Dimerization and DNA binding of the *Salmonella enterica* PhoP response regulator are phosphorylation independent

Philippe Perron-Savard,1 Gregory De Crescenzo2 and Hervé Le Moual1,3

Department of Microbiology and Immunology1, Protein–Protein Interaction Facility, Sheldon Biotechnology Centre2 and Faculty of Dentistry3, McGill University, Montréal, Québec, Canada H3A 2B4

In *Salmonella enterica*, PhoP is the response regulator of the PhoP/PhoQ two-component regulatory system that controls the expression of various virulence factors in response to external Mg\(^{2+}\). Previous studies have shown that phosphorylation of a PhoP variant with a C-terminal His tag (PhoP\(_{\text{His}}\)) enhances dimerization and binding to target DNA. Here, the effect of phosphorylation on the oligomerization and DNA binding properties of both wild-type PhoP (PhoP) and PhoP\(_{\text{His}}\) are compared. Gel filtration chromatography showed that PhoP exists as a mixture of monomer and dimer regardless of its phosphorylation state. In contrast, unphosphorylated PhoP\(_{\text{His}}\) was mostly monomeric, whereas PhoP\(_{\text{His}}\)~P existed as a mixture of monomer and dimer. By monitoring the tryptophan fluorescence of the proteins and the fluorescence of the probe 1-anilinonaphthalene-8-sulfonic acid bound to them, it was found that PhoP and PhoP\(_{\text{His}}\) exhibited different spectral properties. The interaction between PhoP or PhoP\(_{\text{His}}\) and the PhoP box of the *mgtA* promoter was monitored by surface plasmon resonance. Binding of PhoP to the PhoP box was barely influenced by phosphorylation. In contrast, phosphorylation of PhoP\(_{\text{His}}\) clearly increased the interaction of PhoP\(_{\text{His}}\) with target DNA. Altogether, these data show that wild-type PhoP dimerization and interaction with target DNA are independent of phosphorylation, which is in contrast to the previously proposed model.

**INTRODUCTION**

Two-component systems are the prevalent signal transduction pathways in bacteria. They mediate the adaptive response to environmental changes by modulating the expression of specific genes. Two-component systems are typically composed of a histidine kinase sensor and a cognate response regulator (Stock *et al.*, 2000, 1989). Upon autophosphorylation of the histidine kinase sensor on a highly conserved histidine residue, the phosphoryl group is transferred to a specific aspartate residue of the response regulator. Most response regulators are transcription factors that consist of a conserved N-terminal receiver domain and a C-terminal DNA-binding domain. Transcriptional regulation of target genes is modulated through phosphorylation of the response regulator (Stock & West, 2003). Structural studies have shown that phosphorylation of the N-terminal receiver domain promotes a long-range conformational change that is transmitted throughout the receiver domain (Stock & West, 2003). The lifetime of phosphorylated response regulators is limited by both an intrinsic autophosphatase activity of the response regulator and a phosphatase activity of the cognate kinase sensor.

Response regulators are classified into the OmpR/PhoB, NarL/FixJ and NtrC/DctD subfamilies based on sequence similarity within the C-terminal effector domain (Stock *et al.*, 1989). In members of the OmpR/PhoB subfamily, the C-terminal domain consists of a winged helix–turn–helix motif (Kenney, 2002). Although all members of this subfamily share a similar three-dimensional structure and are activated through phosphorylation, they appear to vary in their mechanism of activation. Phosphorylation of *Escherichia coli* PhoB has been shown to induce dimerization and, in turn, increase its affinity for DNA (Fiedler & Weiss, 1995; McCleary, 1996). In contrast, phosphorylation of OmpR was found to enhance its DNA-binding affinity without promoting dimerization of the protein in solution, suggesting that dimerization may occur upon DNA
interaction (Aiba et al., 1989; Jo et al., 1986). The PhoP<sub>BSU</sub> response regulator of the *Bacillus subtilis* PhoP/PhoR two-component system (designated PhoP<sub>BSU</sub> to distinguish it from PhoP of the *Salmonella enterica* PhoP/PhoQ system) has been shown to be dimeric and bind target DNA independently of its phosphorylation state (Liu & Hulett, 1997; Pragai et al., 2004).

In *S. enterica*, PhoP is the response regulator of the PhoP/PhoQ two-component system, which responds to environmental Mg<sup>2+</sup> (Garcia-Vescovi et al., 1996). It is another member of the OmpR/PhoB subfamily. It modulates the expression of more than 40 genes involved in adaptation to Mg<sup>2+</sup>-limiting environments, survival within macrophages, LPS modifications and resistance to antimicrobial peptides (Ernst et al., 2001; Groisman, 2001). Although some genes regulated by PhoP do not display a consensus DNA recognition sequence for PhoP, other PhoP-regulated genes, such as the *mgtA* gene, contain a single conserved PhoP box in their promoter region (Lejona et al., 2003). This PhoP box is located approximately 30 bases upstream of the transcription start site and consists of a direct repeat of the consensus DNA recognition sites. PCR products were purified, digested with EcoRI and Xhol, and inserted into plasmid pGEX-4T1 (Amersham Biosciences) digested with the same enzymes, to generate plasmid pGEX-Q<sub>cyto</sub>.

### METHODS

**Plasmid constructions.** *E. coli* strain XL1-Blue (Stratagene) was used for all DNA manipulations. Plasmid pET-P<sub>His</sub>, which encodes the *S. enterica* PhoP protein fused to a C-terminal His tag (PhoP<sub>His</sub>), has been described previously (Montagne et al., 2001). For the construction of plasmid pET-P, which encodes the wild-type PhoP protein, the *phoP* gene was PCR amplified from plasmid pET-P<sub>His</sub> using the *Pfu* DNA polymerase (Invitrogen) with primer PET-5’ (5’-CGAATTATACGACTCAGTATATAGGC-3’), which hybridizes with the T7 promoter region of pET plasmids, and primer PHOP-BAMHI-3’ (5’-GGGATCCCTGGCGCAATTCCTTACAAAGTAGCTCCCTGTC-3’, underlined nucleotides indicate engineered restriction sites). PCR products were purified using a PCR purification kit (Qiagen), digested with *NdeI* and *BamHI*, and inserted into the expression vector pET-3a (Novagen) previously digested with the same enzymes. The inserts of plasmids pET-P and pET-P<sub>His</sub> were sequenced, and the nucleotide sequences were identical to that of the *S. enterica phoP* gene.

The DNA fragment corresponding to the PhoQ cytoplasmic domain (Arg252–Glu487) was PCR amplified from plasmid pET-Q (Montagne et al., 2001) using primers PHOCYTO-5’ (5’-GTCAGAATTTCGCCACCTTTATCAACTGTCGAAAAATGG-3’) and PHOCYTO-3’ (5’-CTGACTCAAGTATTTACCTCTTCTGTGAGCTG-3’). PCR products were purified, digested with EcoRI and Xhol, and inserted into plasmid pGEX-4T1 (Amersham Biosciences) digested with the same enzymes, to generate plasmid pGEX-Q<sub>cyto</sub>.

**Overexpression and purification of recombinant proteins.** For purification of the PhoP protein, *E. coli* BL21(DE3)/pLySE cells transformed with the pET-P plasmid were grown at 22 °C in Luria-Bertani broth supplemented with ampicillin (100 μg ml<sup>-1</sup>) and chloramphenicol (30 μg ml<sup>-1</sup>). At OD<sub>600</sub> 0.8, transcription of the *phoP* gene was induced overnight with 1 mM IPTG. Cells were harvested by centrifugation and resuspended in buffer A (25 mM NaPO<sub>4</sub>, pH 6.0). After lysis by ultrasonic disruption, cell debris was removed by centrifugation at 216 000 × g for 30 min at 4 °C. The supernatant was applied to a 1 ml Resource Q column (Amersham Biosciences) equilibrated with buffer A. After a 15 ml wash with buffer A, proteins were eluted by applying a 0–1 M NaCl gradient in the same buffer, at a flow rate of 1.5 ml min<sup>-1</sup>. Fractions enriched in PhoP were pooled and dialysed overnight against buffer B (25 mM NaPO<sub>4</sub>, pH 7.0). The dialysed proteins were then applied to a 1 ml HiTrap Heparin HP column (Amersham Biosciences) equilibrated with buffer B. After a 15 ml wash with buffer B, proteins were eluted by applying a 0–1 M NaCl gradient in the same buffer. The PhoP<sub>His</sub> protein was overexpressed as described above for PhoP, and purified by Ni<sup>2+</sup>-NTA (nitrilotriacetic acid) chromatography as described previously (Montagne et al., 2001). Both purified PhoP and PhoP<sub>His</sub> were dialysed overnight against buffer C (20 mM Tris/HCl, pH 7.9, 50 mM NaCl). Proteins were kept at 4 °C and used within 4 days.

For purification of the PhoQ cytoplasmic domain fused to the glutathione S-transferase (GST) protein (GST-PhoQ<sub>cyto</sub>), *E. coli* XL1-Blue cells transformed with the pGEX-Q<sub>cyto</sub> plasmid were grown at 37 °C in LB broth supplemented with ampicillin (100 μg ml<sup>-1</sup>). At OD<sub>600</sub> 0.8, cultures were induced with 1 mM IPTG for 4 h. Cells were harvested and lysed as described above. The supernatant was loaded on a 1 ml GSTrap FF column (Amersham Biosciences) equilibrated in buffer D (20 mM Tris/HCl, pH 7.9, 200 mM NaCl). After extensive wash with buffer D, proteins were eluted with the same buffer supplemented with 10 mM glutathione. Fractions containing GST-PhoQ<sub>cyto</sub> were pooled and dialysed overnight against buffer C. Protein concentrations were determined with the BCA protein assay kit (Pierce), using dilutions of BSA as standards.

**Phosphorylation of PhoP and PhoP<sub>His</sub>.** The purified PhoP or PhoP<sub>His</sub> protein was phosphorylated with either the GST-PhoQ<sub>cyto</sub> protein or *E. coli* membranes containing the overexpressed PhoQ-T48I protein. *E. coli* membranes enriched in the PhoQ-T48I protein were prepared as described previously (Sanowar et al., 2003). Reactions were performed in the presence of 1–1.5 mM ATP to maximize the efficiency of phosphorylation. For gel filtration experiments, PhoP or PhoP<sub>His</sub> (10 μM) was phosphorylated with GST-PhoQ<sub>cyto</sub> (2 μM) in a 2 ml volume of phosphorylation buffer (50 mM Tris/HCl, pH 7.5, 200 mM KC1, 0.1 mM EDTA, 5% v/v glycerol) supplemented with 5 mM MgCl<sub>2</sub>. Reactions were initiated by the addition of 1 mM ATP and incubated for 1 h at 22 °C. Unphosphorylated PhoP or PhoP<sub>His</sub> was subjected to the same procedure except that H<sub>2</sub>O was substituted for ATP. For fluorescence spectroscopy and surface plasmon resonance (SPR) experiments, PhoP or PhoP<sub>His</sub> (15 μM) was incubated for 20 min at 22 °C with *E. coli* membranes containing the overexpressed PhoQ-T48I protein (approx. 1–5 μM) in phosphorylation buffer supplemented with 5 mM MgCl<sub>2</sub> and 1-5 mM ATP. Reactions were stopped by the addition of 0.5 M NaPO<sub>4</sub>.
addition of EDTA to a final concentration of 10 mM. PhoP~P or PhoPHis~P was separated from the PhoQ-containing membranes by centrifugation at 216 000 g for 10 min at 4 °C. Unphosphorylated PhoP proteins were subjected to the same procedure except that H2O was substituted for ATP. For SPR experiments, the PhoP proteins were dialysed overnight at 4 °C against SPR buffer (10 mM HEPES, pH 7-4, 150 mM KCl, 10 mM EDTA, 0-005 % v/v Tween 20). The presence of EDTA in the SPR buffer prevented the autodephosphorylation of PhoP~P or PhoPHis~P.

To assess the extent of PhoP phosphorylation, reactions were performed with 1 mM [γ-32P]ATP (10 Ci mmole−1 (37 × 109 Bq mmole−1)) and stopped by the addition of 4 × Laemmli SDS sample buffer (250 mM Tris/HCl, pH 6-8, 8 % SDS, 40 % (v/v) glycerol, 0-02 % bromophenol blue, 4 % β-mercaptoethanol). Reaction products were heated at 37 °C for 5 min and applied to 10 % SDS-PAGE gels. Gels were dried and exposed to a PhosphorImage (Bio-Rad). The band corresponding to PhoP~P or PhoPHis~P was quantified using Quantity One software (Bio-Rad). This value was then compared to a standard curve generated by using known concentrations of [γ-32P]ATP (5, 25, 50, 75 and 100 pM). The efficiency of the phosphorylation reaction was calculated using the ratio of 1 radiolabelled/100 000 non-radiolabelled phosphoryl groups.

**Gel filtration chromatography.** Samples (2 ml) of PhoP, PhoP~P, PhoPHis, or PhoPHis~P (10 μM solution) were individually applied to a Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences) equilibrated with running buffer (25 mM Tris/HCl, pH 7-5, 150 mM NaCl). Proteins were eluted in the same buffer at a flow rate of 0-5 ml min−1 and collected in 1 ml fractions. Protein concentration was monitored by measuring the OD280 and fractions were analysed by SDS-PAGE. Blue dextran 2000 was used to determine the column void volume. A mixture of protein molecular mass standards, containing β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12-4 kDa), was applied to the column under similar conditions. The elution volumes and molecular masses of the protein standards were used to generate a standard curve from which we determined the apparent molecular mass of the various PhoP proteins.

**Fluorescence spectroscopy.** Fluorescence spectra were obtained at 22 °C using a Varian Cary Eclipse fluorescence spectrophometer. Phosphorylated and unphosphorylated proteins treated with 10 mM EDTA were diluted to the desired concentration using a buffer containing 20 mM Tris/HCl, pH 7-9, 50 mM NaCl. Each spectrum was the mean of four consecutive scans. All spectra were corrected by subtracting the blank spectrum corresponding to buffer alone. For intrinsic fluorescence, protein samples (4 μM) were excited at 295 nm with a slit width of 5 nm. Emission spectra were collected from 300 to 450 nm with a slit width of 5 nm. 1-Anilinonaphthalene-8-sulfonic acid (ANS), a fluorescent hydrophobic dye, was purchased from Fluka. For binding experiments, ANS (500 μM) was incubated with the various PhoP proteins (1-6 μM) in the absence or presence of 70 μM urea for 30 min at 22 °C in the dark. Following incubation, spectra were recorded by exciting the samples at 372 nm, and measuring emission over the range of 400 to 600 nm using 10 nm slit widths for both excitation and emission.

**Surface plasmon resonance.** SPR measurements were performed on a Biacore 2000 using a streptavidin sensor chip (Biacore AB). Each surface of the chip was conditioned as recommended by the manufacturer, and equilibrated with SPR buffer (10 mM HEPES, pH 7-4, 150 mM KCl, 10 mM EDTA, 0-005 % v/v Tween 20). The PhoP box of the mgtA promoter was prepared by annealing a 5′-biotinylated oligonucleotide (5′-biotin-GGGTCTCGTTATYGTTGTTTAATTTGGGG-3′; underlined nucleotides indicate the PhoP box) to a non-biotinylated complementary oligonucleotide (5′-CCCCAAATTTAAACACGTTAAGGCTGAC-CCCCTTGGGG-3′). Equimolar amounts of both oligonucleotides were mixed, heated at 95 °C for 5 min and slowly cooled to room temperature. Similarly, a DNA duplex containing a randomized PhoP box was prepared by annealing the oligonucleotide 5′-biotin-GGGTCTCGTTATYGTTGTTTAATTTGGGG-3′ (underlined nucleotides indicate the randomized PhoP box) to a complementary oligonucleotide (5′-CCCCAAATTTAAACACGTTAAGGCTGAC-CCCCTTGGGG-3′). Biotinylated DNA duplexes were diluted to 20 nM in SPR buffer and injected at a flow rate of 5 μl min−1 until approximately 700 resonance units (RU) were immobilized on the chip. Measurements were performed at 22 °C at a flow rate of 25 μl min−1. Phosphorylated and unphosphorylated proteins were diluted in SPR buffer, containing 10 mM EDTA, prior to injection. Following two 75 μl injections of SPR buffer, PhoP, PhoP~P, PhoPHis or PhoPHis~P (0-1, 0-3 and 1 μM) was injected over the sensor chip surfaces on which the DNA duplexes had been immobilized. Following an injection of 180 s, the kinetics of dissociation were monitored by injecting SPR buffer for 300 s. The sensor surfaces were regenerated by performing two 50 μl injections of 3 M NaCl (100 μl min−1) followed by an EXTRANCLEAN procedure as recommended by the manufacturer. All measurements were performed within 24 h following phosphorylation. Specific binding was calculated by subtracting the sensorgrams obtained with the randomized PhoP box from the sensorgrams obtained with the mgtA PhoP box.

**RESULTS**

**Overexpression, purification and phosphorylation of PhoP and PhoP**

PhoP protein was overexpressed in *E. coli* as both the wild-type protein (PhoP) and a C-terminally His-tagged protein (PhoPHis). PhoP was purified to homogeneity by a two-step procedure involving anion exchange and heparin affinity chromatography, whereas PhoPHis was purified by Ni2+-NTA affinity chromatography. SDS-PAGE analysis showed that PhoP and PhoPHis migrated with apparent molecular masses of approximately 28 and 29 kDa, respectively, which are consistent with the calculated molecular masses of PhoP (25 634 Da) and PhoPHis (26 700 Da) (Fig. 1a). Both PhoP and PhoPHis were phosphorylated to similar extents by the PhoQ protein overproduced in *E. coli* membranes, indicating that both proteins efficiently catalyse the phosphate transfer from PhoQ to PhoP (Fig. 1b).

Purified PhoP and PhoPHis were phosphorylated in the presence of ATP and MgCl2 by using either the PhoQ cytoplasmic domain fused to the GST protein (GST-PhoQcyto) or *E. coli* membranes enriched with the PhoQ-T48I mutant, both of which possess decreased phosphatase activity (Sanower *et al.*, 2003). PhoP and PhoPHis were phosphorylated to similar extents by GST-PhoQcyto, and PhoQ-T48I (data not shown). Under our experimental conditions, approximately 90 % of both PhoP and PhoPHis were phosphorylated (see Methods), which is consistent with previous studies (Lejona *et al.*, 2004).

Most response regulators possess an intrinsic autophosphatase activity that is Mg2+-dependent. The half-life of PhoPHis~P has been reported to be approximately 60 min.
To determine the oligomeric state of PhoP, PhoP<sup>~P</sup>, PhoP<sub>His</sub><sup>~P</sup> and PhoP<sub>His</sub><sup>~P</sup>, these proteins were subjected to gel filtration chromatography on a Superdex 200 column that was calibrated with molecular mass standards. PhoP and PhoP<sup>~P</sup> phosphorylated by GST-PhoQ<sub>cyto</sub> were individually applied to the gel filtration column. PhoP and PhoP<sup>~P</sup> eluted as two peaks (Fig. 2a, b). The earlier- (60 ml) and later-eluted (71 ml) peaks corresponded to masses of 55 and 24 kDa, respectively. These values are consistent with PhoP being in the monomeric (24 kDa) and dimeric (55 kDa) states, and suggest that both PhoP<sup>~P</sup> and PhoP<sup>~P</sup> are in dynamic equilibrium between these two oligomeric states. Strikingly, when PhoP<sub>His</sub><sup>~P</sup> was applied to the column, the elution profile differed greatly. Unphosphorylated PhoP<sub>His</sub> was eluted essentially as a single peak at 67 ml (30 kDa), indicative of a monomer (Fig. 2c). This peak presented a shoulder, towards the higher molecular masses at 57 ml (69 kDa), which may correspond to small amounts of dimer (Fig. 2c). In contrast, phosphorylated PhoP<sub>His</sub><sup>~P</sup> was eluted as two peaks at 67 ml (30 kDa) and 57 ml (69 kDa), indicating that PhoP<sub>His</sub><sup>~P</sup> exists as a mixture of monomer and dimer in solution (Fig. 2d). Overall, these data show that unphosphorylated PhoP and PhoP<sub>His</sub> have different oligomeric states in solution, suggesting that the C-terminal His tag affects the stability of the dimer. They also indicate that phosphorylation has no major effect on dimerization of wild-type PhoP.

**Tryptophan fluorescence and ANS binding**

To further characterize the different behaviours of PhoP and PhoP<sub>His</sub> in solution, we measured the fluorescence of the intrinsic tryptophan residues, which is related to their microenvironment (Lakowicz, 1999). Both PhoP and PhoP<sub>His</sub> contain two tryptophan residues, which are located in the receiver domain (Trp 67 and Trp 85). The fluorescence spectrum obtained for PhoP<sub>His</sub> exhibited a blue-shifted (~8 nm) emission maximum and a 1.7-fold increase in intensity at 346 nm, compared to that of PhoP (Fig. 3). Phosphorylation of PhoP and PhoP<sub>His</sub> resulted in 1.2- and 2-fold decreases in fluorescence intensity at 346 nm, respectively (Fig. 3). These data indicate that the environment of the tryptophan residues is different for PhoP<sub>His</sub> and PhoP. They also indicate that phosphorylation of PhoP<sub>His</sub> results in an important change in the microenvironment of the tryptophan residues. The difference in fluorescence intensity between PhoP<sub>His</sub>, which is essentially monomeric (Fig. 2c), and PhoP, PhoP<sup>~P</sup> and PhoP<sub>His</sub><sup>~P</sup>, which exist as mixtures of monomers and dimers (Fig. 2a, b, d), might be attributed to quenching effects induced by dimerization.

The fluorescent hydrophobic dye ANS binds to solvent-exposed hydrophobic surfaces of proteins (Stryer, 1965). This leads to both a blue-shifted emission spectrum of ANS and increased fluorescence intensity. In the presence of PhoP, the ANS emission spectrum showed a 19 nm blue shift and a 2.3-fold increase in intensity (Fig. 4). In the presence of PhoP<sub>His</sub>, the emission maximum of ANS showed a blue shift of 33 nm and a 4.5-fold increase in fluorescence intensity (Fig. 4). The more drastic changes in
ANS fluorescence induced by PhoP_His, compared to PhoP, indicate that PhoP_His contains more exposed hydrophobic surfaces than PhoP. Phosphorylation of PhoP_His affected the ANS fluorescence spectrum by inducing a red shift of 6 nm and a 1.4-fold decrease in fluorescence intensity (Fig. 4). In contrast, phosphorylation of PhoP did not affect the ANS fluorescence spectrum (Fig. 4). These data indicate that phosphorylation lowers the number of ANS binding sites on PhoP_His suggesting a phosphorylation-induced change of conformation and/or oligomerization state for PhoP_His. As a control, we examined the emission spectra of PhoP and PhoP_His unfolded with 7.0 M urea. The presence of urea

**Fig. 2.** Effect of phosphorylation on the oligomeric state of PhoP and PhoP_His. Gel filtration chromatograms obtained for PhoP (a), PhoP~P (b), PhoP_His (c) and PhoP_His~P (d). PhoP and PhoP_His were phosphorylated as described in Methods. The column was calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) molecular mass standards.

**Fig. 3.** Intrinsic fluorescence emission spectra of the various PhoP proteins. Protein samples (4 μM) were excited at 295 nm and emission spectra were collected from 310 to 450 nm. ●, PhoP; ○, PhoP~P; ■, PhoP_His; □, PhoP_His~P.

**Fig. 4.** Fluorescence emission spectra of ANS incubated with the various PhoP proteins. Samples were excited at 392 nm and emission spectra were recorded from 400 to 600 nm. The emission spectra shown are those of ANS alone (△), ANS incubated with PhoP (●), ANS incubated with PhoP~P (○), ANS incubated with PhoP_His (■) and ANS incubated with PhoP_His~P (□).
resulted in emission spectra similar to that of ANS (data not shown), indicating a complete loss of the ANS binding sites upon unfolding. Altogether, these data strongly suggest that the C-terminal His tag affects the conformation and/or oligomerization state of PhoP, which could explain the unexpected behaviour of unphosphorylated PhoP\textsubscript{His}.

**DNA binding of PhoP proteins by SPR**

A BIACore biosensor was used to examine, in real-time, the binding of the various PhoP proteins to the promoter of mgtA (a PhoP-activated gene that contains a PhoP box in its promoter). Biotinylated oligonucleotide duplexes containing the PhoP box of the mgtA promoter were immobilized on a streptavidin sensor chip, and PhoP, PhoP\sim P, PhoP\textsubscript{His} or PhoP\textsubscript{His}\sim P, at concentrations ranging from 0.1 to 1 μM, was injected over the DNA surface. As a control, we measured the binding of the various PhoP proteins to DNA duplexes containing a randomized PhoP box. Injection of PhoP (0.3 μM) over the DNA surface containing the PhoP box resulted in a net mass accumulation (Fig. 5i) that is 4.5-fold higher than the one detected using a DNA surface containing the randomized PhoP box (Fig. 5ii). A similar pattern was observed for PhoP\sim P (data not shown). This indicates that the PhoP box is required for specific binding of PhoP.

Control-corrected sensorgrams that represent the specific binding of the various PhoP proteins to the PhoP box were obtained by subtracting the control sensorgrams from the matching experimental sensorgrams (Fig. 6). For both PhoP and PhoP\sim P, control-corrected sensorgrams are characterized by a net mass accumulation during the association phase, and similar association rates (Fig. 6a, b). As deduced from the shape of the dissociation phase, complexes between DNA and PhoP or PhoP\sim P are very stable over time (Fig. 6a, b). Thus, phosphorylation of PhoP does not appear to affect the kinetics of association and dissociation of the complex between PhoP and DNA. To assess the effect of phosphorylation on the interaction of PhoP with the PhoP box, we compared the RU values at the end of the association phase. For PhoP at a concentration of 1 μM, phosphorylation decreased specific binding by approximately 150 RU, which corresponds to a 1.3-fold decrease. These results indicate that the interaction between PhoP and the PhoP box of the mgtA promoter is barely affected by phosphorylation.

When the PhoP\textsubscript{His} or PhoP\textsubscript{His}\sim P protein was injected over the DNA surfaces, a greater net mass accumulation was observed for PhoP\textsubscript{His}\sim P than for PhoP\textsubscript{His} (Fig. 6c, d). Phosphorylation of PhoP\textsubscript{His} induced a twofold increase of the binding of PhoP\textsubscript{His} to the PhoP box, as deduced from the RU values at the end of the association phase (Fig. 6c, d). In contrast to what was observed for PhoP, phosphorylation of PhoP\textsubscript{His} significantly increased DNA binding. These data indicate that the C-terminal His tag somehow interferes with the interaction of unphosphorylated PhoP\textsubscript{His} with the PhoP box. They also indicate that PhoP\textsubscript{His} regains its ability to interact with the PhoP box upon phosphorylation.

**DISCUSSION**

In this study, we examined the effect of phosphorylation on the oligomerization state and DNA-binding ability of the *S. enterica* PhoP response regulator. We used the mgtA promoter, which contains a single PhoP box, to study the interaction between PhoP and DNA. We found that phosphorylation of wild-type PhoP barely affects its oligomeric state and its ability to interact with the PhoP box of the mgtA promoter. This indicates that the mechanism of phosphorylation-mediated activation of PhoP is different from that of many other response regulators, including *E. coli* OmpR and PhoB. In addition, we showed that the presence of a His tag at the C-terminus of PhoP greatly affects the biochemical properties of the protein.

Our data clearly showed that both wild-type PhoP and PhoP\sim P exist as a mixture of monomer and dimer in solution (Fig. 2a, b). In contrast to what was observed for the ArcA response regulator (Jeon *et al.*, 2001), higher-order oligomers were not detected under our experimental conditions. In addition, phosphorylation of PhoP did not appear to significantly increase the proportion of dimers (Fig. 2a, b). These data indicate that the oligomeric state of PhoP is phosphorylation independent. They also suggest that PhoP is involved in a monomer–dimer equilibrium that is concentration dependent rather than phosphorylation dependent. We determined the oligomeric state of PhoP and

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**Fig. 5.** Specificity of PhoP binding to the PhoP box of the mgtA promoter. PhoP diluted to 0.3 μM in SPR buffer was injected for 180 s (association phase). This was followed by injection of SPR buffer alone (dissociation phase). The graph shows the binding of PhoP (i) to biotinylated oligonucleotide duplexes containing the PhoP box of the mgtA promoter, and (ii) to biotinylated oligonucleotide duplexes containing a randomized PhoP box.
PhoP\(^{\sim}\)P at a protein concentration of 10 \(\mu\)M (Fig. 2a, b). It has been estimated that the cellular concentration of PhoP ranges between 2 and 11 \(\mu\)M, depending on the presence of Mg\(^{2+}\) in the growth medium (Lejona et al., 2004). A cellular concentration in the same range (6 \(\mu\)M) was estimated for the OmpR response regulator (Cai & Inouye, 2002). At such concentrations, it is most likely that PhoP dimers will form, \(\text{in vivo}\), regardless of the PhoP phosphorylation state. Our results regarding the oligomeric state of PhoP are in contrast to those reported for many response regulators. For example, phosphorylation is required for dimerization of \(E.\ coli\) PhoB (Fiedler & Weiss, 1995; McCleary, 1996), whereas PhoPBSU has been reported to be dimeric independently of its phosphorylation state (Liu & Hulett, 1997).

Our SPR experiments (Fig. 6) clearly showed that both PhoP and PhoP\(^{\sim}\)P bind to the PhoP box of the \(mgtA\) promoter. This is in contrast to those reported for many response regulators. For example, phosphorylation is required for dimerization of \(E.\ coli\) PhoB (Fiedler & Weiss, 1995; McCleary, 1996), whereas PhoPBSU has been reported to be dimeric independently of its phosphorylation state (Liu & Hulett, 1997).

Effect of a C-terminal His tag on PhoP

Recently, it has been shown that the unphosphorylated receiver domain of PhoB inhibits the activity of the DNA-binding domain, and that this inhibition is relieved upon phosphorylation (Ellison & McCleary, 2000). Our data suggest that such a mechanism is most unlikely for PhoP. Overall, this study clearly showed that the \(S.\ enterica\) PhoP protein dimerizes and binds to the \(mgtA\) promoter regardless of its phosphorylation state. Thus, structural rearrangements that occur upon phosphorylation do not significantly affect the oligomeric state and the ability of PhoP to interact with DNA. Although \(S.\ enterica\) PhoP belongs to the same subfamily of response regulators as OmpR and PhoB, their mechanisms of activation appear to be different.

\(S.\ enterica\) or \(E.\ coli\) PhoP response regulators with a C-terminal His tag have been used in previous studies (Lejona et al., 2003, 2004; Minagawa et al., 2003; Yamamoto et al., 2002). Hypothesizing that a His tag at the C-terminus of the DNA-binding domain may interfere with the ability of PhoP to interact with DNA, we systematically compared the \(\text{in vitro}\) biochemical properties of PhoP, PhoP\(^{\sim}\)P, PhoPHis and PhoPHis\(^{\sim}\)P. Strikingly, we found that the His tag specifically affects the properties of unphosphorylated PhoPHis. Both stability of the dimer (Fig. 2c) and binding to target DNA (Fig. 6c) were impaired for unphosphorylated PhoPHis. These results suggest that the C-terminal His tag may affect the function of PhoPHis. This possibility is strongly supported by our fluorescence experiments. Both the intrinsic tryptophan fluorescence measurements (Fig. 3) and the ANS binding experiments (Fig. 4) clearly indicated that unphosphorylated PhoP and PhoPHis have different conformations, and/or oligomerization states. Recently, the crystal structure of the full-length \(T.\ maritima\) DrrD response regulator has been solved (Buckler et al., 2002). This is the first three-dimensional structure of a full-length member of the OmpR/PhoB subfamily. Examination of the DrrD structure suggests that extension of the protein at its C-terminus may introduce a steric hindrance with the
four-stranded β-sheet located at the N-terminus of the DNA-binding domain. This β-sheet appears to be involved in interdomain interactions (Buckler et al., 2002). It is possible that steric hindrance induced by the His tag reduces PhoPHis flexibility and/or affects interdomain communication. In turn, this would result in a locked conformation less permissive for PhoPHis dimerization. Interestingly, our results showed that PhoPHis forms stable dimers and interacts with target DNA upon phosphorylation (Fig. 2d, Fig. 6d). In addition, the tryptophan fluorescence spectrum of PhoPHis ~ P is very similar to that of PhoP ~ P (Fig. 3). This suggests that a phosphorylation-induced conformational change released the steric hindrance imposed by the C-terminal His tag. These results are in good agreement with the proposal that the phosphorylation-induced conformational change is propagated up to the C-terminus of response regulators and promotes a repositioning of the N- and C-terminal domains (Stock & West, 2003).

Using gel mobility-shift assays and DNase I footprinting, a previous study showed that phosphorylation of PhoPHis enhanced its binding to the promoters that contain a consensus PhoP box (Lejona et al., 2003). Another study used chemical cross-linking to show that self-association of PhoPHis is increased by phosphorylation (Lejona et al., 2004). Our data obtained with PhoPHis strongly support these findings. However, our data obtained with PhoP indicate that these conclusions may not be relevant for wild-type PhoP. Thus, we propose that unphosphorylated PhoP binds to the PhoP box of target genes, most likely as a dimer. As in the case of OmpR (Ames et al., 1999), the interaction of PhoP with DNA might stimulate PhoP phosphorylation by its cognate sensor kinase. Since there are only single, if any, PhoP boxes in the promoters of known PhoP-activated genes (Lejona et al., 2003), phosphorylation of PhoP is unlikely to favour cooperative binding between PhoP dimers at the PhoP-activated promoters, as previously observed for OmpR (Harlocker et al., 1995). With very few exceptions, response regulators are active in their phosphorylated forms. Thus, it is possible that the role of PhoP phosphorylation is to favour protein–protein interactions with the transcriptional machinery. This possibility is supported by previous studies showing that PhoB interacts with σ70 (Kumar et al., 1994; Makino et al., 1993), and that OmpR interacts with the C-terminal domain of the RNA polymerase ζ subunit (Slauch et al., 1991).

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