PhoP-PhoP Interaction at Adjacent PhoP Binding Sites Is Influenced by Protein Phosphorylation^{∇‡}

Akesh Sinha,[†] Sankalp Gupta,[†] Shweta Bhutani, Anuj Pathak, and Dibyendu Sarkar*

Institute of Microbial Technology, Sector 39 A, Chandigarh 160036, India

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***Mycobacterium tuberculosis* PhoP regulates the expression of unknown virulence determinants and the biosynthesis of complex lipids. PhoP, like other members of the OmpR family, comprises a phosphorylation domain at the amino-terminal half and a DNA-binding domain at the carboxy-terminal half of the protein. To explore structural effect of protein phosphorylation and to examine effect of phosphorylation on DNA binding, purified PhoP was phosphorylated by acetyl phosphate in a reaction that was dependent on Mg²⁺ and Asp-71. Protein phosphorylation was not required for DNA binding; however, phosphorylation enhanced in vitro DNA binding through protein-protein interaction(s). Evidence is presented here that the protein-protein interface is different in the unphosphorylated and phosphorylated forms of PhoP and that specific DNA binding plays a critical role in changing the nature of the protein-protein interface. We show that phosphorylation switches the transactivation domain to a different conformation, which specifies additional protein-protein contacts between PhoP protomers bound to adjacent cognate sites. Together, our observations raise the possibility that PhoP, in the unphosphorylated and phosphorylated forms, may be capable of adopting different orientations as it binds to a vast array of genes to activate or repress transcription.**

Mycobacterium tuberculosis is a successful intracellular pathogen that encounters a range of environments throughout its life in the human host. Therefore, to adapt and reside within human macrophage phagosome, gene regulation in the bacilli must be tightly controlled. Gene expression with regard to adaptive responses in *M. tuberculosis* likely involves coordinated control by two-component signal transduction systems (4), which in their simplest form utilize a histidine-aspartate phosphorelay between two modular proteins: a sensor kinase and a response regulator (27). Together, these protein pairs sense environmental stimuli and initiate complex transcriptional programs in the bacterium. Although the functions of many of these signaling systems are unknown, recent studies have established a role for *M. tuberculosis* PhoP as a modulator of genes essential for virulence and complex lipid biosynthesis (5, 29).

Previously, it was shown that disruption of *phoP* from *M. tuberculosis* *phoPR* causes a drastic attenuation in virulence of the tubercle bacilli with significantly altered colony morphology and cording properties, suggesting its involvement in the expression of important but unknown virulence factors (22). Furthermore, *phoP* has been shown to exhibit a differential expression pattern during intracellular growth of the bacteria in human macrophages (7, 32). PhoP is a bifunctional response regulator protein containing an N-terminal phosphorylation domain (NTD; also called a receiver domain) and a C-terminal transactivation domain (CTD; also called an effector domain).

Members of this family share a conserved doubly wound α/β fold with a phosphorylation site in their N terminus but are grouped into subfamilies based on the C-terminal domain structure. PhoP belongs to an *Escherichia coli* OmpR subfamily of proteins with a winged-helix-turn-helix DNA binding motif (16; for a review, see reference 12). A BLAST search of *M. tuberculosis* PhoP within a protein data bank shows a highest sequence identity of 45% with *M. tuberculosis* PrrA (PDB ID code 1YS6) and a second highest identity of 33% with OmpR/PhoB homologue (PDB ID code 1KGS) from *Thermotoga maritima*. Although all family members share significant structural homology in their DNA-binding motif (17, 20) and appear to be activated through phosphorylation of receiver domain, members of the subfamily use different mechanisms to regulate their DNA-binding domains and modulate transcription. While phosphorylation of *E. coli* PhoB induces dimerization and, in turn, increases its affinity for DNA sites through relief of inhibition of DNA binding by the amino-terminal domain (1), phosphorylation of OmpR was found to enhance its DNA-binding affinity without promoting dimerization of the protein in solution (10). Furthermore, substitution of Asp-55 of OmpR, the site of phosphorylation, renders OmpR unable to activate transcription, reflecting that phosphorylation at the amino terminus is essential for transcription control (30). Consistently, the isolated C terminus of OmpR binds to DNA only weakly (12) and is unable to activate transcription (30). In contrast, the isolated C terminus of PhoB is constitutively active for transcription (15), suggesting that phosphorylation is not required for transcription regulation. Again, PhoP from *Bacillus subtilis* PhoP-PhoR two-component system has been shown to be dimeric and bind cognate DNA independent of phosphorylation (24). More recently, both dimerization and DNA binding by *Salmonella enterica* PhoP response regulator has been shown to be phosphorylation independent (23).

Although we have previously determined that phosphoryla-

* Corresponding author. Mailing address: Institute of Microbial Technology, Sector 39A, Chandigarh 160036, India. Phone: 091 172 269 5215, ext. 3223. Fax: 091 172 269 0585. E-mail: dibyendu@imtech.res.in.

[†] A.S. and S.G. contributed equally to this study.

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tion of PhoP is not essential for autoregulation of *phoP* (6), the role of the receiver domain and/or its phosphorylation in controlling activity of the regulator remains unknown. We demonstrate here that phosphorylation at the NTD promotes conformational change in the PhoP CTD. Such conformational change in the CTD is likely to be involved in communication between regulator protomers for the formation of a stable protein-DNA complex. We also show for the first time that phosphorylated protein only in the presence of adjacent cognate sites participates in specific protein-protein contacts (that form upon self-association) using a protein surface that appears to be sufficiently different from the unphosphorylated form of the protein.

MATERIALS AND METHODS

Cloning, purification, and mutagenesis of PhoP. *E. coli* DH5 α was used for all cloning procedures. Wild-type and mutant PhoP proteins from *M. tuberculosis* H37Ra were expressed in *E. coli* BL21(DE3) as fusion proteins containing an N-terminal polyhistidine tag (His-Tag; Novagen) as described previously (6). To purify PhoP lacking a His tag (PhoP^{-His}), approximately 1 mg of purified PhoP was cleaved with ~10 μ g of thrombin (Roche) at 25°C for 1 h. The reaction was quenched by adding 1 mM phenylmethylsulfonyl fluoride. Finally, *M. tuberculosis* PhoP^{-His} was extensively dialyzed against 50 mM HEPES (pH 7.5) containing 100 mM NaCl. Purity of the protein preparation was $\geq 95\%$, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent staining with Coomassie blue. Mutations in the *phoP* gene were introduced by the two-stage overlap extension method (9). To construct plasmids encoding single-tryptophan-containing PhoPs, oligonucleotides FPphoPW166Y and RPphoPW166Y for pSG25 and oligonucleotides FPphoPW203Y and RPphoPW203Y for pSG26, respectively, were used with the *phoP* gene from pET15b-*phoP* as templates (6) (Table 1). All enzymatic manipulations of DNA were performed by using standard procedures with reagents purchased from New England Biolabs (restriction endonucleases, T4 DNA ligase, alkaline phosphatase, and *Vent* DNA polymerase). Oligonucleotides were synthesized by Integrated DNA Technologies, USA. For *E. coli* cells, LB media were supplemented with 0.1 mg of ampicillin/ml, when appropriate. Plasmid DNA isolation was performed by using Qiagen spin columns and Qiagen procedures. Sequence analysis with an automated DNA sequencer (Applied Biosystems) with chain termination chemistry revealed that the constructions were error-free.

The protein concentration was determined from the absorbance at 280 nm by using extinction coefficients, calculated from the sequence, of 21,430 M⁻¹ cm⁻¹ for the wild type and 15,930 M⁻¹ cm⁻¹ for single-Trp-containing PhoPs (21). All protein concentrations are given in equivalents of protein monomers.

Phosphorylation of PhoP. PhoP phosphorylation by acetyl phosphate (AcP; $\geq 85\%$ purity; Sigma) was investigated by using two-dimensional (2D) gel electrophoresis. Briefly, purified PhoP was added to phosphorylation buffer (50 mM HEPES [pH 7.5], 100 mM NaCl) supplemented with 50 mM AcP and 10 mM MgCl₂, and the mixtures were incubated at 37°C for 1 h. Reactions were stopped by addition of 3 volumes of 100% ice-cold acetone, and mixtures were incubated at -20°C overnight. Precipitated protein was collected by centrifugation, washed with 1 volume of 100% ice-cold acetone, and allowed to air dry. The protein pellet was resuspended in 120 μ l of rehydration solution (7 M urea, 2 M thiourea, 2% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.002% of bromophenol blue) containing 3 mg of dithiothreitol/ml and 0.5% immobilized pH gradient (IPG) buffer (Pharmacia). Isoelectric focusing resolved proteins according to their pIs in the first dimension by using an 11-cm IPG strip (pH 4.0 to 7.0; Pharmacia) and a Protean isoelectric focusing apparatus (Bio-Rad) according to the manufacturer's recommendations. Protein samples were allowed to undergo passive rehydration for 12 h at 20°C before being focused at 500 V for 250 V · h, 1,000 V for 500 V · h, and 8,000 V for 5,000 V · h at 20°C. Proteins were resolved according to molecular mass in the second dimension by SDS-PAGE using 15% polyacrylamide gels. Focused IPG strips were incubated for 15 min at room temperature in SDS equilibration buffer (75 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue containing 10 mg of dithiothreitol/ml) and subsequently for 15 min at room temperature in SDS equilibration buffer containing 25 mg of iodoacetamide/ml. Equilibrated strips were loaded onto polyacrylamide gels and resolved by SDS-PAGE. The proteins were visualized by silver staining. Densitometric

TABLE 1. Primers, oligonucleotides, and plasmids used in this study

Primer, oligonucleotide, or plasmid	Sequence (5'–3') or description	Source or reference
Primers^a		
phoPstart	GTTTGCCATATGCGGAAAGGGGT	6
phoPstop	TGAT GTGGTGGATCCTCGAGGCTCCCG CAGTAC	6
mphoPstart	GTTTGCGGATCCATGCGGAAAGG GGTTGAT	6
mphoPstop	GGTGGTAAGCTTTCATCGAGGCTC CCGAG	6
FPphoPD71N	GTGATCCTCAACGTGATGATGCC	6
RPphoPD71N	CATCATCACGTTGAGGATCACCGC	6
FPphoPW166Y	GTGATCCTCAACGTGATGATGCC	This study
RPphoPW166Y	CATCATCACGTTGAGGATCACCGC	This study
FPphoPW203Y	GTGATCCTCAACGTGATGATGCC	This study
RPphoPW203Y	CATCATCACGTTGAGGATCACCGC	This study
Oligonucleotides^b		
DR1,2	TGGCAGACTGTTAGCAGACTACT GGCAACGAGCTTT	This study
sDR1-DR2	TGGCAGAAAATTAGCAGACTACT GGCAACGAGCTTT	This study
DR1-sDR2	TGGCAGACTGTTAGCAGACTAAA AGCAACGAGCTTT	This study
NSP	GGTTGGCGCGGGCAATCGTGTC TCGATTCCAGCA	This study
Plasmids		
pET15b	<i>E. coli</i> cloning vector; Amp ^{rc}	Novagen
pSG15	His ₆ -tagged <i>phoP</i> expression plasmid	6
pSG22	D71 codon mutated to N in <i>phoP</i> of pSG15	6
pSG25	W166 codon mutated to Y in <i>phoP</i> of pSG15	This study
pSG26	W203 codon mutated to Y in <i>phoP</i> of pSG15	This study
pAP26	D71 codon mutated to N in <i>phoP</i> of pSG26	This study

^a FP, forward primer; RP, reverse primer.

^b That is, oligonucleotides used in the gel shift experiments represent natural sequences from *phoP* regulatory region. The sequence of the top strand is given, and underlined regions have been identified as PhoP binding sites (6).

^c Amp^r, ampicillin resistance.

scanning of multiple replicates of gels reveals that treatment with AcP under these conditions results in the phosphorylation of 23% $\pm$ 1.2% of the PhoP in the reaction.

Oligodeoxyribonucleotide substrates. The 36-bp DNA fragment used to assess the sequence-specific DNA binding of *M. tuberculosis* PhoP is 5'-ACTGTTAG CagactACTGGCAAC-3' and its complement. This sequence, from position -69 to position -47 of the *M. tuberculosis phoP* promoter region (in relation to the translational start site), is within the PhoP DNase I footprint (6) and is hereafter referred to as the specific DR1,2 DNA. For radiolabeling, the strand shown was 5' end labeled using [γ -³²P]ATP (BRIT, Hyderabad, India) and T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ -³²P]ATP and labeled oligonucleotide were separated by using a Sephadex G-50 quick spin column (GE Healthcare). The labeled strand was annealed to its unlabeled complement by slow cooling after heating it to 90°C.

Electrophoretic mobility shift assays (EMSAs). Binding reactions (10 μ l) contained DNA (20 nM) and PhoP in 20 mM HEPES-Na⁺ (pH 7.5), 50 mM NaCl, 200 μ g of bovine serum albumin/ml, 10% glycerol, 1 mM dithiothreitol, and 200 ng of sheared herring sperm DNA. Incubation was for 10 min at 20°C. Reactions were then put on ice and analyzed immediately by 6% nondenaturing PAGE using 1 $\times$ Tris-EDTA as the running buffer at 4°C. DNA fragments and complexes were visualized by autoradiography of the dried gel. To quantify the sample, the dried gel was placed in contact with an imaging plate, and bands were analyzed in a phosphorimager (Bio-Rad) using QuantityOne software.

DNase I footprinting. Footprinting was carried out essentially as described elsewhere (31). Briefly, a DNA-binding reaction was carried out as described in EMSA experiments by mixing PhoP or P-PhoP with 1 μ l of labeled DNA (~3 pmol/ μ l). Partial DNase I digestion was carried out at 37°C by adding 1.0 U of

DNase I (Amersham Bioscience) into the reaction mixture (final reaction volume, 50 μ l). After 1 min of incubation, the reaction was stopped with 50 μ l of stop solution (3 M ammonium acetate, 0.25 M EDTA, and 15 ng of yeast tRNA). Samples were chilled at -80°C for 1 h and centrifuged for 20 min, and the pellet was rinsed with 150 μ l of 95% chilled ethanol. Purified DNA samples were dried and resuspended in 2.5 μ l of loading buffer (1% bromophenol blue, 1% xylene cyanol, and 10 mM EDTA in 98% formamide), heated to 95°C , and chilled on ice. Reactions were fractionated on a 12% acrylamide–8 M urea (wt/vol) gel alongside a Maxam–Gilbert G sequencing ladder of the same DNA fragment. To quantify extent of protection, bands were analyzed in a phosphorimager (Molecular Imager; Bio-Rad).

Gel filtration. Size-exclusion chromatography was performed at 4°C on a Sephacryl 200 HR (1 by 30 cm; GE Healthcare Biosciences) using an AKTA system. Samples of recombinant PhoP (0.5 mg) purified by Ni^{2+} -chelate affinity chromatography or PhoP phosphorylated by AcP were eluted at a flow rate 0.4 ml/min in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The column was extensively equilibrated with the same buffer prior to each run. Elution profiles were monitored by recording the absorbance at 280 and 220 nm. Elution of β -galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and lysozyme (14 kDa) (all from Sigma) was used to obtain the calibration curve.

CD spectroscopy. Circular dichroism (CD) measurements were carried out with a Jasco spectropolarimeter (J-810; Jasco, Tokyo, Japan). All measurements were performed using a 0.1-cm cell. Residue molar ellipticity $[\theta]$ was defined as $100 \theta_{\text{obs}} (lc)^{-1}$, where θ_{obs} was observed ellipticity, l was length of the light path in centimeters, and c was the residue molar concentration of each protein. The scan speed was 100 nm/min, and 10 scans were signal averaged to increase the signal-to-noise ratio. The buffer used was 10 mM Tris-HCl (pH 7.50)–100 mM NaCl.

Trypsin cleavage of PhoP proteins. Reaction mixtures (20 μ l) containing 4 μ g of wild-type or mutant PhoP proteins (preincubated in phosphorylation buffer with or without AcP) were incubated in the digestion mixture containing 50 mM Tris-HCl (pH 7.90), 100 mM NaCl, 3 mM magnesium acetate, and 400 ng of trypsin at 37°C for the indicated times, keeping the ratio of PhoP to trypsin at 100:1 (wt/wt). Proteolysis was terminated by the addition of SDS, and aliquots from the digestion mixtures were resolved by SDS–12% PAGE and visualized by Coomassie blue staining. To determine the N-terminal sequence of the tryptic fragments, resolved polypeptides from a SDS-polyacrylamide gel were transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore) and visualized by Amido Black (Sigma) staining. Membrane slices containing individual proteolytic products were excised and subjected to automated Edman sequencing using an Applied Biosystems protein sequencer.

Protein cross-linking. Purified PhoP, pretreated in the absence or presence of AcP, was incubated with 1 mM disuccinimidyl suberate (DSS; Sigma) in 50 mM HEPES- Na^{+} (pH 7.5), 150 mM NaCl, 10 mM MgCl_2 , 20% glycerol, and 0.05% Tween 20 at 25°C for 30 min as described elsewhere (14). The reaction was terminated by the addition of SDS sample buffer, and the samples were analyzed by SDS-PAGE (12% polyacrylamide gels), followed by immunostaining using rabbit polyclonal anti-PhoP antibody.

Western blot analysis. Polyclonal antibody to PhoP was raised in rabbit according to a standard procedure and used for the detection of PhoP or mutant protein. The samples were resolved by SDS–12% PAGE and electrotransferred to nitrocellulose membranes for Western blot according to standard procedures (Bio-Rad). The blots were probed with primary (anti-PhoP) and secondary (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G) antibodies and developed with chemiluminescence reagent (ECL Western blotting substrate; Amersham Biosciences).

Fluorescence measurements. All fluorescence spectra were recorded in a Perkin-Elmer LS50B fluorescence spectrophotometer at 25°C using a quartz cell with a 0.3-cm path length. The excitation wavelength was set to 295 nm, and the emission spectra were recorded at between 310 and 410 nm. Bandwidths for both excitation and emission monochromators were set at 5 nm. Acrylamide quenching experiments were carried out by adding freshly prepared solution of acrylamide each time to protein solution. Protein sample was never diluted more than 5%. The effects of dilution and ionic strength were calibrated in parallel experiments. To monitor the fluorescence intensity change for PhoP proteins with the addition of quenching ligand, the emission wavelength was set to 340 nm. In DNA-binding experiments, duplex DNA sites were added to protein solution from a concentrated stock of 600 μM . A Stern–Volmer relationship was utilized to assess the fluorescence accessible to quenching: $F_0/F = 1 + K_{\text{SV}}[Q]$, where F_0 is the fluorescence intensity in the absence of quencher, F is the quenched fluorescence, K_{SV} is the dynamic quenching constant of the accessible fluores-

cence, and $[Q]$ is the quencher concentration. All measurements were carried out in 50 mM HEPES- Na^{+} buffer (pH 7.5) containing 100 mM NaCl.

Data analysis. All data are presented as means $\pm$ the standard error of the mean. Plotting and calculation of the standard deviation was performed in Microsoft Excel. Statistical analysis was performed on crude data by using a paired Student *t* test. *P* values of <0.05 were considered significant.

RESULTS

DNA binding by PhoP and P-PhoP. We have previously identified direct repeat units in the *phoP* promoter region that are important for autoregulation in the presence of PhoP (6). The binding of PhoP to its promoter was investigated here at protein concentrations comparable to those examined previously (6), except that instead of 410-bp *phoP* promoter, a 36-bp double-stranded oligonucleotide (DR1,2) comprising two 9-bp direct-repeat units DR1 and DR2 separated by a 5-bp spacer length (see Materials and Methods) was used. Note that all of the oligodeoxynucleotide constructs used in the present study have 6- and 7-bp extensions of natural sequence at the 5' and the 3' ends, respectively, to stabilize duplex formation (see Table 1). ^{32}P -labeled DR1,2 DNA was incubated at 20°C for 10 min with increasing concentrations of recombinant PhoP, followed by electrophoresis through 6% nondenaturing polyacrylamide gel. Recombinant PhoP strongly bound the DNA probe carrying the wild-type sequence, suggesting that this motif alone is likely responsible for DNA-protein interaction(s). A gel shift pattern consistent with our earlier observation using entire *phoP* promoter was obtained (lanes 2 to 7, Fig. 1A) in which increasing concentrations of PhoP resulted in a progressive decrease in probe mobility until a protein-DNA complex of defined mobility was obtained at higher concentrations of PhoP. Additional gel shift assay to demonstrate PhoP binding specificity examined the ability of excess unlabeled DR1,2 DNA (as specific competitor) and nonspecific DNA (NSP; as a heterologous competitor) to compete with DR1,2 for binding to PhoP (data not shown). NSP represents a 36-bp double-stranded oligonucleotide (5'-GGTTGGCGCGGGCAATCGTGTTCATCGATTCCCAGCA-3') that lacks any PhoP binding site and is derived from a distal region of *phoP* promoter, *phoP4* (6). From these results, we surmise that PhoP binds to 36-bp DR1,2 site in a sequence-specific and concentration-dependent manner.

The effect of phosphorylation by AcP on ability of PhoP to bind to DR1,2 site was examined by EMSA (compare lanes 9 to 14 and lanes 2 to 7 in Fig. 1A). As a control experiment, the presence of AcP alone did not influence the mobility of the labeled DNA fragment (lane 8). The EMSA data suggest that the nature of the shifted bands is largely similar, although somewhat uniform for P-PhoP, suggesting that under the conditions examined the phosphorylation of PhoP does not appear to influence its ability to bind DNA. This observation is consistent with our previous data showing that phosphorylation is not essential for DNA binding.

To gain further insight on DNA binding, DNase I foot printing was carried out using a top-strand labeled 36-bp DR1,2 duplex DNA to monitor binding of PhoP and P-PhoP according to a procedure described by Inouye and coworkers (31). With increasing concentrations of PhoP (lanes 2 to 4, Fig. 1B) and P-PhoP (lanes 7 to 9, Fig. 1B), both DR1 and DR2 sites (vertical bars in Fig. 1B) were protected from DNase I, in a

FIG. 1. Effect of PhoP phosphorylation on DNA binding. (A) EMSA for binding of the indicated concentrations of PhoP (lanes 2 to 7) to 5'-end-labeled duplex DR1,2 (comprising direct repeat units DR1 and DR2 of the *phoP* promoter region). Lane 1 represents the labeled DNA alone. In the right panel, PhoP preincubated with AcP at the indicated protein concentrations (lanes 9 to 14) was analyzed for its DNA-binding ability. The presence of AcP had no effect on the mobility of the DNA duplex (lane 8). In all cases, the position of radioactive material was determined by exposure of dried gel to X-ray film. The open arrow indicates origins of polyacrylamide gel, and the filled arrow indicates band shifts produced in the presence of PhoP. (B and C) DNase I footprinting was carried out with 36-bp DR1,2 DNA as for the gel mobility shift assays. PhoP (lanes 2 to 5) and P-PhoP (lanes 7 to 10) at the indicated protein concentrations were incubated with 10 nM top-strand-labeled (B) and bottom-strand-labeled (C) duplex DR1,2 DNA. Partial digestion of DNA was carried out by DNase I as described in Materials and Methods. Samples were subjected to 12% PAGE-8 M urea gel analysis. The gel is representative of three independent experiments. ND (lanes 5 and 10), naked DNA without DNase I digestion. The residues are indicated by arrows on the left and were positioned with respect to the G ladder of the DR1,2 fragment (data not shown). The vertical bars represent the indicated sites.

protein-concentration-dependent manner. Footprinting experiments revealed two interesting features of PhoP (and/or P-PhoP)-DNA interaction(s). First, protection of DR1 site by both PhoP and P-PhoP was observed to be significantly higher than that of DR2 site. In fact, PhoP showed hardly any protection on DR2 site (lanes 2 to 4, Fig. 1B). However, P-PhoP in a concentration-dependent manner protected both repeat subsites (DR1 and DR2) significantly. Under the conditions examined, at the highest PhoP concentration (6 μM), while PhoP showed a modest (1.8 ± 0.3)-fold protection of DR2 site (compare lane 4 with lane 1, Fig. 1B), phosphorylated PhoP displayed a (4.3 ± 1.0)-fold protection (compare lane 9 with lane 6, Fig. 1B). However, PhoP showed a significant ($10.6 \pm$

0.7)-fold protection of DR1 site (compare lane 4 with lane 1), while phosphorylated PhoP showed an additional at least 10-fold protection of the DR1 site (compare lane 9 with lane 4) (based on the limits of detection in this assay and based on other gels [data not shown]). Interestingly, when a bottom-strand labeled DR1,2 duplex DNA was used in the DNase I footprinting experiments, identical results were obtained, showing P-PhoP displaying a stronger protection of DNase I cleavage compared to the unphosphorylated PhoP (compare lanes 7 to 9 and lanes 2 to 4, Fig. 1C). In contrast, footprinting attempts with PhoP_{D71N} (a mutant PhoP protein that is unable to be phosphorylated in vitro), preincubated with or without AcP, did not show any phosphorylation-specific additional pro-

FIG. 2. Phosphorylation of PhoP. (A) PhoP consists of an NTD and a CTD. The conserved region within the NTD implicated in protein-protein interactions and conserved winged helix-turn-helix (wHTH) DNA-binding motif located within the CTD is indicated (see the text). The previously identified site of phosphorylation (Asp-71) in the NTD (6) and the two tryptophan residues (W166 and W203) located within CTD of PhoP are also shown. (B and C) Phosphorylation of PhoP (B) and PhoP_{D71N} (C) with AcP and resolution by 2D gel electrophoresis. Wild-type and mutant PhoPs were incubated in a phosphorylation mixture in the absence or presence of AcP (as indicated). PhoP was separated according to charge (pI) and molecular mass by 2D gel electrophoresis (see Materials and Methods for details). Unphosphorylated and phosphorylated forms of PhoP are indicated by open and filled arrowheads, respectively. The results are representative of three independent experiments.

tection against DNase I cleavage on an identical DR1,2 substrate (see Fig. S1A in the supplemental material). This observation suggests wild-type PhoP-like DR1,2 binding properties of PhoP_{D71N} and is consistent with our earlier data suggesting the phosphorylation-independent DNA-binding activity of PhoP (6). To further examine the effect of phosphorylation and to rule out any nonspecific effect of phosphorylation, DNase I cleavage studies were carried out using an end-labeled 60-bp oligonucleotide consisting of DR1 and DR2 repeat subunits with identical intervening spacer length (see Fig. S1B in the supplemental material). The results show unambiguously that phosphorylation of *M. tuberculosis* PhoP is accompanied by a stronger protection of the repeat sequences regardless of the length of the DNA. Thus, our results showed that P-PhoP bound to the DR1,2 site with significantly greater affinity than did PhoP, suggesting that phosphorylation contributes to the overall stability of the PhoP-DR1,2 DNA complex.

Phosphorylation of PhoP. The primary site of covalent phosphorylation of PhoP has been mapped to Asp-71 located within the NTD (6) (Fig. 2A). The X-ray crystal structures of

several receiver domains from homologous response regulators have been determined, and they all reveal (α/β)₅ topology (26). The five parallel β -strands form a hydrophobic core surrounded by two α -helices on one side and three on the other. Although *M. tuberculosis* PhoP has been shown to participate in typical phosphotransfer reactions using phosphorylated PhoR as a phosphodonor (6), response regulators are also phosphorylated with small molecule phosphodonor compounds such as AcP (19). To determine whether this was also a characteristic of PhoP, phosphorylation of PhoP with AcP was evaluated using 2D gel electrophoresis, since the addition of a phosphoryl group shifts the pI of a given protein toward the acidic range (11). In the absence of AcP, recombinant PhoP focused predominantly as a single protein species with a calculated pI of 6.9 (left panel, Fig. 2B). This is in good agreement with the predicted pI of 7.0 for unphosphorylated form of PhoP. In contrast, PhoP preincubated with AcP under phosphorylation conditions focused as two predominant protein species that focused at pI 6.9 and another that focused at pI 6.7 were observed. To determine whether this apparent shift in pI of PhoP after incubation with AcP is due to specific phosphorylation of PhoP, PhoP_{D71N} was preincubated with AcP, and the reactions were analyzed by 2D gel electrophoresis. In contrast to wild-type PhoP, PhoP_{D71N} both preincubated with or without AcP focused at pH 6.9 (compare the left and right panels, Fig. 2C) like the unshifted form of the protein. Thus, incubation with AcP results in the specific phosphorylation of PhoP.

Effect of phosphorylation on PhoP structure and fold. To assess quaternary organization of PhoP after phosphorylation, samples of recombinant protein preincubated in the presence or absence of AcP were subjected to gel filtration chromatography using Sephacryl-200 column. Single-peak elutions were observed at 280 nm for multiple replicates of PhoP and phosphorylated PhoP samples (data not shown). Compared to protein standards, the estimated molecular masses of the PhoP proteins were calculated to be 28 ± 2 kDa and 31 ± 1 kDa for the unphosphorylated and phosphorylated PhoPs, respectively. This finding is in good agreement with the predicted molecular mass of 29.5 kDa for the monomeric form of PhoP. Thus, under the conditions examined, PhoP and P-PhoP appear to be monomeric in solution when purified from *E. coli*.

To examine the secondary structural content of phosphorylated and unphosphorylated states of PhoP, far UV-CD spectra of AcP-treated and untreated forms of the protein were compared. Both forms of PhoP exhibit small but significant mean residue ellipticity at 220 nm and a minimum around 208 nm, indicating the solution structure of the protein to be an equilibrium mixture of α -helix and an extended conformation (data not shown). However, there was only a minor change in the absolute CD intensity, with no change in spectrum shape between PhoP and P-PhoP, indicating very little change in the secondary structure.

To identify the distinct structural change and consequence of phosphorylation on the tertiary fold of PhoP, if any, purified PhoP preincubated in the absence or presence of AcP was limit digested with trypsin. Limited proteolysis experiments were analyzed by the time course of protease digestion (Fig. 3A). Gel electrophoresis showed that trypsin yielded two specific fragments of approximately 25 and 19 kDa, respectively (ar-

FIG. 3. Effect of phosphorylation on limited proteolysis of PhoP proteins. (A) Wild-type PhoP, preincubated in phosphorylation buffer with or without AcP, was limit digested with 400 ng of trypsin at 37°C for 15 min (lanes 2 and 5), 30 min (lanes 3 and 6), or 45 min (lanes 4 and 7) as described in Materials and Methods. Proteolysis was terminated by the addition of SDS, and the samples were resolved by SDS-12% PAGE and visualized by Coomassie blue staining. As a reference, untreated PhoP is resolved in lane 1. (B) PhoP and PhoP_{D71N} (4 μ g) preincubated in phosphorylation mixture with or without AcP were limit digested for 30 min with trypsin in a 20- μ l reaction mixture. The products of digestion were resolved by SDS-12% PAGE and transferred to a polyvinylidene difluoride membrane. The trypsin cleavage sites were determined by N-terminal sequencing of the stained bands excised after transfer to Immobilon (Millipore). As a reference, untreated PhoP and PhoP_{D71N} were resolved in lanes 1 and 4, respectively. The sizes of the molecular mass markers (in kilodaltons) are indicated on the right of the figure. See Results for a description of the proteolytic fragments.

rows in Fig. 3A). Although both of the fragments were observed regardless of the phosphorylation of PhoP, phosphorylation stimulated subsequent cleavage of 25-kDa fragment into smaller fragments (compare lanes 2 to 4 and lanes 5 to 7, Fig. 3A). The fact that phosphorylation-specific stimulation of cleavage of 25-kDa fragment is not accompanied by a corresponding increase in the 19-kDa band is presumably due to the involvement of more than one trypsin cleavage site in PhoP. Thus, phosphorylation influenced the cleavage pattern of wild-type PhoP. It was of interest, therefore, to examine the cleavage pattern of PhoP_{D71N} preincubated in the absence or presence of AcP. To this effect, PhoP and PhoP_{D71N} preincubated with or without AcP were limit digested with trypsin at 37°C for 30 min, keeping the ratio of PhoP to trypsin at 100:1 (Fig. 3B). The structural significance of these proteolysis products was suggested by the fact that proteolysis of PhoP_{D71N} with trypsin yielded the same profile as that observed with PhoP. Densitometric analysis of multiple replicates of gels revealed that the relative intensities of the top band (representing undigested full-length PhoP) and the bottom band (representing the 19-

kDa fragment) of lane 2 and lane 3 are comparable, with a <5% difference, thus ruling out the possibility of loading artifact of proteins in lane 2 and lane 3. However, the middle band (representing the 25-kDa fragment) showed a significant (2.5 ± 0.1)-fold difference in band intensity, as estimated from at least three independent experiments. Strikingly, for PhoP_{D71N} that is not phosphorylated in vitro there was no phosphorylation-specific stimulation of cleavage of 25-kDa fragment (compare lanes 5 and 6, Fig. 3B). Quantifying each band of lane 5 and lane 6 of PhoP_{D71N} panel showed that all of the three bands of lane 5 were comparable to the corresponding three bands of lane 6, with a variation of intensity by <3%. From these results we surmise that specific phosphorylation at Asp-71 of the NTD exposes single or multiple trypsin cleavage site(s) in PhoP.

To probe the trypsin cleavage sites, peptide fragments of approximately 25 and 19 kDa were transferred from the gel by electroblotting the samples onto polyvinylidene difluoride (Millipore) membrane and subjecting them to N-terminal sequencing. Both fragments yielded an identical N-terminal sequence (VLVVDDE), showing unambiguously that these are generated by trypsin cleavage of the same peptide bond between Arg-22 and Val-23. Trypsin digest fragments were further analyzed by mass spectroscopy, and the sites of protease cleavage were identified. While the 25-kDa limit digest product was shown to consist of residues 23 to 245, the 19-kDa fragment consisted of residues 23 to 197. Thus, the 19- and 25-kDa fragments share identical N termini but extend to variable C termini of PhoP. From these results we conclude that clearly NTD-specific phosphorylation of *M. tuberculosis* PhoP at Asp-71 exposes one or multiple trypsin cleavage site(s) in the PhoP CTD.

Phosphorylation of PhoP enhances protein-protein interaction(s). We next investigated whether a phosphorylation-coupled conformational change of purified PhoP could influence its self-association. To study protein-protein interaction(s), PhoP or PhoP_{D71N} preincubated with AcP was subjected to cross-linking using DSS, which cross-links two amino groups that are in close proximity to each other. The samples were analyzed by SDS-PAGE and visualized by Western blotting using rabbit anti-PhoP polyclonal antibody. A band that migrated as a dimer of PhoP was observed for both untreated and AcP-treated PhoP (lane 2 and lane 3, Fig. 4). A densitometric analysis to quantify and compare cross-linking efficiency of PhoP and P-PhoP, based on three independent experiments showed that under the conditions examined, phosphorylated PhoP is (3.8 ± 0.2)-fold more effective than unphosphorylated protein in forming a stable cross-linked dimer. However, PhoP_{D71N} mutant, as a control, did not show any phosphorylation-dependent stimulation of cross-linking (compare lane 5 and lane 6, Fig. 4). From the in vitro cross-linking results, we conclude that PhoP is able to self-associate independently of phosphorylation, although protein-protein interaction(s) is significantly enhanced upon phosphorylation.

Effect of phosphorylation on the fluorescence of W166 and W203. A number of studies have established that tryptophan fluorescence in proteins is a sensitive probe of folding and tertiary structure (13). One of the fluorescence properties sensitive to environment and solvent accessibility is the Stern-Volmer constant for collisional quenching. To assess effect of

FIG. 4. Phosphorylation of PhoP enhances self-association. Affinity-purified PhoP or PhoP_{D71N} (15 μ M) previously incubated with or without AcP was subjected to cross-linking with 1 mM DSS as described in Materials and Methods. Samples were analyzed by SDS-12% PAGE and electrotransferred to nitrocellulose membranes for Western blotting using rabbit polyclonal anti-PhoP antibody according to a standard procedure. The numbers at the right are molecular masses in kilodaltons. The results are representative of at least three independent experiments.

phosphorylation, we measured the fluorescence properties of two tryptophan residues present within the CTD (W166 and W203, Fig. 2A). Whereas W166 is proximal to the NTD, W203 belongs to the central part of the winged helix-turn-helix DNA-binding motif spanning residues R196-K224 (Fig. 2A). Structural studies have shown that phosphorylation of the amino-terminal receiver domain promotes either a long-range conformational change that enhances DNA binding affinity or induces multimerization and, in turn, increases its affinity for DNA (27). Thus, the environment of these tryptophan residues may shed light on important aspects of structure and function as a consequence of phosphorylation. Figure 5A shows the tryptophan fluorescence emission spectra of PhoP incubated in phosphorylation buffer in the absence or presence of AcP. The observed emission maximum wavelength of recombinant PhoP both in the unphosphorylated and in the phosphorylated form at pH 7.5 was 340 nm (curves 1 and 2; Fig. 5A), consistent with tryptophans being in a relatively nonpolar environment. However, as a consequence of phosphorylation, PhoP displayed a significant quenching of tryptophan fluorescence. To examine whether phosphorylation induces a specific change in the environment of tryptophan residues, acrylamide quenching experiments were carried out for both untreated and AcP-treated PhoP. The acrylamide quenching data in both cases are linear and can be fitted well to the Stern-Volmer equation with a single class of fluorophore (Fig. 5B and C). The Stern-Volmer constants, K_{SV} , calculated from the plots, are 6.9 and 3.7 M^{-1} for unphosphorylated and phosphorylated PhoP, respectively. Thus, phosphorylation of PhoP resulted in statistically significant shielding of tryptophan residues [(1.9 $\pm$ 0.2)- fold, $P < 0.01$]. In contrast, acrylamide-quenching profiles of PhoP_{D71N} preincubated in the absence or presence of AcP displayed almost overlapping plots with comparable K_{SV} values for untreated and AcP-treated mutant. This observation also rules out the possibility of any influence of the N-terminal His tag of PhoP in phosphorylation induced conformational change of protein. To further show that the phosphorylation-coupled conformational change of the PhoP is independent of an N-terminal His₆ tag, *M. tuberculosis* PhoP lacking a His tag (PhoP^{-His}) was purified by cleavage of the tag using thrombin.

The purified proteins with or without His tag were resolved by SDS-PAGE in lanes 1 and 2, respectively (inset of Fig. 5D). Subsequently, PhoP^{-His} was phosphorylated with AcP, and the effect of phosphorylation was assessed using acrylamide quenching of tryptophan fluorescence. Essentially identical results with PhoP^{-His} and phosphorylated PhoP^{-His} were obtained (Fig. 5D), suggesting that the N-terminal His tag does not influence the structure of PhoP. It is noteworthy that, compared to the unphosphorylated protein, the K_{SV} value is significantly reduced in the phosphorylated form, suggesting that W166 and/or W203 residues shift to a more inaccessible environment. Although emission maxima are similar for both the unphosphorylated and the phosphorylated PhoP ($\sim$ 340 nm in both cases); clearly, the K_{SV} of tryptophan undergoes a significant reduction as a result of phosphorylation. A decrease of the K_{SV} may be the result of long-range interactions of phosphorylated PhoP in a folded conformation, which shields W166 and/or W203 from the solvent. We attribute this altered quenching pattern to a phosphorylation-coupled conformational change which appears to enhance protein-protein contacts.

To determine which tryptophan residue is undergoing a change around its environment, sequential substitution of each of the tryptophan residue was carried out by site-directed mutagenesis (see Materials and Methods). Each protein was expressed and purified by Ni²⁺-affinity chromatography. The introduction of D71N mutation in either W166Y or W203Y yielded phosphorylation-defective single-tryptophan-containing PhoP proteins. Since the conformational state of a protein can influence the exposure of its tryptophan residues to solvent (13), we undertook a biochemical approach to examine and compare the microenvironment around each tryptophan residue in unphosphorylated and phosphorylated forms of the protein. Figures 6A and B show Stern-Volmer plots of the acrylamide quenching of W166Y and W203Y mutants, respectively, preincubated in the absence or presence of AcP. For mutant W166YPhoP, which probes the environment around W203, the plots of PhoP and P-PhoP are almost identical within experimental error. In contrast, mutant W203Y showed a sixfold decrease in slope (K_{SV} declined from 4.2 to 0.7 M^{-1} , $P < 0.01$) for the phosphorylated protein compared to the unphosphorylated protein (Fig. 6B), suggesting that the environment around W166 is a sensitive probe for monitoring phosphorylation. As a control experiment, D71NW203Y PhoP did not show any phosphorylation-dependent alteration in the quenching profile (Fig. 6C). Thus, we conclude that the phosphorylation of PhoP results in enhanced protein-protein contacts, as a result of which W166 shifts to a more inaccessible environment. The lack of a detectable difference in the Stern-Volmer analysis of tryptophan fluorescence from W203 (located within the DNA-binding domain) between the unphosphorylated and phosphorylated forms of the protein is consistent with phosphorylation-independent DNA-binding ability of PhoP (6; the present study).

Phosphorylation influences DNA-mediated protein-protein interaction. In the DNase I footprinting experiment using a 36-bp DR1,2 DNA, PhoP displayed a stronger protection of DR1 compared to the DR2 site (Fig. 1B). Thus, we next investigated the importance of each of the sequence motifs (direct repeats) for DNA binding. To this effect, we radiolabeled

FIG. 5. Fluorescence emission of PhoP proteins. (A) The intrinsic tryptophan fluorescence of untreated PhoP was observed to decrease significantly after preincubation with AcP (compare curve 1 to curve 2), suggesting that quenching of tryptophan fluorescence is a consequence of PhoP phosphorylation. (B) Stern-Volmer plot of acrylamide quenching of tryptophan fluorescence of PhoP preincubated in the absence (circles) or presence (triangles) of AcP. (C) Stern-Volmer plot of acrylamide quenching of PhoP_{D71N} preincubated in the absence (circles) or presence (triangles) of AcP. (D) Acrylamide quenching of tryptophan fluorescence of PhoP^{-His}, preincubated under phosphorylation conditions in the absence (circles) or presence (triangles) of AcP. The inset shows a Coomassie blue-stained SDS-PAGE gel of ~ 2.5 μ g of purified *M. tuberculosis* PhoP and PhoP^{-His}, recovered after thrombin cleavage of affinity-purified PhoP, as described in Materials and Methods. All measurements were carried out in 50 mM HEPES- Na^+ buffer (pH 7.5) containing 100 mM NaCl at $25 \pm 1^\circ\text{C}$. The excitation wavelength was set to 295 nm, and the emission was noted at 340 nm. Each point is an average of three independent experiments.

the 36-bp DR1,2 oligonucleotide (Fig. 7) or sequence variants that were altered in either of the repeat subunits (sDR1-DR2 and DR1-sDR2, respectively, Table 1) with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, annealed them with their complementary oligonucleotides, and used them in binding reactions with PhoP. Since, all of the first four nucleotides of DR1 and DR2 sites are conserved and appeared to be critical for DNA binding, the first four nucleotides from either of the repeat units were replaced with adenine to construct sDR1-DR2 or DR1-sDR2 (Fig. 7). When the binding of PhoP to these probes was investigated by EMSA, in the range of protein concentrations examined, only a single retarded complex was observed with sDR1-DR2 probe (Fig. 7B; see also Fig. S2 in the supplemental material). However, clearly more than one retarded species (as with wild-type DR1,2; Fig. 7A) were observed with DR1-sDR2 probe (Fig.

7C; see Fig. S2 in the supplemental material). These data suggest that DR1 site is essential for the formation of multiple retarded species. From these results we surmise that DNA subsites play a critical role in the formation of multiply bound PhoP-DNA complex.

Since phosphorylation of PhoP was observed to influence protein-protein self-association and since direct-repeat DNA sites appeared to be essential for DNA-mediated protein-protein contacts, we sought to probe protein-protein interactions of DNA-bound protein in the unphosphorylated or phosphorylated form. If the nature of protein-protein association is different in bound PhoP based on the phosphorylation status of the protein, then the nature of the residues involved may also be significantly different. In order to obtain structural information on the DNA-bound state of PhoP, we studied the acryl-

FIG. 6. Properties of single-tryptophan-containing PhoPs. Stern-Volmer plots of acrylamide quenching of W166Y PhoP (A), W203Y PhoP (B), and D71NW203Y PhoP (C) proteins preincubated under phosphorylation conditions in the absence (circles) or presence (triangles) of AcP are shown. Solution conditions were as described in the legend to Fig. 5. The excitation and emission wavelengths were 295 and 340 nm, respectively. The protein concentration was 9 μ M for each mutant. Each point is an average of at least three independent determinations.

amide quenching of both PhoP and P-PhoP bound to the DR1,2 site. Since, acrylamide may have an effect on promoter-regulator interaction(s), we attempted to measure the quenchable fluorescence of PhoP-DNA complex as a function of acrylamide concentration. Although the phosphorylation of W203YPhoP was shown to primarily influence the environment around W166, subsequent DNA-binding experiments

were not carried out with W203Y since the conserved W203 appeared to be important for DNA binding. Upon DNA (DR1,2 site) binding, PhoP displayed a different quenching pattern of tryptophan residue, suggesting that specific DNA binding affects the environment around the tryptophan residues (Fig. 8A). The calculated K_{SV} values are 6.9 and 3.5 M^{-1} , respectively, for unbound and DNA bound PhoP, suggesting a (1.9 ± 0.1) -fold decrease in W166 exposure ($P < 0.01$). In contrast, upon DNA binding, phosphorylated PhoP showed significantly enhanced K_{SV} (Fig. 8B). The calculated K_{SV} values were 3.7 and 8.7 M^{-1} , respectively, for unbound and DNA bound phosphorylated PhoP, reflecting a statistically significant $[(2.4 \pm 0.2)$ -fold, $P < 0.01$] increase in the exposure of tryptophan residue(s) upon DNA binding. These data showing dramatically opposite effects of DR1,2 binding for PhoP and P-PhoP clearly suggest that DNA-mediated protein-protein interaction(s) are phosphorylation dependent. Interestingly, in the presence of the sDR1-DR2 duplex DNA, where PhoP generates a single retarded complex (Fig. 7B), the acrylamide quenching profile of PhoP and P-PhoP becomes overlapping within experimental error (Fig. 8C). In another control experiment, PhoP and P-PhoP incubated with DR1-sDR2 oligonucleotide generated almost overlapping Stern-Volmer plots (Fig. 8D), suggesting a phosphorylation-specific, target DNA motif-dependent variation of protein-protein interactions of PhoP.

DISCUSSION

Phosphorylation-coupled conformational change of PhoP. It is becoming increasingly clear that regulation of gene expression, in addition to correct recognition of DNA sequences by regulatory proteins, involves homologous and heterologous protein-protein interactions. Previous studies with OmpR, a member of the PhoP subfamily of proteins, have shown that N-terminal phosphorylation influences C-terminal DNA binding (18, 31) and that C-terminal DNA binding affects N-terminal phosphorylation (28). These studies reflect the importance of bidirectional intradomain communication(s) in the functionality of this group of proteins. Recently, X-ray structures of DrrD and DrrB, the PhoP homolog from *T. maritima*, revealed that the NTD of the molecule interacts with its CTD (3, 25). Although the conserved $\alpha 4$ - $\beta 5$ - $\alpha 5$ region of NTDs of response regulator family have been shown to contribute to protein-protein interactions (see above), in the present study we show that phosphorylation of *M. tuberculosis* PhoP at the NTD influences protein-protein self-association through CTDs. We further show here that protein-protein interaction interfaces appear to be different for phosphorylated and unphosphorylated forms of the protein, and the presence of specific sequence motifs of target DNA sites clearly influences protein-protein interaction(s). Although one can argue how phosphorylation of $\sim 23\%$ of the PhoP molecules would translate into a significant conformational change, our results are consistent with a relatively low efficiency of phosphorylation leading to significant stimulation of DNA binding by *M. tuberculosis* MprA (8).

Phosphorylation does not appear to affect secondary structure and protein oligomerization in solution, as judged by gel filtration chromatography and CD studies (data not shown).

FIG. 7. DNA-mediated protein-protein interactions of PhoP. The results of mobility shift assays for the binding of the indicated concentrations of PhoP to 5'-end-labeled 36-bp DR1,2 (A) and sDR1-DR2 (B) and DR1-sDR2 (C) duplex oligodeoxynucleotides (see Materials and Methods) are shown. In all cases, the position of the radioactive material was determined by exposure of the dried gel to X-ray film as described in the legend to Fig. 1. The sequences of the repeat units in the substrate used are shown below each panel. Open arrows indicate the origins of the native polyacrylamide gel, and filled arrows indicate the band shifts produced in the presence of PhoP. The gels are representative of three independent experiments.

However, phosphorylation of the PhoP NTD induces a conformational change in the PhoP CTD (Fig. 3). Again, phosphorylated PhoP shows enhanced protein-protein self-association in DSS cross-linking experiments (Fig. 4). Although the present study does not show any structural effect of phosphorylation at the PhoP NTD, our tryptophan fluorescence data suggest shielding of the tryptophan residue as a consequence of phosphorylation (at the NTD) and confirm the localization of the

conformational change to part of the PhoP CTD. It appears that the enhanced protein-protein interaction(s) events and phosphorylation-coupled conformational transition are related to each other. Perhaps this explains why the phosphorylated form of the protein shows enhanced protein-protein contacts that lead to the movement of indole moieties of the tryptophan residues toward an inaccessible environment (Fig. 5).

Since both phosphorylation and DNA binding affect the

FIG. 8. DNA-mediated PhoP-PhoP interaction is influenced by phosphorylation. Stern-Volmer plots of acrylamide quenching of unbound and DR1,2 bound proteins in the unphosphorylated (open and filled circles) (A) or phosphorylated forms (open and filled triangles) (B), respectively, are shown. The data for unphosphorylated PhoP (open circles) and phosphorylated PhoP (open triangles) from Fig. 5B are included for clarity. (C and D) Plots of acrylamide quenching data for sDR1-DR2- and DR1-sDR2-bound unphosphorylated (open squares) and phosphorylated (open diamonds) PhoP, respectively, as indicated in the figure. In all cases, duplex DNA was added from a concentrated stock of 600 μ M at a protein/DNA molar ratio of 2:1. The solution conditions were as described in the legends to Fig. 5 at 25°C. The excitation wavelength was 295 nm, and emission was noted at 340 nm. The error bars were derived from at least three independent measurements.

environment of tryptophan residues of PhoP (Fig. 6 and 8), part of the PhoP CTD seems to be sensitive to both phosphorylation and DNA-binding domain occupancy. This is consistent with values of Stern-Volmer quenching constants of free phosphorylated PhoP (3.7 M^{-1}) and DNA-bound PhoP (3.5 M^{-1}) compared to unphosphorylated, unbound protein (6.9 M^{-1}). Thus, it is likely that the PhoP CTD undergoes a major structural change in response to phosphorylation and/or DNA binding. However, this mechanistically important structural change would be difficult to detect by low-resolution structural methods such as CD. As an alternate possibility, protein-protein interactions resulting from the phosphorylation of PhoP may promote an intermolecular reaction causing a change of the PhoP CTD conformation.

Phosphorylation of PhoP stimulates DNA binding. In the present study, we demonstrated that PhoP molecules bound to 36-bp DR1,2 site in a sequence-specific manner. Although gel retardation analysis of PhoP and P-PhoP with DR1,2 site did not reveal any significant differences, footprinting studies clearly showed that P-PhoP bound to DR1,2 with an overall higher binding affinity than did PhoP. Interestingly, in footprinting experiments both PhoP and P-PhoP protected the upstream DR1 site more strongly than the downstream DR2 site, suggesting a hierarchy of DNA-binding affinity ($\text{DR1} > \text{DR2}$). To examine the role of each repeat unit in PhoP binding, DR1 and DR2 sites were scrambled to generate sDR1-DR2 or DR1-sDR2 (Fig. 7). Scrambling of DR1 site obliterated multiple PhoP binding to the modified substrate. Over the entire range of protein concentrations tested, a single retarded complex was observed (Fig. 7B; see also Fig. S2 in the supplemental material). In contrast, scrambling of the DR2 site did not significantly alter PhoP binding of DR1-sDR2 oligonucleotide (Fig. 7C and Fig. S2 in the supplemental material). Thus, these results unambiguously established that DR1 site is the initial contact point essential for DR1,2 DNA binding by multiple PhoP molecules. From these results we surmise that additional protection by P-PhoP is presumably due to enhanced protein-protein interaction(s) by phosphorylated protomers bound to the two adjacent repeat units, DR1 and DR2.

In the crystal structure of another two-domain response regulator, NarL, the linker region is disordered and the DNA-binding HTH motif is occluded by NTD (2). Since phosphorylation in the N terminus of NarL allows DNA binding, phosphorylation must alter the exposure of the DNA-binding surface. It has been proposed that phosphorylation decreased interdomain interactions to render the DNA-binding surface accessible. This model is difficult to reconcile with our finding since unphosphorylated PhoP can bind to DR1,2 on its own. Thus, in the case of PhoP, the phosphorylation seems to primarily affect its ability to extend protein-protein contact(s) along the DNA helix to downstream sites. Since intradomain interaction(s) in PhoP do not occlude the DNA-binding site, the conformational change induced by phosphorylation might enhance the interaction(s) of PhoP monomers on the DNA surface. This possibility is consistent with our data showing an altered environment around tryptophan moieties for unphosphorylated and phosphorylated PhoP while bound to DNA sites containing two adjacent repeat subunits (see below).

DNA-mediated protein-protein interaction(s). Formation of multiprotein complexes by binding of PhoP molecules to DR1,2 DNA (consisting of two direct-repeat units) indicates that PhoP oligomer formation is mediated by DNA. Multiple binding of PhoP is not due to preformed PhoP oligomers that subsequently bind to DNA, since scrambling the DR1 site abolishes multiple binding to the sDR1-DR2 site (Fig. 7B). It therefore appears that PhoP oligomer formation occurs only on DNA such that binding of a PhoP molecule to the upstream (stronger) DR1 site assists the binding of a second PhoP molecule to the downstream (weaker) DR2 site.

Acrylamide quenching profile of PhoP and P-PhoP clearly shows sharp contrast in the quenching pattern with a more than $(1.8\text{-fold} \pm 0.2)\text{-fold}$ lower K_{SV} for P-PhoP (Fig. 5B). Interestingly, the addition of specific DNA sites displayed drastically opposite effects on the fluorescence quenching pattern of PhoP compared to its phosphorylated counterpart (Fig. 8A and B). While the presence of DR1,2 DNA shielded the tryptophan residue of PhoP with a decrease of K_{SV} from 6.9 to 3.5 M^{-1} (Fig. 8A), the addition of DR1,2 DNA significantly exposed the tryptophan residue of P-PhoP with an increase in the K_{SV} from 3.7 to 8.7 M^{-1} (Fig. 8B). However, neither sDR1-DR2 or DR1-sDR2 DNA substrates showed a similar effect. Thus, these data suggest that (i) protein-protein interaction(s) are mediated by the DNA substrate comprising two direct-repeat units and (ii) the nature of interaction(s) and residues involved therein strongly depends upon the phosphorylation status of the protein. The protein-protein contacts exhibited at adjacent direct repeat units might result from direct interactions between neighboring PhoP protomers or changes in the DNA structure upon binding to PhoP. Either possibility would be consistent with the fact that multiple PhoP binding to DNA is extremely sensitive to the presence of two direct-repeat units relative to each other. We cannot exclude the possibility that PhoP and P-PhoP at two different orientations involve similar protein-protein interaction interface while bound to the DR1,2 site. However, because the target sites are direct repeats, it is likely that two different protein surfaces (for PhoP and P-PhoP) comprise the interface between proteins bound to adjacent repeat units. While the highly conserved $\alpha 4\text{-}\beta 5\text{-}\alpha 5$ region of NTD has been proposed to act as an interface to stabilize the dimer formation on DNA (25), only a region relevant to the above-mentioned protein-protein contacts could involve residue(s) from the CTD analogous to OmpR (28). Thus, the results presented here suggest phosphorylation-dependent target sequence motif-specific protein-protein contacts between PhoP transactivation domains that contribute additional stability to the DNA-protein complex. Also, interaction(s) between the two phosphorylated PhoP protomers in the presence of adjacent cognate sites appears to vary significantly with that of unphosphorylated PhoP molecules, suggesting multiple orientation of DNA binding by the complex regulator.

Conclusion. It has been appreciated that structural complexity of bifunctional response regulators (like PhoP) is related to the need for the regulation of an array of genes. There is accumulating evidence that *M. tuberculosis* PhoP regulates many additional genes outside the repertoire of virulence and complex lipid biosynthesis (29). The results reported here suggest additional layers of complexity that have evolved in order

to further tune gene expression in response to the continuously changing physiologies of their bacterial hosts. This may have broader applications in the prokaryotic world since a single transcription factor often controls multiple genes under various conditions.

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