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Mutational Analysis of the Phosphate-Binding Loop of *Rhizobium meliloti* DctD, a σ^{54}-Dependent Activator

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The phosphate-binding loop of σ^{54}-dependent activators is thought to participate in ATP binding and/or hydrolysis. Alanine substitutions at positions 3, 4, 6, 7, and 8 of this motif in *Rhizobium meliloti* DctD disrupted transcriptional activation and ATP hydrolysis. Interestingly, substitution of alanine at position 7 also affected DNA binding.

Transcription by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) requires an activator protein (16, 20). The activator binds to upstream activation sequences (UAS) that are generally located 100 to 200 bp upstream of the transcriptional start site and contacts σ^{54}-holoenzyme bound to the promoter in a closed complex through DNA looping (3, 17, 24, 28). The activator catalyzes the isomerization of the closed complex to an open complex that is transcriptionally active in a reaction that requires ATP hydrolysis by the activator (10, 12, 13, 16, 20, 30). The barrier to open complex formation by σ^{54}-holoenzyme is thought to be both kinetic and thermodynamic, and the activator is believed to act as a simple molecular machine that couples the energy from ATP hydrolysis to open complex formation (29).

σ^{54}-dependent activators contain a phosphate-binding loop (also referred to as P loop or Walker A sequence) which occurs in other GTP- and ATP-binding proteins and binds the phosphate moiety of the nucleotide (19, 23, 25). The consensus sequence for the P loop is GXXXXGK(T/S), where X denotes various amino acids and the parentheses enclose alternative amino acids at one position (19, 25). The P loop often has distinctive features within protein families. Sequence comparisons of over 60 σ^{54}-dependent activators indicates the consensus sequence GE(S/T)G(T/S/V)GK(E/D) for the P loop of this family of proteins (Fig. 1).

Substitution of asparagine for glycine at position 6 in the P loops of the σ^{54}-dependent activators NtrC of *Salmonella typhimurium* and XylR of *Pseudomonas putida* interfered with the abilities of these proteins to hydrolyze ATP and activate transcription (14, 15). It is unclear from these studies, however, if other amino acid residues are critical for ATP hydrolysis or if amino acid substitutions in the P loop affect other functions of the activator, such as DNA binding or interaction with σ^{54}-holoenzyme. To understand better the function of the P loop in σ^{54}-dependent activators, we systematically changed each amino acid residue in the P loop of *Rhizobium meliloti* DctD (C_4-dicarboxylic acid transport protein D) by site-directed mutagenesis and biochemically characterized several of the mutant proteins. DctD activates transcription from *dctA*, which encodes a permease for C_4-dicarboxylic acids. It forms a two-component regulatory system with DctB that positively regulates expression from *dctA* (2, 7, 8, 18). In this study, we used $DctD_{(\Delta 1\text{-}142)}$, which is a truncated, constitutively active form of DctD that lacks the N-terminal regulatory domain (12). Unlike the full-length DctD protein, this truncated protein does not need to be phosphorylated to hydrolyze ATP or activate transcription (12).

Alanine substitutions at five of the eight positions in the P loop of DctD result in loss of transcriptional activation. The P loop of *R. meliloti* DctD has the amino acid sequence ^{173}GETGSGKE^{180} (8). Alanine substitutions were introduced into this motif by site-directed mutagenesis, as described previously for $DctD_{(\Delta 1\text{-}142)}$ (27). Alanine was chosen because it is generally thought to have minimal effects on protein structure (6).

Alanine substitutions at positions 3 (Thr-175), 4 (Gly-176), 6 (Gly-178), 7 (Lys-179), and 8 (Glu-180) resulted in loss of the ability of $DctD_{(\Delta 1\text{-}142)}$ to activate transcription from a *dctA′-′lacZ* reporter gene in *Escherichia coli* (Table 1). The eighth amino acid of the P loop is usually a hydroxyl-containing residue that serves as one of the protein ligands for the divalent metal cation associated with the nucleotide (23). Substitution of a threonine for Glu-180 resulted in a protein that was unable to activate transcription (Table 1). If Glu-180 is a ligand to the divalent cation associated with ATP, then the failure of threonine to replace Glu-180 suggests that it may not be properly positioned to ligate the cation. Alternatively, Glu-180 may have other roles that cannot be accomplished by threonine.

Introducing alanine at position 5 (Ser-177) did not affect the ability of $DctD_{(\Delta 1\text{-}142)}$ to activate transcription, but this position is not well conserved in σ^{54}-dependent activators (Fig. 1). Alanine substitutions at positions 1 and 2 of the P loop (Gly-173 and Glu-174, respectively) did not seriously affect the ability of the protein to activate transcription either, despite the fact that these amino acids are well conserved in σ^{54}-dependent activators.

Mutant proteins that fail to activate transcription are deficient in the ability to hydrolyze ATP. Mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were purified as described previously (26) so that we could characterize their activities in vitro. We were unable to purify two of the mutant proteins, $DctD_{(\Delta 1\text{-}142,\ G176A)}$ and $DctD_{(\Delta 1\text{-}142,\ G178A)}$, both of which behaved differently in the purification protocol than $DctD_{(\Delta 1\text{-}142)}$. All of the other mutant proteins behaved similarly to $DctD_{(\Delta 1\text{-}142)}$ in the purification protocol, suggesting that the amino acid substitutions in these proteins did not cause major structural changes.

Mutant proteins that failed to activate transcription from the *dctA′-′lacZ* reporter gene in vivo also failed to activate transcription from the *dctA* promoter regulatory region in an in

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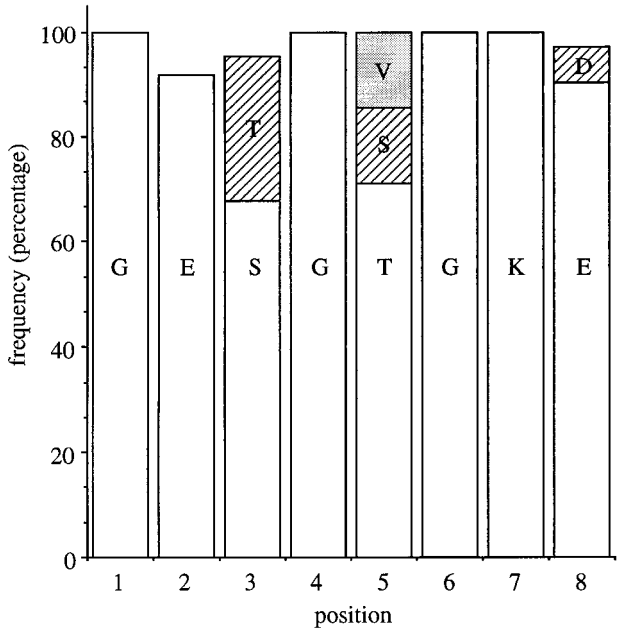

FIG. 1. Consensus sequence for P-loop motif of σ^{54}-dependent activators. The frequency at which a given amino acid occurs at each position within the P-loop motif was calculated after comparisons of the amino acid sequences of 62 σ^{54}-dependent activators. The amino acid sequence of the P-loop motif of *R. meliloti* DctD is GETGSGKE and spans positions 173 through 180.

vitro transcription assay (data not shown). No transcripts were produced with these mutant proteins even when the concentration of ATP in the assay was increased from 3 to 10 mM.

For the mutant proteins that were purified, we examined their ATPase activities as described previously (26). ATPase activities were determined in the presence of a plasmid that carries the *dctA* UAS, which was shown to stimulate ATP hydrolysis by $DctD_{(\Delta 1\text{-}142)}$ (12). The mutant proteins that activated transcription in vivo, $DctD_{(\Delta 1\text{-}142,\ G173A)}$, $DctD_{(\Delta 1\text{-}142,\ E174A)}$, and $DctD_{(\Delta 1\text{-}142,\ S177A)}$, exhibited ATPase activities that ranged from 54 to 140% of that observed with $DctD_{(\Delta 1\text{-}142)}$ (Table 2). In contrast, the mutant proteins that failed to activate transcription were severely affected in their abilities to hydrolyze ATP, which likely accounted for their failure to activate transcription.

We predicted that some substitutions in the P loop might affect the affinity of the protein for ATP, so we examined the abilities of the purified proteins to bind ATP. ATP binding

TABLE 1. Transcriptional activation from a *dctA'-'lacZ* reporter gene by mutant forms of $DctD_{(\Delta 1\text{-}142)}$

Protein	β-Galactosidase activity (Miller units)	% Activity of $DctD_{(\Delta 1\text{-}142)}$
None	10	
$DctD_{(\Delta 1\text{-}142)}$	4,210	100
$DctD_{(\Delta 1\text{-}142,\ G173A)}$	3,630	86
$DctD_{(\Delta 1\text{-}142,\ E174A)}$	2,970	70
$DctD_{(\Delta 1\text{-}142,\ T175A)}$	14	<0.1
$DctD_{(\Delta 1\text{-}142,\ G176A)}$	13	<0.1
$DctD_{(\Delta 1\text{-}142,\ S177A)}$	3,800	90
$DctD_{(\Delta 1\text{-}142,\ G178A)}$	11	<0.1
$DctD_{(\Delta 1\text{-}142,\ K179A)}$	14	<0.1
$DctD_{(\Delta 1\text{-}142,\ E180A)}$	12	<0.1
$DctD_{(\Delta 1\text{-}142,\ E180T)}$	14	<0.1

TABLE 2. ATPase activities of mutant forms of $DctD_{(\Delta 1\text{-}142)}$

Protein	Phosphate released[a] (pmol)	Incubation period (min)	% Activity of $DctD_{(\Delta 1\text{-}142)}$[b]
$DctD_{(\Delta 1\text{-}142)}$	4,010	10	100
$DctD_{(\Delta 1\text{-}142,\ G173A)}$	2,160	10	54
$DctD_{(\Delta 1\text{-}142,\ E174A)}$	3,070	10	77
$DctD_{(\Delta 1\text{-}142,\ S177A)}$	5,650	10	140
$DctD_{(\Delta 1\text{-}142)}$	7,760	15	100
$DctD_{(\Delta 1\text{-}142,\ T175A)}$	50	15	0.6
$DctD_{(\Delta 1\text{-}142,\ K179A)}$	<15	15	<0.2
$DctD_{(\Delta 1\text{-}142,\ E180A)}$	150	15	1.9
$DctD_{(\Delta 1\text{-}142,\ E180T)}$	160	15	2.1

[a] Assay mixtures contained 1.5 μM $DctD_{(\Delta 1\text{-}142)}$ protein (dimer) and 50 nM pJHL2, which carries the *dctA* UAS. Each value is the amount of phosphate released over the indicated incubation period.

[b] ATPase activities of the mutant proteins are compared to the activities observed for $DctD_{(\Delta 1\text{-}142)}$ for the corresponding incubation periods.

assays were carried out essentially as described previously (5). DctD proteins were spotted onto 5-mm-diameter nitrocellulose discs and dried at room temperature for 20 min. The nitrocellulose discs were incubated for 5 min in a solution of 0.5% (wt/vol) bovine serum albumin in the transcription assay buffer (29) and then transferred to tubes that contained 0.5 ml of the same buffer with 2 μCi of [γ-^{32}P]ATP (~3,000 Ci/mmol; 1.3 nM ATP) and incubated on ice for 50 min. The buffer was removed, and the discs were quickly washed with 1 ml of buffer and then analyzed for radioactivity by liquid scintillation counting. Controls in which no DctD protein was applied to the nitrocellulose disc were included. For reasons that we do not understand, the background radioactivity from these control discs was unchanged when unlabelled ATP was included in the binding assay. Cold ATP did, however, reduce the amount of radioactivity associated with bound DctD, indicating that the proteins were specifically binding ATP (data not shown). Because the background counts were unaffected by cold ATP, we had to use ATP with a high specific activity. As indicated in Fig. 2, most of the mutant proteins bound ATP less efficiently than did $DctD_{(\Delta 1\text{-}142)}$.

Despite the fact that the ATPase activity of $DctD_{(\Delta 1\text{-}142,\ T175A)}$ was severely diminished, it appeared to bind ATP almost as well as did $DctD_{(\Delta 1\text{-}142)}$. These data indicate that Thr-175 may have a direct role in ATP hydrolysis. Like DctD, substitution of an alanine at the corresponding position (Ser-170) in *Klebsiella pneumoniae* NtrC resulted in loss of transcriptional activation (1). In contrast, mutant forms of *K. pneumoniae* NifA with either glycine or alanine substitutions at this position (Ser-242) retained activity in vivo (4).

It seemed unusual that $DctD_{(\Delta 1\text{-}142,\ G173A)}$, $DctD_{(\Delta 1\text{-}142,\ E174A)}$, and $DctD_{(\Delta 1\text{-}142,\ S177A)}$ hydrolyzed ATP but showed reduced affinities for ATP in the binding assay. These binding assays, however, were done at nanomolar ATP concentrations, whereas millimolar concentrations of ATP were present in the ATPase assays. We infer that these mutant proteins have reduced affinities for ATP but that the affinities are not reduced enough to affect ATP hydrolysis at physiological ATP concentrations.

Mutant proteins appear to interact normally with σ^{54}-holoenzyme. $DctD_{(\Delta 1\text{-}142)}$ can be cross-linked to both σ^{54} and the β subunit of RNA polymerase, suggesting that it interacts with these subunits to activate transcription (11). We had previously isolated mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that failed to activate transcription and were also deficient in their abilities to cross-link to σ^{54} and the β subunit (27). To determine if any of the mutant proteins generated in this study were similarly defec-

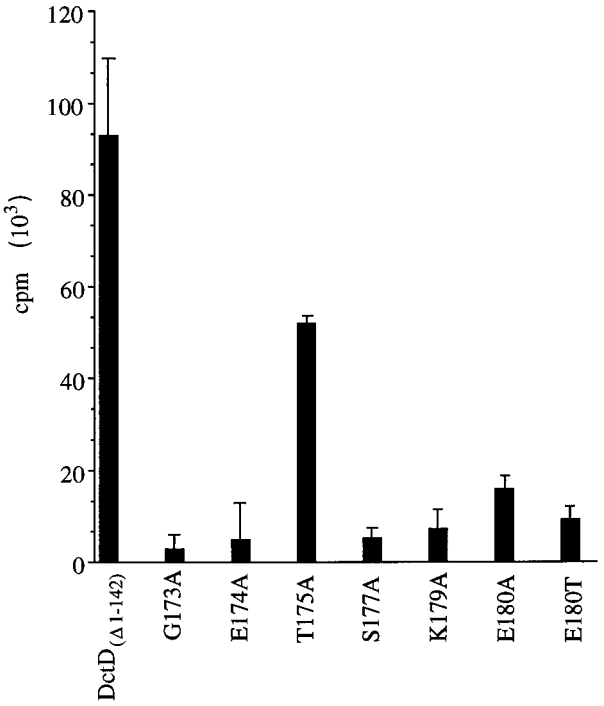

FIG. 2. ATP binding assays for $DctD_{(\Delta 1\text{-}142)}$ and mutant forms of the protein. Purified proteins were spotted onto nitrocellulose discs and incubated with [γ-^{32}P]ATP for 50 min on ice. After washing of the discs, the amount of radioactivity associated with each disc was determined by liquid scintillation counting. Background counts were determined from discs on which no DctD had been applied, and these counts were subtracted from the counts determined for the filters with the various $DctD_{(\Delta 1\text{-}142)}$ proteins. The data are averages of four independent assays; error bars show the 95% confidence limits for these averages.

tive in interaction with σ^{54}-holoenzyme, mutant proteins were cross-linked to either *S. typhimurium* σ^{54} or the β subunit of *E. coli* core RNA polymerase with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, as described previously (11). All of the mutant proteins that we purified in this study cross-linked normally to σ^{54} and the β subunit, suggesting that the amino acid substitutions did not significantly interfere with interactions between $DctD_{(\Delta 1\text{-}142)}$ and σ^{54}-holoenzyme (data not shown).

Substitution of alanine for Lys-179 affects DNA binding. The UAS of *R. meliloti dctA* contains two DctD-binding sites, referred to as sites A and B. DctD has a 50- to 100-fold-higher affinity for site B (the site that is proximal to the *dctA* promoter) than for site A when the sites are separated (9), but it binds cooperatively to these sites when they are together in the native UAS (21). We compared the DNA-binding activities of the mutant proteins that failed to activate transcription with that of $DctD_{(\Delta 1\text{-}142)}$ in a DNase I footprinting assay.

DNase I footprinting patterns observed with three of the four mutant proteins, $DctD_{(\Delta 1\text{-}142,\ T175A)}$, $DctD_{(\Delta 1\text{-}142,\ E180A)}$, and $DctD_{(\Delta 1\text{-}142,\ E180T)}$, were very similar to that observed with $DctD_{(\Delta 1\text{-}142)}$ (Fig. 3). For each of these proteins, sites A and B appeared to be equally protected from DNase I digestion at the various protein concentrations, suggesting that these proteins bound cooperatively to the UAS. Binding of $DctD_{(\Delta 1\text{-}142,\ K179A)}$ to the native UAS, however, was somewhat different. Site A was not fully protected from DNase I digestion even though site B was fully protected. These data indicate that substitution of alanine for Lys-179 interferes with binding of $DctD_{(\Delta 1\text{-}142)}$ to the low-affinity site of the UAS, either by reducing the affinity of the protein for this site or by disrupting cooperative binding to the UAS.

Conclusions. We have shown that Thr-175, Gly-176, Gly-178, Lys-179, and Glu-180 are important for the structural or functional integrity of the P loop of DctD. It is surprising that Gly-173 and Glu-174 (positions 1 and 2, respectively) are not essential for activity given the high degree to which these residues are conserved among σ^{54}-dependent activators. Moreover, examination of the P loops of diverse protein families reveals that glycine at position 1 is essentially invariant (19). In general, conserved glycines help to maintain the structure of the loop and allow main-chain hydrogen bonds between adjacent amino acids and the β and γ phosphates of the nu-

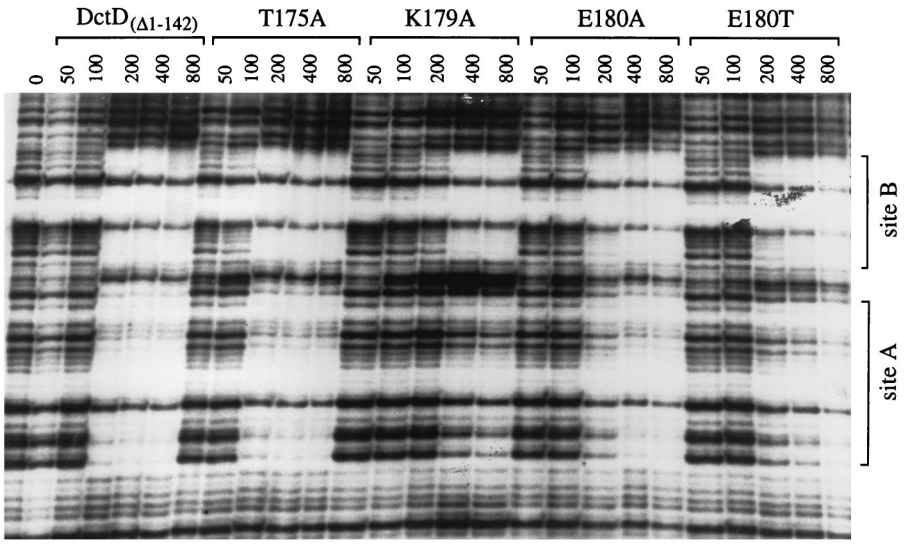

FIG. 3. DNase I footprinting of $DctD_{(\Delta 1\text{-}142)}$ and mutant forms of the protein at the *R. meliloti dctA* UAS. DNase I footprinting assays were performed with a 5′-end-labelled DNA fragment carrying the *dctA* UAS and various purified proteins, as indicated at the top of the figure. Final protein concentrations were varied from 50 to 800 nM (dimer), as indicated for each lane. The two DctD binding sites (sites A and B) are labelled at the side of the figure. Footprints were visualized by subjecting the reactions to denaturing gel electrophoresis and then exposing X-ray film to the resulting gel.

cleotide (23). If Gly-173 has a similar function, it appears that substitutions of at least small amino acids, such as alanine, are tolerated at this position. Like σ^{54}-dependent activators, other protein families often show conservation at position 2. For example, position 2 in the β subunit of the F_1-ATPase protein family is glycine. This glycine in the F_1 β subunit from yeast, however, can be replaced with virtually any amino acid and still produce an active enzyme (22). While the alanine substitutions at Gly-173 and Glu-174 of DctD appeared to affect the affinity of the protein for ATP, this was not enough to interfere with ATP hydrolysis or transcriptional activation at physiological ATP concentrations. Taken together, our data suggest that Gly-173 and Glu-174 play only a minor role in the structure and function of the P loop of DctD.

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