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

Denise F. Jones, Rachelle A. Stenzel and Timothy J. Donohue

Department of Bacteriology, University of Wisconsin–Madison, Room 390B, 420 Henry Mall, Madison, WI 53706, USA

The *Rhodobacter sphaeroides* response regulator PrrA directly activates transcription of genes necessary for energy conservation at low O$_2$ 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 $^{32}$P-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.

**INTRODUCTION**

Facultative bacteria use a variety of strategies to control expression of pathways needed to conserve energy under low O$_2$ 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 O$_2$ 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 O$_2$ 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 O$_2$ 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

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**Correspondence**

Timothy J. Donohue
tdonohue@bact.wisc.edu
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* DH5α 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 µg 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 GCC 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 (pPC407) and amplification with Pfu polymerase (containing 1% Triton X-100 and DMSO) (Strategene). 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 7.5, 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 µM 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 µl. 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 µl 3× 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 (pRK146) or puc template plasmid (pC412) in a 20 µl 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 µM UTP) plus 10 µCi *[^32P]UTP*. After incubation for 20 min at 30°C, the reaction was terminated by adding 10 µl 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 µM 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 pRK128 with HindIII and NotI followed by labelling the HindIII restriction site with *[^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 µl 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.,...
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 $^{32}$P-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 ($t_{0.5} \sim 330$ min) to monitor this modification by SDS-PAGE (Comolli et al., 2002). Under our assay conditions, most of the mutant PrrA proteins were not shown). However, PrrA-N168G and PrrA-L174A were obtained at ~2-fold lower yields (~20 μM final concentration) relative to wild-type PrrA or the other mutant PrrA proteins.

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 μM final concentration) relative to wild-type PrrA or the other mutant PrrA proteins.

### Table 1. Bacterial strains and plasmids

<table>
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<td><strong>Plasmids</strong></td>
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<td>prrA cloned into Ncol/Smal sites of pTYB4</td>
<td>Comolli et al. (2002)</td>
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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.
Table 2. Properties of mutant PrrA proteins

Phosphorylation is scored as: ++++, ≥50% activity compared with wild-type PrrA; ++, ~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 μM PrrA; +, severely reduced activation at 5 μM PrrA; −, no detectable activation at any concentration tested. Binding to cycA P2 is scored as: +++, binding activity comparable to wild-type PrrA; +, detectable footprint at any concentration; NT, footprinting was not tested with this mutant PrrA protein.

<table>
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<th>Binding to cycA P2</th>
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<td></td>
<td>puc</td>
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<td>+++</td>
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<tr>
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<td>Turn</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<td>Turn</td>
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<td>+</td>
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<td>Turn</td>
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labelled to ≥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 ~2.5 μM 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-L177A (30%) displayed partial activation of _cycA_ P2 even when using 5 μM 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 μM 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 ~2.5 μM 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 (~5%), but detectable, levels of _cycA_ P2 transcript when adding 5 μM 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 μM. 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 μM. 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...
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

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**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.

**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;
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 cya P2 transcription detectably and to protect cya 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 cya 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 cya 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 cya P2 or puc transcription. In addition, PrrA-R171A could not protect cya 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 cya 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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