Mutational analysis of the C-terminal domain of the *Rhodobacter sphaeroides* response regulator PrrA

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The *Rhodobacter sphaeroides* response regulator PrrA directly activates transcription of genes necessary for energy conservation at low O₂ tensions and under anaerobic conditions. It is proposed that PrrA homologues contain a C-terminal DNA-binding domain (PrrA-CTD) that lacks significant amino acid sequence similarity to those found in other response regulators. To test this hypothesis, single amino acid substitutions were created at 12 residues in the PrrA-CTD. These mutant PrrA proteins were purified and tested for the ability to be phosphorylated by the low-molecular-mass phosphate donor acetyl phosphate, to activate transcription and to bind promoter DNA. Each mutant PrrA protein accepted phosphate from ³²P-labelled acetyl phosphate. At micromolar concentrations of acetyl phosphate-treated wild-type PrrA, a single 20 bp region in the PrrA-dependent *cycA* P2 promoter was protected from DNase I digestion. Of the mutant PrrA proteins tested, only acetyl phosphate-treated PrrA-N168A and PrrA-I177A protected *cycA* P2 from DNase I digestion at similar protein concentrations compared to wild-type PrrA. The use of *in vitro* transcription assays with the PrrA-dependent *cycA* P2 and *puc* promoters showed that acetyl phosphate-treated PrrA-N168A produced transcript levels similar to that of wild-type PrrA at comparable protein concentrations. Using concentrations of acetyl phosphate-treated PrrA that are saturating for the wild-type protein, PrrA-H170A and PrrA-I177A produced <45 % as much transcript as wild-type PrrA. Under identical conditions, the remaining mutant PrrA proteins produced little or no detectable transcripts from either promoter *in vitro*. Explanations are presented for why these amino acid side chains in the PrrA-CTD are important for its ability to activate transcription.

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INTRODUCTION

Facultative bacteria use a variety of strategies to control expression of pathways needed to conserve energy under low O₂ or anoxic conditions (Bauer *et al.*, 1999; Patschkowski *et al.*, 2000). We are interested in determining how the response regulator PrrA (Elsen *et al.*, 2004; Eraso & Kaplan, 1994; Zeilstra-Ryalls *et al.*, 1998) controls the synthesis of energy-conserving pathways in the facultative photosynthetic bacterium *Rhodobacter sphaeroides*.

R. sphaeroides is an alphaproteobacterium that grows by respiration in the presence or absence of O₂ or by photosynthesis when light is present under anaerobic conditions (Zeilstra-Ryalls *et al.*, 1998). In this or related photosynthetic bacteria, PrrA target genes encode components of the photosynthetic apparatus (Eraso & Kaplan, 1994), cytochrome oxidases that conserve energy at low O₂ tensions (Eraso & Kaplan, 2000), electron carriers like cytochrome *c*₂ that function in respiration and photosynthesis (Comolli *et al.*, 2002; Karls *et al.*, 1999; Swem *et al.*, 2001) and proteins that use reducing power under anoxic conditions (Dubbs *et al.*, 2000; Joshi & Tabita, 1996; Qian & Tabita, 1996).

Homologues of PrrA exist in alpha- and gammaproteobacteria, where they act in homeostatic control circuits to regulate gene expression in response to changes in the oxidation–reduction state of the aerobic electron transport chain (Bauer *et al.*, 1998; Comolli *et al.*, 2002; Comolli & Donohue, 2002; Elsen *et al.*, 2004). PrrA is phosphorylated by the membrane-bound histidine kinase PrrB (Bird *et al.*, 1999; Comolli *et al.*, 2002; Eraso & Kaplan, 1995, 1996; Oh *et al.*, 2004; Potter *et al.*, 2002). A terminal oxidase within the aerobic respiratory chain, the cytochrome *cbb*₃ oxidase, is able to control the phosphatase activity of PrrB (Oh *et al.*, 2004), increasing the amount of phosphorylated PrrA when O₂ is limiting (Oh & Kaplan, 2000, 2001; Oh *et al.*, 2004).

Response regulators functioning as transcription factors often contain a C-terminal DNA-binding domain related to *Escherichia coli* OmpR or NarL (Hakenback & Stock, 1996; Kenney, 2002; Stock *et al.*, 2000). However, the PrrA C-terminal domain (PrrA-CTD) does not contain significant amino acid sequence similarity to the DNA-binding domains of well-studied response regulators (Elsen *et al.*, 2004; Eraso & Kaplan, 1994). Computational analysis of the PrrA-CTD suggests that there is amino acid sequence

			t	helix 1	t	helix 2	
Rs	PrrA	153	YEMC	DRN	VSETARRL	NMH	RRTLQRILAK RSPR 184
Rc	RegA	153	YEQC	DRN	VSETARRL	NMH	RRTLQRILAK RSPR 184
Rd	RegA	153	YELC	DRN	VSETARRL	NMH	RRTLQRILAK RSPR 184
Bj	RegR	153	YEMC	NRN	VSETARRL	NMH	RRTLQRILAK RSPR 184
Pa	RoxR	153	LAEH	EGN	ISATARAL	GMH	RRTLQRILAK RSPR 184
Ec	Fis	67	MQYT	RGN	QTRAALMM	GIN	RGTLRKKLKK YGMN 98

Fig. 1. Amino acid sequence comparison of the C-terminal domains of PrrA homologues and *E. coli* Fis. Amino acids are aligned according to the structure of PrrA-CTD (t indicates the two turns, one before helix 1 and one between helices 1 and 2; Laguri *et al.*, 2003). Amino acids shaded are shared among PrrA homologues (Elsen *et al.*, 2004). Rs, *R. sphaeroides* 2.4.1 (GenBank accession no. AAA86723); Rc, *Rhodobacter capsulatus* B10 (P42508); Rd, *Roseobacter denitrificans* OCh114 (AB010723); Bj, *Bradyrhizobium japonicum* 110spc4 (CAA06859); Pa, *Pseudomonas aeruginosa* PA01 (AAT50683); Ec, *E. coli* MG1655 Fis (P0A6R3).

similarity to the DNA-binding domain of *E. coli* Fis (Fig. 1). Indeed, NMR analysis of the PrrA-CTD revealed that it forms a three-helix bundle with a helix–turn–helix DNA-binding domain (Laguri *et al.*, 2003). In addition, the helix–turn–helix of the PrrA-CTD showed the greatest degree of structural similarity to Fis when compared to other transcription factors for which structures are available (Laguri *et al.*, 2003).

The similarity between PrrA-CTD and Fis predicts that the amino acid side chains of residues 168–180 of PrrA recognize DNA target sites. To test this hypothesis, we generated mutant PrrA proteins, each with a single amino acid substitution in these residues. Mutant PrrA proteins were purified and analysed for their ability to be phosphorylated, bind target DNA and activate transcription *in vitro*. This analysis identified amino acid side chains in the PrrA-CTD that are important for its ability to activate transcription.

METHODS

Strains and growth conditions. *E. coli* DH5 α was used for cloning, while ER2566 was used for expression of recombinant PrrA proteins (Table 1). *E. coli* strains were grown at 37 °C in Luria–Bertani medium (Sambrook *et al.*, 1989) containing 100 μ g ampicillin ml⁻¹.

Mutagenesis of *prrA*. To generate amino acid substitutions in PrrA, the respective codon was changed to GCC (residue L167, N168, H170, R176 and I177) or GCG (residues M169, R171, R172, T173, L174, Q175, L178 and K180); a GGC codon was used to generate PrrA-N168G. Site-directed mutagenesis was performed by annealing 33 nt overlapping primers to a *prrA* gene that encodes a PrrA–intein fusion protein (pJC407) and amplification with *Pfu* polymerase (containing 1% Triton X-100 and DMSO) (Stratagene). Each *prrA* gene was sequenced to confirm that only the desired mutation was present.

PrrA purification. PrrA proteins were expressed as intein fusions in ER2566 (Comolli *et al.*, 2002). An 800 ml culture was grown (37 °C) until an OD₆₀₀ of 0.5–0.8, 0.3 mM IPTG was added and the culture was then shaken at 30 °C for 3 h. Cells were harvested by centrifugation, resuspended in 15 ml column buffer (20 mM Tris/HCl,

pH 8.0, 0.1 mM EDTA, 0.5 M KCl, 0.1% Triton X-100, 5 mM MgCl₂) and lysed by sonication. The extract was centrifuged for 30 min at 13 000 g (4 °C) and the supernatant was loaded onto a 10 ml chitin column previously equilibrated with 10 vols column buffer. The column was washed with 10 vols column buffer before overnight treatment (4 °C) with 35 ml column buffer supplemented with 0.3 mM DTT to stimulate cleavage of the intein domain. The column was washed with column buffer and 4 ml fractions were collected. An aliquot of each fraction was analysed on 12% Bis-Tris SDS gels (Invitrogen). PrrA-containing fractions were pooled, dialysed into PrrA buffer (40 mM Tris/HCl, pH 7.9, 5 mM MgCl₂, 50 mM KCl, 0.1 mM DTT) and then into PrrA buffer containing 25% glycerol for storage at –80 °C. Protein concentrations were determined by the Bradford assay using BSA as a standard (Bio-Rad).

PrrA phosphorylation. PrrA proteins (15 μ M final concentration) were added to transcription buffer (10 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 0.3 mM DTT), supplemented with 0.1 mg BSA ml⁻¹ and 0.1 mM DTT in a final volume of 25 μ l. ³²P-labelled acetyl phosphate was added to 25 mM and the mixture was incubated at 30 °C for 45 min (McCleary & Stock, 1994). To assay PrrA phosphorylation, 10 μ l 3 $\times$ SDS stop solution (Comolli & Donohue, 2002) was added, the samples were analysed on 12% Bis-Tris SDS gels (Invitrogen) and radioactivity in PrrA was measured by phosphorimaging (Molecular Dynamics).

In vitro transcription. *R. sphaeroides* RNA polymerase holoenzyme was prepared by heparin agarose chromatography (Anthony *et al.*, 2003). Multiple-round *in vitro* transcription assays used ~70 nM RNA polymerase, acetyl phosphate-treated PrrA, 20 nM *cycA* P2 template plasmid (pRKK146) or *puc* template plasmid (pJC412) in a 20 μ l reaction containing transcription buffer, 0.1 mM DTT and 0.1 mg BSA ml⁻¹. These components were incubated for 20 min at 30 °C and the reaction was initiated by adding nucleoside triphosphates (0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP and 50 μ M UTP) plus 10 μ Ci [α -³²P]UTP. After incubation for 20 min at 30 °C, the reaction was terminated by adding 10 μ l gel-loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were placed at 95 °C for 3 min and analysed on a 6% polyacrylamide, 7 M urea gel alongside DNA sequencing reactions to map the transcription start site for individual promoters. Transcript abundance was quantified by phosphorimaging. PrrA proteins were phosphorylated by mixing 25 μ M PrrA with 25 mM acetyl phosphate in transcription buffer for 60 min at 30 °C.

DNase I footprinting. A ³²P-labelled *cycA* P2 DNA fragment (extending from –228 to +22 relative to the known transcription initiation site; Karls *et al.*, 1999; Newlands *et al.*, 1991) was obtained by digesting pRKK128 with *Hind*III and *Not*I followed by labelling the *Hind*III restriction site with [α -³²P]dATP. The DNA fragment was gel-purified and then treated with DNase I (approx. 5–10 nM) for 30 s in transcription buffer in the absence or presence of acetyl phosphate-treated PrrA (Karls *et al.*, 1999; Newlands *et al.*, 1991). Prior to electrophoresis, 10 μ l gel-loading buffer was added to the samples and they were heated at 95 °C for 45 s. Samples were analysed on a 6% polyacrylamide, 7 M urea gel alongside DNA sequencing reactions to localize the relative position of PrrA-binding sites. Radioactivity was visualized and quantified by phosphorimaging.

RESULTS

Generation and properties of mutant PrrA proteins

The model for PrrA function predicts that the PrrA-CTD is involved in DNA binding (Comolli *et al.*, 2002; Elsen *et al.*,

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/phenotype	Source
<i>E. coli</i> strains		
DH5 α	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR</i> $\Delta(lacZYA-argF)U169/\lambda$ (Φ 80d <i>lacZ</i> Δ M15)	Invitrogen
ER2566	F ⁻ λ ⁻ <i>fhuA2 lon ompT lacZ::T7gene1 gal sulA11</i> $\Delta(mcrC-mrr)114::IS10$ R (<i>mcr-73::miniTn10-TetS</i>)2 R(<i>zgb-210::Tn10</i>) (Tet ^s) <i>endA1(dcm)</i>	New England Biolabs
Plasmids		
pTYB4	C-terminal intein/chitin-binding domain expression vector; Ap ^r	New England Biolabs
pRKK128	<i>HindIII-NlaI</i> <i>cycA</i> P2 fragment (−228 to +22) cloned into <i>SmaI</i> site of pUC21	Karls <i>et al.</i> (1999)
pRKK146	<i>In vitro</i> transcription template containing <i>cycA</i> P2 (positions −73 to +22) −14C→T fused to the <i>spf'</i> terminator	Karls <i>et al.</i> (1999)
pJC407	<i>prpA</i> cloned into <i>NcoI/SmaI</i> sites of pTYB4	Comolli <i>et al.</i> (2002)
pJC412	<i>NsiI-StuI</i> fragment of <i>puc</i> promoter (−629 to +33) in <i>NsiI/StuI</i> of pRKK96	Laboratory strain
pDJ120	pJC407 with alanine substitution at position R172	This study
pDJ121	pJC407 with alanine substitution at position L167	This study
pDJ131	pJC407 with alanine substitution at position H170	This study
pDJ144	pJC407 with alanine substitution at position R171	This study
pDJ146	pJC407 with alanine substitution at position Q175	This study
pDJ147	pJC407 with alanine substitution at position R176	This study
pDJ149	pJC407 with alanine substitution at position K180	This study
pDJ160	pJC407 with alanine substitution at position T173	This study
pDJ162	pJC407 with alanine substitution at position I177	This study
pDJ177	pJC407 with alanine substitution at position L178	This study
pDJ181	pJC407 with alanine substitution at position N168	This study
pDJ184	pJC407 with glycine substitution at position N168	This study
pDJ186	pJC407 with alanine substitution at position L174	This study
pDJ202	pJC407 with alanine substitution at position M169	This study

2004; Laguri *et al.*, 2003). The recently reported NMR structure of the PrrA-CTD shows that it contains a helix–turn–helix motif in which amino acids 168–170 form a turn before a putative DNA-binding helix (helix 2) that extends from residue 171 to 180 (Laguri *et al.*, 2003). To test the predicted role of these amino acids in PrrA function, we individually substituted an alanine for 11 amino acids between residues 168 and 180 (residue 179 of wild-type PrrA is an alanine). Because many helix–turn–helix transcription factors, including Fis and other PrrA homologues, contain a glycine at the first residue in the turn before helix 2 (Fig. 1), we also generated PrrA-N168G.

To analyse the mutant PrrA proteins, each was expressed as an intein fusion and purified by a protocol used previously for purification of wild-type PrrA (Comolli *et al.*, 2002). The yield and purity (>95 % pure by SDS-PAGE) of most of the mutant proteins was comparable to wild-type PrrA (data not shown). However, PrrA-N168G and PrrA-L174A were obtained at ~2-fold lower yields (~20 μ M final concentration) relative to wild-type PrrA or the other mutant PrrA proteins.

Acetyl phosphate-treated wild-type or mutant PrrA proteins, when analysed by molecular sieve chromatography (Superdex 200; 25 mM Tris/HCl, pH 7.9, 150 mM NaCl), eluted at the apparent molecular mass expected for a PrrA monomer (data not shown). Thus, there is no evidence that any of these amino acid substitutions caused significant aggregation or alterations in the apparent oligomeric state of the protein (data not shown).

Many response regulators acting as transcription factors require phosphorylation to activate transcription (Kenney, 2002; Stock *et al.*, 2000). If any amino acid substitutions in the PrrA-CTD caused dramatic changes in the overall conformation of the protein, they might alter the capacity of the protein to be phosphorylated. To test for such changes, we monitored the ability of the mutant PrrA proteins to accept phosphate from ³²P-acetyl phosphate, a low-molecular-mass phosphate donor. Wild-type PrrA is phosphorylated by acetyl phosphate *in vitro* and the phosphorylated protein is sufficiently stable (*t*_{0.5} ~330 min) to monitor this modification by SDS-PAGE (Comolli *et al.*, 2002). Under our assay conditions, most of the mutant PrrA proteins were

Table 2. Properties of mutant PrrA proteins

Phosphorylation is scored as: + + +, $\geq 50\%$ activity compared with wild-type PrrA; + +, $\sim 33\%$ activity compared with wild-type PrrA. Activation of *cycA* P2 and *puc* is scored as: + + +, activity comparable to wild-type PrrA; + +, slightly reduced activation at $5\ \mu\text{M}$ PrrA; +, severely reduced activation at $5\ \mu\text{M}$ PrrA; –, no detectable activation at any concentration tested. Binding to *cycA* P2 is scored as: + + +, binding activity comparable to wild-type PrrA; –, no detectable footprint at any concentration; NT, footprinting was not tested with this mutant PrrA protein.

PrrA protein	Region	Phosphorylation	Activation of:		Binding to <i>cycA</i> P2
			<i>cycA</i> P2	<i>puc</i>	
Wild-type	–	+ + +	+ + +	+ + +	+ + +
N168A	Turn	+ + +	+ + +	+ + +	+ + +
N168G	Turn	+ + +	+	+	NT
M169A	Turn	+ + +	+	+	NT
H170A	Turn	+	+	+	NT
R171A	Helix 2	+ + +	–	–	–
R172A	Helix 2	+ + +	–	–	NT
T173A	Helix 2	+ + +	+	+	–
L174A	Helix 2	+	–	–	–
Q175A	Helix 2	+ + +	–	–	NT
R176A	Helix 2	+ + +	–	–	NT
I177A	Helix 2	+ + +	+ +	+ +	+ + +
L178A	Helix 2	+ + +	+	+	–
K180A	Helix 2	+ + +	+	+	NT

labelled to $\geq 50\%$ of the level observed for wild-type PrrA (Table 2). The only exceptions were PrrA-H170A and PrrA-L174A, which were labelled at a level ~ 3 -fold lower than wild-type PrrA (Table 2).

Function of mutant PrrA proteins in *in vitro* transcription assays

To test the ability of the mutant PrrA proteins to function as transcription factors, we assayed the ability of acetyl phosphate-treated proteins to activate transcription of two known PrrA-dependent promoters, *cycA* P2 and *puc*, in the presence of *R. sphaeroides* RNA polymerase. Previous analysis of PrrA activation at *cycA* P2 *in vitro* showed that the product of this promoter is not detected in multiple-round transcription assays unless this activator is present (Comolli *et al.*, 2002; Karls *et al.*, 1999). The addition of acetyl phosphate-treated wild-type PrrA increases the abundance of the *cycA* P2 transcript in a concentration-dependent manner relative to the control RNA1 transcript (Fig. 2). Under these assay conditions, activation of *cycA* P2 was maximal at $\sim 2.5\ \mu\text{M}$ acetyl phosphate-treated wild-type PrrA (Fig. 2; throughout the paper, we report the amount of acetyl phosphate-treated protein added to each assay).

Only acetyl phosphate-treated PrrA-N168A produced amounts of *cycA* P2 transcript similar to those generated by the same concentration of wild-type protein (Fig. 2). Under identical conditions, PrrA-H170A (15%) and PrrA-I177A (30%) displayed partial activation of *cycA* P2 even when using $5\ \mu\text{M}$ protein (no detectable *cycA* P2 transcript

was produced in this assay at lower protein concentrations of this or the remaining mutant PrrA proteins; data not shown). The extent of activation of *cycA* P2 transcription using $5\ \mu\text{M}$ acetyl phosphate-treated mutant PrrA proteins (scored by comparing the increase in this transcript relative to that of the control RNA1 product) suggested that these mutant proteins have considerably less activity than their wild-type counterpart, especially when considering that $\sim 2.5\ \mu\text{M}$ acetyl phosphate-treated wild-type PrrA typically produces near maximal promoter activity under these assay conditions. The PrrA-N168G, PrrA-M169A, PrrA-T173A, PrrA-Q175A, PrrA-L178A and PrrA-K180A mutant proteins reproducibly produced low ($\sim 5\%$), but detectable, levels of *cycA* P2 transcript when adding $5\ \mu\text{M}$ acetyl phosphate-treated PrrA (Fig. 2) when compared to the amount of product generated from the same concentration of wild-type PrrA. All the other mutant PrrA proteins tested had no detectable activation of *cycA* P2 transcription at PrrA concentrations as high as $5\ \mu\text{M}$. Because higher concentrations of wild-type PrrA lower the amount of *cycA* P2 transcript (data not shown), we did not compare the ability of wild-type and mutant proteins to activate transcription at concentrations of acetyl phosphate-treated proteins above $5\ \mu\text{M}$. From these results, it appears that many of these single amino acid substitutions produced a large negative effect on the ability of acetyl phosphate-treated PrrA to activate *cycA* P2 transcription.

We also assayed the ability of PrrA proteins to stimulate transcription from the *puc* promoter (Eraso & Kaplan, 1994), which is directly activated by RegA, a PrrA homologue from

Fig. 2. *In vitro* transcription assays using the PrrA-dependent *cycA* P2 promoter. The *cycA* P2 promoter fragment extends from -73 to +22 relative to the transcription start site (Karls *et al.*, 1999). The RNA1 transcript (~108 nt) was produced by the PrrA-independent *oriV* promoter. The concentration of acetyl phosphate-treated wild-type (WT) or mutant PrrA protein is indicated above the panel. Numbers below each lane report the relative amount of *cycA* P2 transcript (~176 nt) produced after correction for background and normalization to the amount of RNA1 transcript. ND, None detected.

Rhodobacter capsulatus (Bowman *et al.*, 1999). Unlike *cycA*, there was detectable *puc*-specific transcript produced in the absence of acetyl phosphate-treated wild-type PrrA (Fig. 3). Transcript abundance increased in the presence of acetyl phosphate-treated wild-type PrrA, with maximal levels at ~2.5 μM protein when compared to the control RNA1 product (Fig. 3).

When testing the ability of acetyl phosphate-treated mutant PrrA proteins to activate *puc* transcription *in vitro*, their function often mimicked what was observed with *cycA* P2. For example, comparable levels of *puc* transcript were produced by acetyl phosphate-treated wild-type PrrA and PrrA-N168A at each concentration tested (Fig. 3). In addition, PrrA-H170A and PrrA-I177A, at 5 μM, produced reduced amounts of the *puc* transcript (32 and 46%, respectively) compared to wild-type PrrA. At 5 μM acetyl phosphate-treated protein, PrrA-N168G (~16%), PrrA-M169A (~11%), PrrA-L178A (~15%) and PrrA-K180A

(~10%) gave low, but reproducible, activation of the *puc* promoter (Fig. 3). In addition, when tested at 5 μM acetyl phosphate-treated protein, each of the remaining mutant PrrA proteins produced levels of *puc* transcript comparable to that found in assays containing only *R. sphaeroides* RNA polymerase with no PrrA protein added (Fig. 3), suggesting that they are severely defective in activating this promoter.

DNA binding by mutant PrrA proteins

To test whether any of these amino acid substitutions altered DNA binding, we assayed the ability of acetyl phosphate-treated PrrA proteins to bind *cycA* P2 by DNase I footprinting. Previous experiments demonstrated that a mutant form of the *R. capsulatus* PrrA homologue, RegA*, which has increased activity in the absence of phosphorylation (Du *et al.*, 1998; Karls *et al.*, 1999), protected a single region of *cycA* P2 from DNase I digestion (Karls *et al.*, 1999). Analysis of wild-type PrrA binding to *cycA* P2 showed that ~1–2 μM

Fig. 3. *In vitro* transcription assays using the PrrA-dependent *puc* promoter. The *puc* promoter fragment extends from -629 to +33 relative to the transcription initiation site (Lee & Kaplan, 1992). The RNA1 transcript (~108 nt) was produced by the PrrA-independent *oriV* promoter. The concentration of acetyl phosphate-treated wild-type (WT) or mutant PrrA protein is indicated above the panel. Numbers below each lane report the relative amount of *puc* transcript (~187 nt) produced after correction for background and normalization to the amount of RNA1 transcript.

acetyl phosphate-treated protein was sufficient to protect a ~20 bp region on the top strand that is centred at -51 (Fig. 4). The region of *cycA* P2 protected from DNase I digestion matches the region protected by RegA* previously (Karls *et al.*, 1999).

To test whether the relative ability of an acetyl phosphate-treated mutant PrrA protein to activate *cycA* P2 transcription correlated with its DNA-binding capability, we assayed six of the mutant PrrA proteins by DNase I footprinting. PrrA-N168A activated *cycA* P2 transcription at levels similar to wild-type PrrA. PrrA-I177A only weakly activated *cycA* P2 transcription, while the other mutant proteins tested (PrrA-R171A, PrrA-T173A, PrrA-L174A and PrrA-L178A) had major defects in activation of this promoter even when using ~2-fold more protein than needed for near-maximal *in vitro* transcription activation by wild-type PrrA.

Acetyl phosphate-treated PrrA-N168A and PrrA-I177A proteins each protected the same ~20 bp region from DNase I digestion as seen when using acetyl phosphate-treated wild-type PrrA protein (Fig. 4). In addition, DNase I protection of *cycA* P2 by PrrA-N168A protein was detectable at ~1 µM, whereas protection by PrrA-I177A was detectable at ~2 µM. When comparing the change in intensity of DNase I cleavage products in a region of the promoter that was not protected by acetyl phosphate-treated PrrA (-92 to -72) to that protected by acetyl phosphate-treated protein (-61 to -45), it appeared that PrrA-N168A, PrrA-I177A and wild-type PrrA each saturated *cycA* P2 at concentrations between ~2 and 4 µM (Fig. 4). The similar apparent affinity of PrrA-N168A for *cycA* P2 is not surprising, since it has essentially wild-type activity for activation of this promoter. However, a similar apparent affinity of PrrA-I177A and wild-type PrrA for *cycA* P2 is surprising, since this mutant protein was defective in activating this promoter *in vitro* (see above).

The other acetyl phosphate-treated mutant PrrA proteins tested (PrrA-R171A, PrrA-T173A, PrrA-L174A and PrrA-L178A) were unable to protect the *cycA* P2 promoter from DNase I digestion even when using ~4-fold more acetyl phosphate-treated protein than was needed to occupy this

site with wild-type PrrA. The inability to detect significant binding of PrrA-R171A, PrrA-T173A, PrrA-L174A or PrrA-L178A to this promoter is consistent with their inability to activate *cycA* P2 transcription at any of the concentrations tested (see above).

DISCUSSION

The helix–turn–helix region within the PrrA-CTD was recently proposed to be important for DNA binding (Laguri *et al.*, 2003). In this study, we generated a series of mutant PrrA proteins containing a single amino acid change to test the role of the helix–turn–helix region in the function of this protein as a transcription factor. Below, we summarize the findings derived from analysing this set of mutant PrrA proteins *in vitro* (Table 2).

Amino acids in the turn of the PrrA C-terminal helix–turn–helix motif

PrrA N168, M169 and H170 form the turn between helices 1 and 2 (Laguri *et al.*, 2003). We found the side chains at residues 169 and 170 to be important for PrrA function, since PrrA-M169A and PrrA-H170A each exhibited significant defects in activating transcription at the two target promoters tested. In contrast, PrrA-N168A produced transcripts from both PrrA-dependent promoters at levels comparable to that produced by similar concentrations of the wild-type protein. However, even at high concentrations, PrrA-N168G only partially activated both of the PrrA-dependent promoters. The diminished activity of PrrA-N168G is surprising, since glycine is often found in the first position of the turn between helices 1 and 2 of helix–turn–helix DNA-binding proteins (Brennan & Matthews, 1989). While many PrrA homologues contain an asparagine at the position analogous to PrrA N168, some, including *Pseudomonas aeruginosa* RoxR (Fig. 1), contain a glycine at the analogous position (Comolli *et al.*, 2002; Comolli & Donohue, 2002; Elsen *et al.*, 2004). Indeed, amino acid sequence alignments propose that family members containing a glycine at the position equivalent to PrrA N168 are in a second group of PrrA homologues (Comolli *et al.*, 2002;

Fig. 4. DNase I protection of the PrrA-dependent *cycA* P2 promoter by wild-type or mutant PrrA proteins. Indicated concentrations of acetyl phosphate-treated wild-type or mutant PrrA proteins (0, 0.5, 1, 2 or 4 µM) were incubated with end-labelled *cycA* P2 promoter DNA and treated with DNase I. Hatched bars represent the region of *cycA* P2 DNA protected by wild-type or mutant PrrA proteins.

Comolli & Donohue, 2002; Elsen *et al.*, 2004). The group 2 PrrA homologue *P. aeruginosa* RoxR substitutes for *R. sphaeroides* PrrA *in vivo* and *in vitro* (Comolli *et al.*, 2002; Comolli & Donohue, 2002; Elsen *et al.*, 2004), so the amino acid side chain at this position is not absolutely required for function in either organism. However, there may be other, unknown features of PrrA homologues that allow the protein to function with either a glycine or asparagine at the first position in the turn before helix 2. For example, side-chain differences at other positions between group 1 and group 2 PrrA homologues may allow proper helix 1–helix 2 positioning in other members of this family. In this regard, the residue analogous to PrrA Y153, which makes side-chain interactions with both L178 and L174 (see below), is a leucine in *P. aeruginosa* RoxR (Fig. 1).

The effects of alanine substitutions in residues that stabilize helix–helix interactions in the PrrA-CTD

The structure of the PrrA-CTD predicts that the L174 and L178 side chains (helix 2) make hydrophobic interactions with side chains of residues T163 and Y153 (helix 1), respectively (Laguri *et al.*, 2003). Both PrrA-L174A and PrrA-L178A failed to activate *cycA* P2 transcription detectably and to protect *cycA* P2 from digestion by DNase I. Because of this, we propose that the loss of a single leucine side chain is sufficient to decrease high-affinity DNA binding at *cycA* P2, presumably by destabilizing helix 1–helix 2 interactions.

We also found that PrrA-I177A only partially activated transcription of both promoters tested, even though this protein protected *cycA* P2 from DNase I digestion at concentrations identical to wild-type PrrA. The I177 side chain is predicted to make hydrophobic contacts with W146, a residue within the three-helix bundle of the PrrA-CTD (Laguri *et al.*, 2003). PrrA I177 is also located near the C terminus of the DNA-binding helix, so the PrrA-I177A substitution could cause a partial activation defect by altering a protein–protein interaction, either within the PrrA-CTD itself, with DNA or by altering some other step in transcription.

The effects of alanine substitutions in the potential DNA-binding residues of the PrrA-CTD

Models of PrrA–target site interactions predict that surface-exposed side chains of residues R171, R172, Q175 and R176 make sequence-specific contacts with DNA (Laguri *et al.*, 2003; Pan *et al.*, 1994, 1996). In the case of Fis, the analogous side chains of residues R85, K89 and K90 are mapped in close proximity to DNA based on copper(I) *ortho*-phenanthroline cleavage (Pan *et al.*, 1994, 1996). PrrA proteins with alanine substitutions in R171, R172, Q175 or R176 exhibited little or no detectable activation of *cycA* P2 or *puc* transcription. In addition, PrrA-R171A could not protect *cycA* P2 from DNase I protection at any concentration tested. Thus, the properties of PrrA-R171A, PrrA-R172A, PrrA-Q175A and

PrrA-R176A support the notion that these residues constitute part of the DNA-recognition helix (Laguri *et al.*, 2003).

It is not known how residues T173 and K180 contribute to PrrA function, but the analogous residues in Fis (T87 and K94) are proposed to interact with the DNA phosphate backbone (Pan *et al.*, 1994, 1996; Yuan *et al.*, 1991). Based on the structural similarity between the PrrA-CTD and Fis, we propose that the inability of PrrA-K180A to activate transcription reflects the loss of a positively charged side chain required to make phosphate backbone contacts. We also propose that the T173 side chain makes polar contacts with DNA, since PrrA-T173A only partially activated transcription and did not protect *cycA* P2 from DNase I digestion at the concentrations tested.

In summary, our findings support the recent proposal for function of the helix–turn–helix motif in the PrrA-CTD in DNA binding (Laguri *et al.*, 2003). However, the behaviour of individual mutant PrrA proteins suggests that features in the turn preceding helix 2 are different within other members of this family or atypical compared with other helix–turn–helix transcription factors. Our data also pose additional questions about the interaction of amino acid side chains within helix 2 of the PrrA-CTD and either target DNA or RNA polymerase. The ability to analyse the structure and function of mutant PrrA proteins makes it possible to obtain molecular insights into how this global regulator of energy-conserving pathways binds DNA and activates transcription. The presence of PrrA homologues in many facultative bacteria (Comolli *et al.*, 2002; Comolli & Donohue, 2002; Elsen *et al.*, 2004) means that this information can provide new insights into how free-living bacteria or those that interact with plant or animal cells control expression of energy-conserving pathways.

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