sub>2</sub>.

**DctD<sub>2</sub> Binds the dctA UAS Cooperatively—** We used DNase I footprinting to measure fractional occupancy of DctD<sub>2</sub> at the dctA-UAS, and at two mutant forms designed to serve as "reduced-valency" templates (see Fig. 1). First, a 5-base insertion was placed between the two sites orienting them on opposite faces of the DNA helix, which in theory eliminates any protein-protein interaction between molecules bound at the two sites. Second, an A site deletion construct was made so that DctD<sub>2</sub> can only occupy the B site. The data from two independent repetitions of each experiment were then analyzed by global, nonlinear regression to extract estimates for the intrinsic binding and cooperativity free energies (Table II, row 1) as well as
the upper and lower protection limits for each titration (data not shown). The estimated parameter values were used to recast the observed fractional protection data as fractional occupancies, which were then plotted as the familiar binding isotherms for the wild type template (Fig. 3A), for the insertion template (Fig. 3B), and for the site A deletion mutant (Fig. 3C). The results suggest that sites A and B have different intrinsic affinities, and that binding to these two sites is cooperative. For the wild type template, the binding isotherms overlapped for both the A and B sites. In the case of the insertion mutant, however, occupancies were lower than for the wild type DNA, with site A showing considerably less affinity than site B. The site A deletion mutant showed only occupancy at the B site. The square root of the variance for the global analysis was 0.053, and the residuals between the observed data and the model, plotted below the isotherms in Fig. 3, were typically less than 0.2.

Analyses of the data for each DNA template and all other combinations of them are also shown in Table II. Combining the data sets for wild type and 5-bp insertion templates provided enough information for the analysis to converge on estimates of intrinsic binding and cooperativity that were essentially identical to those of the complete combination. Removing the information about binding to site A by combining data from wild type and site A deletion templates, or just considering the data from the wild type template, however, resulted in replacing most of the cooperativity energy with increased intrinsic binding to site A. For these data sets, holding either the cooperativity or site A intrinsic binding free energies constant restored the corresponding estimate to values derived in the more complete analyses. Analyses of the site A deletion and 5-bp insertion templates together or singly considered the data from the wild type template, however, resulted in replacing most of the cooperativity energy with increased intrinsic binding to site A. For these data sets, holding either the cooperativity or site A intrinsic binding free energies constant restored the corresponding estimate to values derived in the more complete analyses. Analyses of the site A deletion and 5-bp insertion templates together or singly yielded parameter estimates that were very consistent with those of the global analysis. The square root of the variances for all of these analyses were between 0.060 and 0.046.

To provide a different context for measuring the free energy of cooperativity and intrinsic binding to site A, a point muta-

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**Fig. 2.** Purified DctD has a molecular mass of 120,000 daltons. A, proteins in a DctD preparation were separated on an SDS-polyacrylamide gel and stained with Coomassie Blue. B, elution profiles of gel filtration studies. UV absorbance absorption tracings are shown for DctD run at total monomer concentrations of 2.3 μM (solid line) and 230 nM (dashed line). The peak at 58 ml elution volume is for blue dextran, marking the void volume for the column. Separate runs (data not shown) showed no material in the void volume when only DctD was run. The top elution profile (filled circles) represents the immunodetected material from a sample loaded at 2.3 μM total monomer concentration. The arrows indicate elution volumes of protein standards. Note that the elution volumes are identical in all cases indicating that DctD is in the same multimeric state at concentrations relevant to this study, probably existing as a dimer.

**Fig. 3.** DctD binding curves derived from DNase I footprint titrations of WT, +5, and ΔA templates. Open and closed symbols represent different experiments. Circles, site A; triangles, site B. A, wild type template; note that the A and B sites are both occupied at the same DctD concentrations. B, 5-base pair insertion template. C, site A deletion template; note that occupancy at the B site is identical with the B site isotherms in the 5-base pair insertion mutant.
TABLE I
DNA configurations and associated free energy states for DctD<sub>2</sub> binding to the wild type and mutant dctA UAS elements

<table>
<thead>
<tr>
<th>State</th>
<th>DNA configuration</th>
<th>Site B&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Site A</th>
<th>Site B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Gibbs free energy</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Reference state</td>
<td></td>
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<tr>
<td>2</td>
<td>O</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O</td>
<td>ΔG&lt;sub&gt;A&lt;/sub&gt;</td>
<td></td>
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<tr>
<td>3</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>O</td>
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<td>ΔG&lt;sub&gt;B&lt;/sub&gt;</td>
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<td>4</td>
<td>O</td>
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<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ΔG&lt;sub&gt;AB&lt;/sub&gt;</td>
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<td>5</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↔</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ΔG&lt;sub&gt;AB&lt;/sub&gt; + ΔG&lt;sub&gt;1D&lt;/sub&gt;</td>
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<td>6</td>
<td>O</td>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>↔</td>
<td>ΔG&lt;sub&gt;AB&lt;/sub&gt; + ΔG&lt;sub&gt;D2B&lt;/sub&gt;</td>
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</tbody>
</table>

ΔG<sub>1D</sub> and ΔG<sub>D2B</sub> are the intrinsic free energies for binding to the corresponding sites; ΔG<sub>AB</sub> is the free energy of cooperative interactions between liganded sites in the wild type and mutant UAS constructs, respectively.

To some extent, DctD<sub>Δ(1-142)</sub> thus represents the activated state of DctD. The UAS-binding properties for DctD<sub>Δ(1-142)</sub> were determined using the wild type and the 5-bp insertion templates to assess potential differences between the states of active and inactive activator (Table III and Fig. 4C). The data were analyzed assuming that DctD<sub>Δ(1-142)</sub> also exists in a single state of dimer over the concentrations tested. The global analysis yielded Gibbs free energies for intrinsic binding to sites A and B that were indistinguishable from those of wild type protein; however, the estimate of cooperativity free energy was significantly elevated from −2.7 ± 0.4 kcal/mol for wild type protein to −3.8 ± 0.6 kcal/mol for the truncated protein. Separate analysis of the data for the wild type UAS converged upon a consistent set of parameter estimates, but the confidence intervals were very large and asymmetric. Fixing the cooperativity parameter at −3.5 kcal/mol yielded estimates of intrinsic binding free energies that were consistent with the global analysis, but these estimates still had rather large errors. Separate analysis of the data from the UAS bearing a 5-bp insertion between sites yields estimates in excellent agreement with those of the combined analysis. The square root of the variances of fit for these analyses were between 0.46 and 0.69.

DISCUSSION

The results reported here confirm that DctD binds specifically to DNA at two sites that make up the dctA upstream activation sequence. They also confirm that the intrinsic affinity of DctD<sub>B</sub> is ~20-fold lower for the A site than for the B site. A and decreased for site B, compared to those estimated for the combined data. Separate analysis of data from the 5-bp insertion template yielded free energy states consistent with the combined analysis. The square root of the variances of fit for these analyses were between 0.41 and 0.49. The data from these two templates were also combined with that of the previously discussed templates. The global analysis of all five templates yielded results that were in excellent agreement with the prior analyses (Table II).

**The Truncated, Constitutively Active Mutant DctD<sub>Δ(1-142)</sub> Displays More Cooperativity than DctD**—We have previously characterized a truncated DctD mutant, DctD<sub>Δ(1-142)</sub>, which lacks the NH<sub>2</sub>-terminal 142 residues of the wild type protein. Unlike DctD<sub>Δ(1-142)</sub>, is not regulated by the two-component receiver function, and it is constitutive for both transcriptional activation and the required ATP hydrolysis (19). The truncated, constitutively active mutant DctD<sub>Δ(1-142)</sub> displays more cooperativity than DctD.
More importantly, they strongly suggest that a cooperative protein/DNA-protein/DNA interaction is involved in binding of DctD$_2$ to the dctA UAS, and provide an estimate of this cooperativity. This cooperativity in effect allows occupancy at each site to be roughly equal at a given concentration of DctD$_2$. 

Finally, the results show that the truncated, constitutively active mutant DctD$_{Δ(1–142)}$ binds to the dctA UAS with greater cooperativity than does the wild type protein.

Concluding that cooperativity contributes to binding of DctD$_2$ to the dctA UAS is based in part on the observation that DctD was apparently a dimer in solution at concentrations as low as 2 nM total monomer, and that it did not show any evidence of further multimerization up to concentrations of 2 μM total monomer. Since the binding isotherm changed most between 1 nM and 10 nM DctD$_2$, dimerization can probably be ruled out as a contributing factor for binding to the dctA UAS. 

This conclusion has to be tempered with the realization that elution volumes for small zone gel filtration experiments are not always correlated with molecular weight (36). It was also apparent from the DNase I titrations that DctD$_2$ is capable of binding independently to single sites. The fact that the single site binding data for both sites A and B are described well by the Langmuir binding polynomial also suggests that the oligomeric state of DctD is stable.

We analyzed binding of DctD$_2$ to the dctA UAS with a simple twosite, cooperative model, as previously developed for bacteriophage λ repressor cl (37). Under the conditions tested and so modeled, DctD$_2$ binds to the A and B sites of the dctA UAS with respective intrinsic affinities of $−9.4 \pm 0.3$ kcal/mol and $−11.2 \pm 0.2$ kcal/mol, with a cooperativity free energy of $−2.5 \pm 0.5$ kcal/mol (75-fold increased binding). The separate analyses of all five UAS templates are internally consistent with this model. First, the ΔA and +5 templates give the same estimate for Δ$G_{ΔA}$, and the insertion mutation does so for Δ$G_{ΔI}$ in both wild type and site BG attenuation templates. Second, the attenuation mutant appears to only change the intrinsic binding energy for site B. In this analysis, it is possible that we have underestimated the intrinsic binding free energies, as we do not know the fraction of DctD$_2$ that is competent to bind to DNA. 

Our estimate of cooperativity is not affected by assuming that all of DctD$_2$ is active for DNA binding, provided that the true fraction of active protein is uniformly competent to participate in cooperative interactions.

The above analysis is crucially dependent upon the assumption that the 5-bp insertion mutation does not disturb intrinsic binding to site A, site B, or site BG. The importance of this assumption is indicated by the inability of the regression analyses to determine the same intrinsic binding and cooperativity estimates when only data from the wild type or attenuated site B DNA templates are considered. This means that the shape of the isotherms obtained for these UAS elements are not able to verify the cooperativity indicated by the global analyses. Instead, invoking cooperativity is entirely dependent upon including the data from the 5-bp insertion templates, which provide the information about intrinsic binding to site A in the absence of cooperativity. The fact that deleting site A yielded a similar estimate for intrinsic binding to site B provides some confidence for the assumption; however, the deletion template is itself subject to the assumption that its mutation does not affect intrinsic binding to site B. Studies of λ cl binding to O$_R$1

5-base pair insertion (closed symbols, two experiments) are shown, titrated with DctD$_{Δ(1–142)}$. In comparing these data with that of Fig. 3, note that occupancy on the 5-base pair insertion template is identical with that of DctD$_2$; however, occupancy of the wild type UAS configuration requires less (DctD$_{Δ(1–142)}$ than DctD$_2$, indicating higher cooperativity for the deletion mutant.

**Fig. 4.** Binding of DctD$_2$ to B$_{G–C}$ and B$_{G–C}+5$ templates, and of (DctD$_{Δ(1–142)}$)$_2$ to WT and +5 templates. Circles, site A; triangles, site B. A, template B$_{G–C}$; note that the A and B$_{G–C}$ site isotherms overlap and require slightly higher protein concentrations for occupancy than was true for WT template in Fig. 3A. Open and closed symbols represent two separate experiments. B, template B$_{G–C}$ with 5-base pair insertion; occupancy at the B$_{G–C}$ site requires more protein than the wild type site B (see Fig. 3, B and C), while occupancy at the A site is identical when separated by the 5-base pair insertion from site B$_{G–C}$ or site B (see Fig. 3B). Open and closed symbols represent two separate experiments. C, both the wild type template (open symbols, two experiments) and the template with wild type sites separated by a
have illustrated the importance of examining DNA binding interactions in the native context with flanking DNA present (38). If point mutants can be identified which effectively reduce the valency of the dctA UAS without disturbing binding to adjacent sites, then DNase I studies of them might address this problem.

Implicit in the above assumption is the general presumption that the intrinsic and cooperative energies for DctD binding to the dctA UAS are unlinked, allowing them to be studied in each other’s absence. This simplifying assumption has certainly been questioned for other systems (39–42). For example, non-additivity of thermodynamic energies (40, 41) and direct evidence of conformational changes in λ O24 upon binding of cI (42) imply a role for DNA sequence-dependent conformational changes in the mechanisms of both intrinsic and cooperative binding of cI to O24. We observed no evidence of hypersensitivity within or between the DctD binding sites that might indicate such conformational changes. Further evidence that is consistent with intrinsic binding and cooperative binding being unlinked in the dct system was provided by comparing data for the wild type and B43–44 mutation templates. The global and individual analyses for these data indicated that at the 67% confidence level, the point mutation reduced DctD’s intrinsic affinity for mutant site B but left unchanged its affinity for site A and the apparent cooperativity between the two protein-DNA complexes.

There are several possible functions for a cooperative DctD-DctA UAS interaction. First, DctD may have a dual function as an activator and as a corepressor, repressing the transcription of dctBD. Occupancy of the UASs would be greater than 95% at a concentration of 10 nM, allowing repression at presumed minimal cellular concentrations of DctD. Although little is known about transcription of dctBD, the position of the UAS is such that it could block binding of RNA polymerase at the dctBD promoter. Also, strong binding of DctD to the dctA UAS even when DctD is in an inactive form may insulate the dctA promoter from spurious activation by other σ54-dependent transcriptional activators (43). A third possible function of cooperativity is that DctD2 may need to be in an oligomeric form to be transcriptionally active. This has been suggested for the similar two-component transcriptional activator protein, NtrC (28, 29), which has also been shown to bind cooperatively to the glnA UAS (26–28). It has been previously shown that DctD2(1–142) has an ATPase activity (19). ATP hydrolysis was only seen at high protein concentrations and was increased in the presence of DNA. These observations are consistent with the active form of the protein being a higher oligomeric form.

One of the more interesting observations of these studies is that DctD2(1–142) showed increased cooperativity relative to the transcriptionally inactive form. Such an increase in cooperativity may allow the active form of DctD to displace the inactive form from the dctA UAS. Alternatively, this additional interaction may be responsible for actually converting the inactive form to the active form. There may thus be two mechanisms mediating cooperativity in DctD, one detected in transcriptionally inactive protein, and the other appearing in transcriptionally active protein. DctD2(1–142) may present only the latter, or both of these activities. If DctD2(1–142) truly represents the active state typical of phosphorylated DctD, then the amino-terminal domain would not be needed for such cooperativity. However, an artificial protein-protein interaction may have been caused by deleting the amino-terminal domain from DctD in making DctD2(1–142). The increased cooperativity of DctD2(1–142) is formally similar to the observation that cooperativity is increased in NtrC when it is phosphorylated (26–28). In that case, the increase is proposed to be mediated by properties of the phosphorylated amino-terminal domain of NtrC (44), and/or its central domain (27). In the latter report, based on DNase I footprint data, the authors also suggest that binding of ATP to the NtrC central domain influences cooperativity by 2-fold. While that might be true, estimating cooperativity effects of less than 3–4-fold may well be meaningless given the difficulty inherent to precise measurement of apparent constants (33).

It is also worth noting that the previous studies of E. coli and S. typhimurium NtrC that first demonstrated cooperative binding by a σ54-dependent transcription activator utilized filter binding (26) and gel shift assays (28). Both studies reported about a 20-fold cooperative binding by NtrC, which was observed to increase upon phosphorylation of NtrC; however, the estimates of the increase in cooperativity due to phosphorylation were 50,000-fold versus 12-fold. While several features of the studies differed, the magnitude of this discrepancy remains to be explained. DNase I has a distinct advantage over other methods of studying multiple site DNA-protein interactions because occupancy at each site can be monitored separately in the same experiment. This is particularly important when it is believed that the two sites have different intrinsic binding affinities because it may be difficult, or even impossible, to distinguish between site heterogeneity and cooperativity using filter binding (31) or gel shift (45, 46) approaches. In a more recent DNase I footprint study of E. coli NtrC binding to the glnA UAS region (27), 20-fold cooperativity for binding of unphosphorylated protein was estimated to increase 12-fold upon phosphorylation. These values are more similar to those reported for Salmonella NtrC (28).

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REFERENCES
R. meliloti DctD Binds the dctA UAS Cooperatively

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