Decay of Activated *Bacillus subtilis* Pho Response Regulator, PhoP∼P, Involves the PhoR∼P Intermediate†

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ABSTRACT: PhoR of *Bacillus subtilis* is a histidine sensor-kinase belonging to the family of two-component signal transduction systems. PhoR is responsible for processing the phosphate-starvation signal and providing phosphate input to regulate the level of phosphorylated response regulator, PhoP, which activates/represses Pho regulon gene transcription. The catalytic domain of PhoR is sufficient for the low-phosphate inducible expression of Pho regulon genes since removing the *N*-terminal membrane-associated domain did not alter the kinetics of Pho induction, albeit the total level of induction was decreased (*1*). In this study we showed that the complete *B. subtilis* PhoR protein produced in *Escherichia coli* can be reverse phosphorylated by PhoP-phosphate. We also used a *C*-terminal fragment of the PhoR protein, *PhoR, to demonstrate that the phosphoryl group on phospho-PhoP was transferred back to *PhoR in the reverse phosphorylation reaction or released as inorganic phosphate to the reaction mixture. The reverse phosphorylation of the PhoR protein likely occurs at the same histidine residue (His360) that is utilized for the autokinase reaction by the same protein. In the presence of ADP, the phosphoryl group is further transferred to ADP to form ATP. While the autokinase reaction, the forward phosphotransfer reaction from PhoR∼P to PhoP, and the release of inorganic phosphate from PhoP∼P in the presence of PhoR require Mg^{2+}, the reverse phosphotransfer from PhoP∼P to PhoR does not. These results indicate that the energy levels of the phosphoryl groups on PhoP and PhoR are very similar. The reversible autokinase reaction and/or the reversible phosphotransfer reaction between PhoR∼P and PhoP may have a role in PhoP∼P decay thus influencing the PhoP∼P concentration in the cell.

Bacterial cells are capable of responding to and transducing environmental signals from outside of the cell to the cytoplasm in order to alter their metabolism. *Bacillus subtilis* responds to phosphate deprivation by regulating Pho regulon gene transcription. This regulation is modulated by a network of at least three two-component signal transduction systems (*2*−*4*). In the late stage of vegetative growth if phosphate becomes limited, *B. subtilis* cells respond with increased expression of the alkaline phosphatase genes *phoA*, *phoB* (*2*, *3*), and *phoD* (*5*), the high affinity phosphate transport genes in the *pstS* operon (*6*), the techuronic acid synthesis genes *tuaABCDE* (*7*), and the decreased expression of teichoic acid biosynthesis genes *tagA* and *tagD* (*8*).

The low phosphate-inducible expression of these Pho regulon genes is directly regulated by the PhoP−PhoR two-component system (*2*, *6*, *9*−*11*), in which PhoP is phosphorylated by PhoR∼phosphate as a result of phosphate starvation in postexponential growth. PhoP-phosphate is the active form of PhoP protein that not only binds to Pho regulon gene promoters but also activates the σ^A-dependent transcription of *phoA*, *phoB* (*12*, *13*), *phoD* (*13*), *pstS* (*11*, *14*), and *tuaA* (*10*) and represses the transcription of the *tagA*/*D* divergens (*8*, *10*).

The formation of PhoP-phosphate is the result of phosphotransfer from the membrane-bound sensor-kinase, PhoR. PhoR, by analogy to other sensory kinases, receives the low phosphate signal, which results in the autophosphorylation of the conserved histidine residue. The phosphorylated PhoR can subsequently phosphorylate PhoP. However, it was recently reported that the C terminal catalytic domain of the PhoR protein possesses all the relevant activities necessary for low phosphate induction of the Pho regulon (*1*).

PhoR belongs to the EnvZ family of transmitters (*15*). The cytoplasmic catalytic domain of many kinases, including EnvZ (*16*, *17*), CpxA (*18*) and KdpD (*19*, *20*), can dephosphorylate the phosphorylated response regulator (for a review, see refs *15* and *21*). However, the signal decay mechanism for PhoP∼P is not clear. It is known that the Pho response of *B. subtilis* is controlled by at least three two-component signal transduction systems (*2*, *3*). Phosphate limitation is required but not sufficient to induce the Pho response in *B. subtilis* (*3*) since double mutations in *resD* (a response regulator for respiration) and *abrB* (a transitional phase regulator) genes completely abolish the induction of the Pho regulon genes (*2*, *4*). Therefore, it seems that other factors antithetical to the Pho response must be controlling the phosphorylation state of histidine kinase and/or the response regulator protein, possibly at the autophosphorylation or the phosphotransfer reaction.

In this report, we studied the dephosphorylation of *B. subtilis* PhoP∼P by PhoR in order to identify the possible

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points for the regulation of PhoP−PhoR phosphotransfer. Our results showed two possible pathways for the dephosphorylation of PhoP∼P. PhoR protein may have a weak phosphatase activity to dephosphorylate PhoP∼P, which generated inorganic phosphate. PhoR can also be reverse phosphorylated by the PhoP∼P protein, which, in the presence of ADP, also generated ATP through the reverse autophosphorylation reaction. Unlike autophosphorylation, the forward phosphotransfer from PhoR∼P to PhoP, or the formation of inorganic phosphate in the presence of *PhoR and PhoP∼P, the reverse phosphorylation from PhoP∼P to PhoR does not require magnesium. In fact, the latter reaction is inhibited by magnesium. Our data presented here suggest that control of the phosphorylation state of both PhoR and PhoP may be achieved, at least in part, by regulation of the transphosphorylation reaction between these two proteins.

EXPERIMENTAL PROCEDURES

Site-Specific Mutagenesis. The PCR amplification method (*22*) was used to construct a **phoR* gene with a His360Gln mutation on a GST-fusion expression plasmid pLS28. The oligonucleotides were purchased from Operon Technologies. Primers FMH204 (TTGGATCCCCGGGAATTCA^{759}GACGCGAA), which contains *Bam*HI, *Sma*I, and *Eco*RI site at its 5′, and FMH206 (^{1170}CAGCTC**T**TGAGAAAC^{1156}), which contains a single base pair change from T to A (shown as in bold), were used in the first round PCR amplification to obtain the DNA fragment containing the H360Q mutation from pES5 (*23*) by PCR. The first round PCR product was then used together with pES5 as template, along with primers FMH205 (TTGGATCCCCGGGAATTCA), which has the sequence identical to that of the 5′ restriction sites on FMH204 but has no *phoR* sequence, and FMH198 (CGGGATCC^{1882}ACATCCTACCAGCATAGG^{1865}) containing a *Bam*HI site to get the secondary PCR product which was ligated to pCRII vector (Invitrogen) to yield pLS27. pLS27 was then digested with *Bam*HI. The 1.1 kb fragment containing *phoRH360Q* was ligated to pGEX-2T expression vector (Pharmacia) digested with *Bam*HI to yield pLS28. The accuracy of the cloned sequence was verified by sequencing. The number in the sequence refers to the number in the operon sequence of GenBank database accession number M23549.

Overexpression and Purification of PhoR and PhoP Proteins. E. coli BL21(DE3) was used as a host for all plasmids overexpressing *phoR* or *phoP* genes. The overexpression and purification of truncated *PhoR and *PhoRH360Q proteins was performed as described previously in ref *1*. Overproduction and purification of PhoP protein was performed according to procedures described previously in ref *12*. To obtain the insoluble fraction of *E. coli* cells overproducing the complete PhoR protein, the cells harboring the *phoR* plasmid pLS6 were grown at 30 °C until the optical density at 600 nm (OD_{600})[1] of the culture reached 0.6. Then 1 mM IPTG (isopropylthio-β-D-galactoside) was added to the culture, and growth was continued for another 3 h. The cells were harvested by centrifugation at 4 °C and washed with P buffer [50 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$ (pH 8.0)]. The cells were then suspended in P buffer and disrupted by sonication. Disruption of the cells was checked by microscopic observation. After centrifugation for 1 h at 100 000*g* in a Beckman 50Ti rotor, the insoluble fraction containing mostly membrane protein was suspended in P buffer.

Phosphorylation and Transphosphorylation Assays. Unless otherwise indicated, reaction mixtures contained various additions of protein in P buffer [50 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$ (pH 8.0)] at room temperature. A 10 mM EDTA solution was used where appropriate. Aliquots were removed at various times, added to an equal volume of 6× sodium dodecyl sulfate (SDS) sample buffer, and subjected to SDS−polyacrylamide gel electrophoresis (PAGE) on 10% or 12.5% polyacrylamide gels (*24*). The radioactivity of the proteins resolved on gels was determined qualitatively by autoradiography of dried gels with X-Omat AR film (Kodak). A PhosphorImager (Molecular Dynamics) was used for quantitative analyses. See specific experiments for details.

Purification of Phosphorylated PhoP. A 27 μg amount of purified GST-*PhoR plus 50 μL of glutathione−Sepharose beads (Pharmacia) were mixed for 10 min. The 600 μL MicroSpin disposable column (Pharmacia) was spun briefly once. A 200 μL volume of P-buffer with 1 mM (50 μCi) [γ-^{32}P]ATP was added to the column. The reaction was allowed to proceed for 15 min. The beads were then washed extensively with P-buffer until the radioactive signal in the wash buffer became constant. A 30 μg amount of PhoP was then added to the beads and incubated for 2 min. The column was then spun once to collect the flow-through fraction containing PhoP∼P, which was applied to a disposable G-25 column (Pharmacia) equilibrated with P-buffer and centrifuged in an Eppendorf microfuge at maximum speed for 20 s to remove small radioactive molecules.

*Stability of Reverse-Phosphorylated *PhoR∼P.* GST-*PhoR-phosphorylated PhoP (40 μg) was incubated with *PhoR (10 μg) in the presence of 1 mM ATP at room temperature for 1 min. The reaction was stopped by the addition of 1/5 vol. of 6× SDS loading buffer and aliquoted. To each aliquot, either HCl or NaOH was added at two final concentrations: 0.1 or 1 N. After incubation at room temperature for 20 min, the samples were neutralized with the same concentration of NaOH or HCl and subjected to SDS−PAGE analysis. After electrophoresis, the gel was dried and was exposed to X-Omat AR film (Kodak).

Detection of Protein Concentration. Protein concentration was detected by the Bradford method (*25*), using the Bio-Rad protein assay kit as instructed by the manufacturer.

Thin-Layer Chromatography. Cellulose polyethyleneamine plates (Selecto Scientific) were prerun in water and air-dried. A 2 μL volume of each reaction mixture was spotted onto plates. When the plates are developed in 0.25 M potassium phosphate buffer (pH 8.0), the phosphorylated protein stays at the origin, while ATP migrates with an R_f of 0.33 and inorganic phosphate (P_i) migrates with an R_f of 0.7. After development the plates were air-dried and analyzed by a PhosphorImager.

[1] Abbreviations used in the paper: OD, optical density; IPTG, isopropylthio-β-D-galactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GST, glutathion-*S*-transferase; P_i, inorganic phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid, ATP, adenosine 5′-triphosphate; ADP, adenosine-5′-diphosphate.

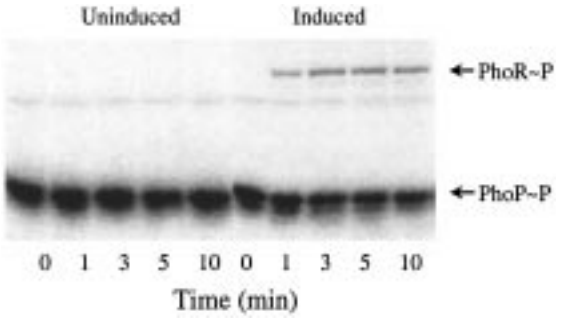

FIGURE 1: Reverse phosphorylation of PhoR by PhoP∼P. Purified PhoP∼P (0.35 μM) was mixed with the insoluble membrane fraction (approximately 12 μg total protein) of uninduced (left) or induced (right) *E. coli* cells containing the complete *phoR* gene in a 90 μL reaction mixture. The 15 μL aliquots of the reaction were taken at the indicated time 0, 1, 3, 5, and 10 min, stopped by the addition of 5 μL of 6× SDS sample buffer, and subjected to SDS−PAGE. The dried gel was exposed to a PhosphorImager.

RESULTS

Reverse Phosphorylation of Wild-Type PhoR by PhoP∼P. Many members of the histidine kinases family have been shown to possess a phosphatase activity to the phosphorylated cognate response regulator, in additional to their autokinase and phosphotransfer capability. To examine whether PhoR catalyzes the dephosphorylation of PhoP∼P, we performed an experiment using the insoluble fraction of *E. coli* cells producing the complete *B. subtilis* PhoR protein as a membrane protein. The result showed, in Figure 1, that when purified PhoP∼P was mixed with *E. coli*-produced *B. subtilis* PhoR, PhoR was reverse phosphorylated, albeit the fraction of the total label transferred from PhoP∼P was small. In the experiment using the same fraction of uninduced *E. coli* cells, the reverse phosphorylation was not observed (Figure 1, left panel). From these results, it appeared that PhoR had a very low level of phosphatase activity, if any, for PhoP∼P since the level of PhoP∼P phosphate did not decrease appreciably over the assay time of 10 min.

**PhoR Is Reverse Phosphorylated by PhoP∼P.* An *N*-terminal truncated form of the PhoR protein, *PhoR, was used in subsequent studies because, having had its two transmembrane segments removed, it is easily purified as a soluble protein and it was shown to retain the ability to correctly initiate the low phosphate starvation-inducible Pho response in vivo (*1*, *12*). When we assayed for the potential phosphatase activity of *PhoR protein on PhoP∼P, we also observed phosphorylation of a protein the size (41 KD) of the *PhoR protein. It has been observed in a kinase$^-$/phosphatase$^+$ mutant of EnvZ, EnvZ·347D(C), that the phosphoryl group was reverse transferred from response regulator to the histidine kinase, a reaction which required Mg^{2+} but was inhibited by ADP (*26*). The accumulation of *PhoR∼P is rapid, but the percentage of *PhoR being reverse phosphorylated is only approximately 15% (Figure 2A,C) of total initial label in PhoP∼P. Inclusion of ATP (Figure 2A) or ADP (Figure 2A) causes the rapid disappearance of labeled PhoP∼P (Figure 2B). In both cases, PhoR∼P was formed at a similar initial reaction rate but the disappearance of PhoR∼P was much more rapid when ADP was added (Figure 2A,C). Adding buffer containing inorganic phosphate did not affect the level or rate of PhoR∼P formation (Figure 2A,C).

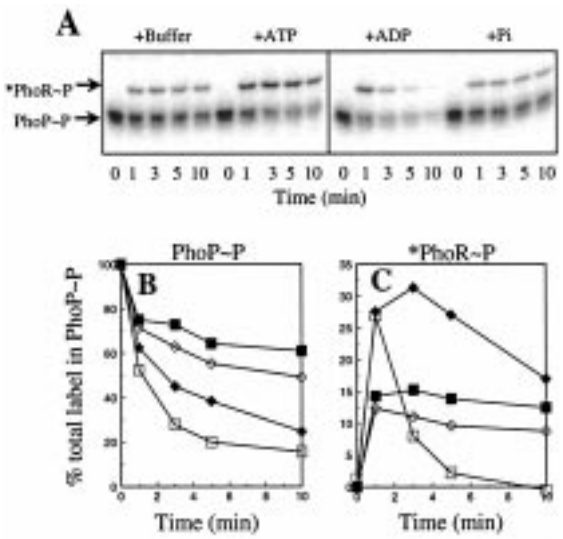

FIGURE 2: Reverse phosphotransfer from PhoP∼P to *PhoR. Panel A: PhoP∼P was purified according to Experimental Procedures. PhoP∼P (0.31 μM) was mixed with an equal molar concentration of *PhoR in a 120 μL reaction mixture containing only P-buffer, P-buffer containing 1 mM ATP, 1 mM ADP, or 1 mM K_2HPO_4−KH_2PO_4 (pH8.0). The 20 μL aliquots of each reaction were taken at 0, 1, 3, 5, and 10 min, as indicated and stopped by addition of 6× SDS sample buffer. The phospho proteins were separated by SDS−PAGE. Panel B: Quantitation of radioactivity on PhoP∼P separated by SDS−PAGE in A. The total radioactivity on PhoP∼P at the initiation of reaction time (0 min) was considered as 100%. Symbols represent the reactions containing only P-buffer (■), or P-buffer plus 1 mM ATP (◆), 1 mM ADP (□), or 1 mM K_2HPO_4−KH_2PO_4 (pH 8.0) (◇). Panel C: Quantitation of radioactivity on *PhoR∼P separated by SDS−PAGE in (A). Symbols as in panel B.

Reverse Phosphorylation Reaction Yields both ATP and Inorganic Phosphate. The effect of adding ADP to the mixture of *PhoR and phospho-PhoP, which resulted in the rapid disappearance of radioactivity on both of the proteins, led us to examine the destination of radioactive phosphoryl group in the reverse phosphotransfer reaction by using thin-layer chromatography. In an experiment similar to that in Figure 2, aliquots of the reaction mixture were taken at various times and the reaction was stopped by adding SDS sample buffer. Samples were spotted on Cellulose PEI plates and developed in 0.25 M potassium phosphate buffer (pH 8.0). From our results shown in Figure 3, it is clear that ATP was generated in the reaction containing PhoP∼P, *PhoR, and ADP but not in the reactions containing either ATP or inorganic phosphate, in the absence of ADP (Figure 3A). This suggests that ADP serves as a receptor for the radioactive phosphoryl group released from the reaction upon mixing PhoP∼P and *PhoR. The reaction also generated inorganic phosphate over time (Figure 3B), suggesting that *PhoR may have a phosphatase activity for PhoP∼P, since either PhoP∼P or *PhoR∼P alone is stable with a half-life well over 2 h (*12*). When PhoP∼P was mixed with equal molar amount of *PhoR, about 30% of the total label on phosphorylated PhoP was released as P_i within 10 min. The rate of the inorganic phosphate release into the reaction mixture containing both PhoP∼P and *PhoR was independent of the inclusion of ATP or P_i. Inclusion of ADP in the reaction slightly decreased the rate of P_i release, possibly due to the competition between the reverse autokinase reaction and the phosphatase reaction of *PhoR (see below).

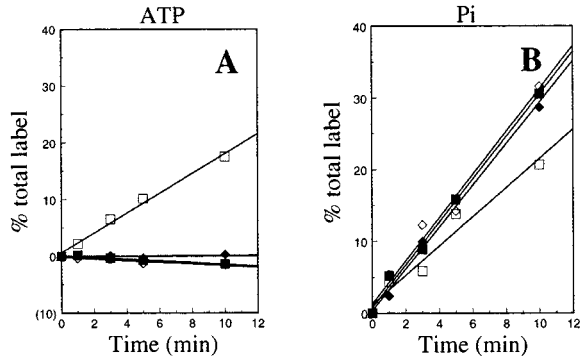

FIGURE 3: In addition to *PhoR∼P, the reactions containing PhoP∼P and PhoR yield Pi and, in the reaction with ADP, ATP. The reverse phosphorylation reaction was carried out as described in Figure 2, panel A. A 2 μL volume of the reaction mixture was used in thin-layer chromatography experiment described in experimental procedure. Formation of radioactively labeled ATP and P_i were quantified by a PhosphorImager. Amounts in the lanes containing the 0 min reaction were used as background. The total radioactivity in the reaction at the initiation of reaction time (0 min) was considered as 100%. Panel A. ATP formation with symbols as in Figure 2B. Panel B. P_i leased in the reverse phosphorylation reaction with symbols as in Figure 2B.

*ATP Is Generated in the Reverse Reaction of *PhoR Autophosphorylation.* To confirm that the source of the ATP generated from the reverse phosphotransfer in the presence of ADP was through *PhoR∼P, *PhoR∼P was purified free of nucleotide and mixed with ADP. Aliquots of the reaction were stopped by the addition of SDS sample buffer and spotted on Cellulose PEI paper. After being developed in 0.25 M potassium phosphate buffer (pH 8.0), the TLC plate was quantitated by a PhosphorImager. The results showed that (Figure 4) inclusion of ADP with *PhoR∼P initiated the reverse autokinase reaction to generate unphosphorylated PhoR and ATP. Mixing PhoP∼P with ADP, however, did not lead to the formation of ATP (data not shown). This result suggested that the ATP observed in Figure 3 was generated by a two-step reaction: the reverse phosphorylation of *PhoR by PhoP∼P, followed by the phosphorylation of ADP by the PhoR∼P.

Effects of Magnesium Ion on the Reverse Phosphotransfer and Dephosphorylation of PhoP∼P. It has been reported that Mg^{2+} is a necessary cofactor of the phosphatase reaction in EnvZ·N347D(C) (*26*). The presence of EDTA chelated Mg^{2+} so that the dephosphorylation of OmpR was inhibited. We examined the role of Mg^{2+} in the reverse phosphotransfer from PhoP∼P to *PhoR by running the reaction in P-buffer in the presence of 10 mM EDTA, with either ATP, ADP, or P_i added. The results differed from those reported for EnvZ·N347D(C). As shown in Figure 5, 10 mM EDTA significantly increased the ratio of PhoR∼P to PhoP∼P. Over 80% of the initial label in PhoP∼P was transferred to *PhoR. All reactions showed similar final levels of reverse transfer from PhoP∼P to *PhoR, which is quite different from the result obtained without EDTA (Figure 2). The presence of ADP in the reaction mixture failed to show a difference from the reactions containing P-buffer, ATP, or inorganic phosphate. In a similar experiment in which the samples were spotted on Cellulose PEI and developed in potassium phosphate buffer, we measured the formation of radioactive ATP and inorganic phosphate. The results showed that the addition of EDTA inhibited the formation of ATP in the reaction

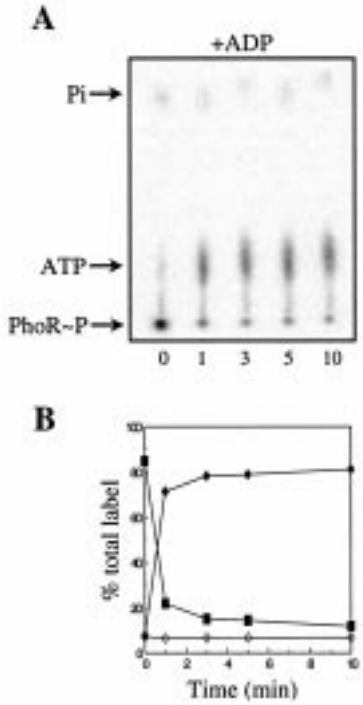

FIGURE 4: Reverse autokinase reaction of *PhoR. Panel A. 0.14 μM radio-labeled *PhoR∼P was mixed with 1 mM ADP in a 120 μL reaction mixture. Aliquots of 20 μL were removed at 0, 1, 3, 5, and 10 min and stopped by adding 5 μL of 6× SDS sample buffer. A 2 μL volume was used in thin-layer chromatography to separate *PhoR∼P, ATP, and P_i. Panel B: Quantitation of (A). Percentages of labeled *PhoR∼P (■), P_i (◇), and ATP (◆) were calculated using total label in the reaction as 100% and plotted.

containing ADP (data not shown). Further, no increase in inorganic phosphate was observed during the 10 min reaction in the presence of EDTA (data not shown). These results showed that the absence of Mg^{2+} did not inhibit the reverse phosphotransfer reaction from PhoP∼P to PhoR, but that Mg^{2+} was required for the reverse autokinase reaction, the forward phosphotransfer reaction (data not shown), and the phosphatase reaction.

Involvement of the PhoR Conserved Autophosphorylation Site, His360, in the Dephosphorylation of PhoP∼P. The reverse phosphorylation from PhoP∼P to *PhoR may or may not occur at the conserved autophosphorylation site, His360. It has been reported that the same histidine residue which was autophosphorylated by ATP was phosphorylated by the cognate phosphorylated response regulator in EnvZ·N347D-(C) (*26*). To examine whether this is also true with *PhoR, we first examined the pH stability of the reverse phosphorylated *PhoR. In a reverse phosphorylation experiment using PhoP∼P and *PhoR, the reaction mixture containing Phospho-*PhoR was subjected to 0.1 or 1 N HCl, 0.1 or 1 N NaOH, or P-buffer only, for 20 min prior to SDS−PAGE analysis and quantitation by a PhosphorImager. The result showed that the phosphor-*PhoR was more stable at alkaline than acidic pH (Figure 6A), which is consistent with the characteristics of a phosphohistidine. The slightly decreased level of ^{32}P-labeled *PhoR phosphate in 1 N NaOH was probably due to peptide bond cleavage than to hydrolysis of the phosphoramidate linkage (*21*). To examine the role of the conserved His360 residue in reverse phosphorylation, we mutated this histidine to glutamine in *PhoR. The mutant protein, *PhoRH360Q, was mixed with purified PhoP∼P in

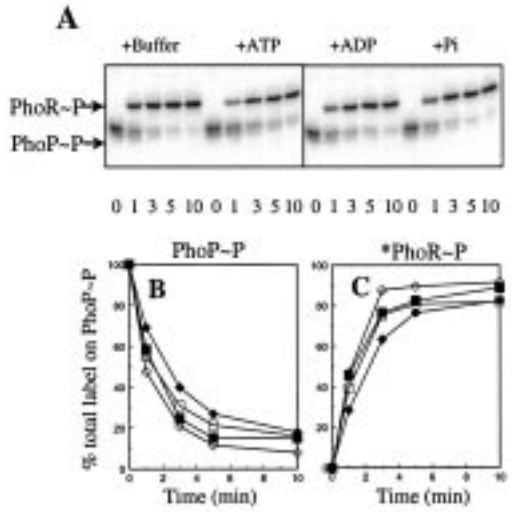

FIGURE 5: Effect of EDTA on the reverse phosphorylation of *PhoR by PhoP∼P. Panel A: PhoP∼P was purified according to Experimental Procedures. PhoP∼P (0.31 μM) was mixed with an equal molar concentration of *PhoR in a 120 μL reaction mixture, in the presence of EDTA (10 mM), containing only P-buffer, P-buffer with 1 mM ATP, with 1 mM ADP, or with 1 mM $K_2HPO_4-KH_2PO_4$ (pH 8.0). The 20 μL aliquots of the reaction were taken at 0, 1, 3, 5, and 10 min, as indicated, and stopped by addition of 5 μL of 6× SDS sample buffer. The phospho proteins were separated by SDS−PAGE. Panel B: Quantitation of radioactivity on PhoP∼P separated by SDS−PAGE in (A). The total radioactivity on PhoP∼P at the initiation of reaction time (0 min) was considered as 100%. Symbols are as in Figure 2B. Panel C: Quantitation of radioactivity on *PhoR∼P separated by SDS−PAGE in (A). Symbols are as in Figure 2B.

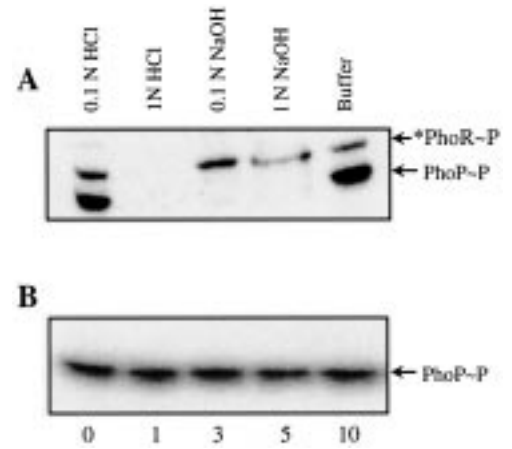

FIGURE 6: Involvement of the conserved His360 in the reverse phosphorylation of *PhoR by PhoP∼P. Panel A: Stability of reverse phosphorylated *PhoR. Purified PhoP∼P (40 μg) (free of GST-*PhoR) was incubated with *PhoR (10 μg) in the presence of 1 mM ATP for 1 min. The reaction was stopped by the addition of 1/5 vol. of 6× SDS sample buffer containing P-buffer, HCl, or NaOH at final concentration (indicated above the lanes) for 20 min. The samples were neutralized with the same concentration of NaOH or HCl and were subjected to SDS−PAGE. The dried gel was exposed to an X-ray film. Panel B: Reverse phosphorylation of *PhoRH360Q. PhoP∼P (0.37 μM) was mixed with an equal concentration of PhoRH360Q in a 90 μL reaction mixture. Aliquots of the reaction were taken at 0, 1, 3, 5, and 10 min, as indicated, and stopped by addition of 5 μL of 6× SDS sample buffer. The phospho proteins were separated by SDS−PAGE. The dried gel was exposed to an X-ray film.

the reverse phosphorylation reaction. In this experiment (Figure 6B), no reverse phosphotransfer was observed with the mutant protein *PhoRH360Q, suggesting that the conserved histidine is important for the reverse phosphotransfer reaction and that the reverse transfer possibly occurs at the same histidine residue. Thin-layer chromatography of a similar experiment did not show any significant difference in inorganic phosphate released in the reaction containing PhoP∼P alone compared to the reaction containing both PhoP∼P and *PhoRH360Q (data not shown), suggesting that the *PhoRH360Q mutant is also inactive in phosphatase function. This latter result is consistent with the observations made in previous studies using similar mutations at the conserved histidine residue in NarX, NarQ (*27*), and EnvZ proteins of *E. coli* (*28*).

DISCUSSION

Our previous study showed that, unlike PhoR of *E. coli*, *B. subtilis* strains carrying *phoR* mutations leading to the deletion of the *N*-terminal membrane-associated domain of PhoR did not cause a constitutively elevated level of Pho regulon gene expression under high phosphate conditions in vivo, nor did it lead to the loss of Pho inducibility under phosphate deprivation conditions (*1*). The mechanism for *B. subtilis* PhoR signal-sensing is not clear as the nature of the signal has not been defined. All reported cases of Pho regulon induction are correlated with low environmental phosphate concentrations. Perception is that the signal from the extracellular environment is received by the *N*-terminal domain of the histidine kinase, as shown in proteins NarX, NarQ (*29*), and FixL (*30*). The recent observation that the *C*-terminal catalytic domain is sufficient for the low phosphate-inducible expression of Pho regulon genes (*1*) suggested that the transmembrane domain of the PhoR protein is not essential for the Pho response, implying either that the *N*-terminal of PhoR may not be the sensor or that there is additional regulation exerted on the *C*-terminal catalytic domain in conjunction with the *N*-terminal sensing. It is therefore possible that the autophosphorylation of the PhoR kinase domain or the phosphotransfer between PhoR and PhoP is controlled by regulation from cellular factors other than, or in addition to, a low phosphate signal.

Three biochemical activities have been shown in the *C*-terminal domain of various sensor histidine kinases (*15*, *21*, *31*): the autokinase, phosphotransfer, and phosphatase activities. The kinase activity of PhoR has been examined in a previous study (*1*, *12*). In this study, we showed that the autophosphorylation and phosphotransfer reactions between PhoR and PhoP are reversible and that PhoR also has a weak PhoP∼P phosphatase activity. Our results clearly showed the formation of reverse phosphorylated PhoR∼P by PhoP∼P and, in the presence of ADP, further phosphotransfer from PhoR∼P to ADP, forming ATP.

Reverse phosphorylation has been observed in several transmitter-receiver pairs. Our result showing reverse phosphorylation leading to the formation of ATP was consistent with the studies of KinA, in which phosphate is transferred from KinA∼P to form ATP in the presence of ADP (*32*). The CheA protein of *Rhizobium meliloti* has also been shown to be reverse phosphorylated by CheY2∼P (*33*). However, our results differed from observations made with several other systems including some of the EnvZ/OmpR family, the family which includes PhoR/PhoP. Attempts to reverse phosphorylate the wild-type EnvZ protein have not succeeded

although certain kinase$^-$/phosphatase$^+$ mutant forms of EnvZ are able to be reverse phosphorylated by OmpR∼P (*26*) and serve as intermediates in the phosphatase reaction. The NR_I−NR_{II} transphosphorylation is considered reversible as ATP is formed in the reaction containing NR_I∼P, NR_{II}, and ADP (*34*). However, the accumulation of reverse phosphorylated NR_{II} is only observed when Mg^{2+} is chelated by EDTA, because the formation of ATP from NR_{II}∼P plus ADP requires Mg^{2+}. This reverse phosphorylated NR_{II} is observed in reactions after nearly 1 h, whereas the reverse phosphorylation of *PhoR or PhoR by PhoP∼P with or without ADP was observed in less than 1 min.

The reverse phosphorylation of the *PhoR protein likely occurs on the same histidine residue that is used for the autophosphorylation of the *PhoR protein. A His360Gln mutant *PhoR not only caused loss of the autophosphorylation activity (data not shown) but also lost the ability to be reverse phosphorylated by PhoP∼P, suggesting the conserved histidine residue is at a minimum important for the reversibility of phosphotransfer, if not the site of reverse phosphorylation. These observations are consistent with the result for EnvZ·N347D(C), in which the conserved histidine was suggested as the site for reverse phosphorylation by limited proteolysis (*26*).

The above examples of phosphotransfer from an aspartyl phosphate to a histidine residue suggest that such transfers are thermodynamically possible between certain cognate two component proteins, but not all. Perhaps this is not surprising when one considers that the environment of the acyl phosphate strongly influences the free energy of hydrolysis of that bond (*35*, *36*). In compounds such as acetyl phosphate the free energy of hydrolysis of the acyl−phosphate bond is −10 to −13 kcal/mol (*37*), whereas the phosphoaspartyl group of response regulators is believed to be considerably more stable due to the conformation change induced by phosphorylation (*35*). The accumulating data seem to suggest that the degree of stabilization achieved upon phosphorylation of the aspartate residue of a response regulator varies with the protein in question. Perhaps this best illustrated in *R. meliloti* which has two CheY proteins, one which can reverse phosphorylate CheA and one which cannot (*33*).

The Mg^{2+} requirement of the PhoR reverse phosphorylation reaction is summarized in Figure 7. Unlike the autophosphorylation of *PhoR or the phosphotransfer from *PhoR∼P to PhoP, the reverse phosphotransfer from PhoP∼P to *PhoR did not require Mg^{2+}. The removal of Mg^{2+} from PhoP∼P and PhoR resulted in a higher percentage of labeled phosphate being transferred to *PhoR. Indeed, Mg^{2+} played an inhibitory role in the reverse phosphorylation from PhoP∼P to PhoR (signal decay), suggesting the possibility that Mg^{2+} levels in vivo could have a regulatory role in Pho signal transduction. A regulatory role for Mg^{2+} has recently been suggested in several other cellular processes (*38*, *39*).

The catalytic domain of many histidine kinases also have a phosphatase activity for the phosphorylated cognate response regulator. These proteins include NarX (*40, 41*), DegS (*42*, *43*), and FixL (*30*). Members of the EnvZ family, including EnvZ, CpxA, and KdpD, also show phosphatase activity in vitro (*16*−*20*). The reverse phosphorylation demonstrated in the recent study of the ArcA−ArcB phosphorelay shows that the signal decay of ArcA∼P proceeds

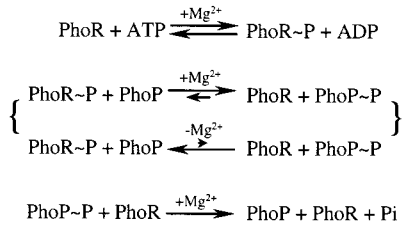

FIGURE 7: Pathways of phosphoryl transfer in the two-component system PhoP/PhoR.

through reverse phosphorylation of the H2 and D1 residues ArcB, leading to the release of inorganic phosphate from D1 (*44*). Our results showed that the phosphatase activity of PhoR is substantially weaker than those above-mentioned histidine kinases known to have a strong phosphatase activity. Adding equal molar amounts of unphosphorylated *PhoR to PhoP∼P led to a slow rate of release of inorganic phosphate into the reaction. This apparent phosphatase activity is likely attributable to the *PhoR protein since the half-life of PhoP∼P or *PhoR∼P alone is more than 2 h (*1*, *12*). Since the stability of purified PhoP∼P (*12*) is similar to acyl−phosphate stability (*21*), it can be argued that the dephosphorylation of PhoP∼P is not achieved by stimulating an existing PhoP autophosphatase activity. The phosphatase activity of *PhoR requires Mg^{2+} as inclusion of EDTA inhibits the release of phosphate (Figure 5), a phenomenon observed in other systems (*16*, *28*, *45*−*47*).

It is not known how phosphate is released from PhoP∼P to achieve the signal decay in vivo during Pho regulation in *B. subtilis*. A mutation that changes the conserved H360 residue to Q led to an inactive *PhoR protein defective in the reverse phosphotransfer (Figure 6) and phosphatase activities (data not shown). A similar mutation in the conserved histidine in EnvZ, NarX, and NarQ inactivate or impair the phosphatase activity in vitro (*27*, *28*). Significantly, further mutagenesis of the histidine residue of EnvZ, NarX, and NarQ (*27*, *28*, *48*) has shown that the conserved histidine residue is important but not required for dephosphorylation of the cognate response regulator, suggesting that the conserved histidine is not a phosphorylated intermediate in the phosphatase reaction (*28*, *48*).

By focusing only on PhoP and PhoR, our results suggest that two different pathways may exist for the signal decay of phosphorylated response regulator PhoP∼P (Figure 7). One involves the reverse reaction of the transphosphorylation between the PhoR and PhoP proteins, which, in the presence of ADP, will lead to the formation of ATP. The other may involve the interaction between PhoP∼P and PhoR, which results in a slow release of inorganic phosphate either via protein−protein interaction that destabilizes the acyl−phosphate bond or via a weak PhoP∼P phosphatase activity of PhoR, although this phosphatase specific activity is quite low compared to that of other sensor-kinases.

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