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Genetic and Biochemical Studies of Phosphatase Activity of PhoR

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In *Escherichia coli*, PhoR is the histidine kinase of the phosphate regulon. It has been postulated that PhoR may function as a phospho-PhoB phosphatase. Experiments with four precise *phoR* deletion mutants supported this hypothesis and suggested that this activity resides within the histidine phosphorylation domain. This biochemical activity was confirmed by using a separately expressed histidine phosphorylation domain.

Two-component signal transduction systems enable many bacteria to adapt to changes in their environments (5, 17). In their simplest forms, these systems are composed of a histidine kinase that receives sensory input and a response regulator protein that controls output. As a stimulus is sensed, the histidine kinase autophosphorylates and donates its phosphoryl group to the response regulator, which becomes phosphorylated on a conserved aspartate residue. The phosphorylated response regulator then effects changes in the cell's physiology, most frequently by modulating transcription.

The Pho system is the two-component system by which *Escherichia coli* responds to changing environmental phosphate levels (23). In response to low-phosphate conditions, more than 30 genes encoding various proteins such as alkaline phosphatase are upregulated. The two components of this system are PhoR (histidine kinase) and PhoB (response regulator). Phospho-PhoB binds to conserved DNA sequences called *pho* boxes (7, 10) and interacts with the σ^{70} subunit of RNA polymerase to control transcription (8, 9). CreC is an additional histidine kinase, not part of the Pho regulon, which can also act as a phosphodonor to PhoB (1, 24).

PhoR is a member of the class I family of histidine kinases in which the site of phosphorylation is adjacent to the conserved catalytic region (4). Another member of this family is the well-studied EnvZ protein, which is involved in the sensory response to changes in environmental osmolarity (14). Structural studies on EnvZ have elucidated the topology of its functional domains (20, 22). By comparing the genetic sequences of PhoR and EnvZ, several homologous domains have been identified (Fig. 1). A catalytic and ATP-binding (CA) domain has been identified between amino acid residues 267 and 431. The dimerization and histidine phosphorylation (DHp) domain extends from amino acid residue 193 to residue 267. Functional regions within these domains have previously been identified from conserved sequences (13, 18, 19).

In addition to the CA and DHp domains, a PAS domain has been identified, comprising amino acids 109 to 196 (21). PAS domains have primarily been observed in signal transduction proteins, such as histidine kinases, in which these domains have been shown to mediate signal transduction through substrate or protein-protein interactions.

The membrane-spanning region of PhoR was previously identified between amino acid residues 10 and 60 (15). It has been suggested that this hydrophobic region spans the membrane twice and does not contain a substantial periplasmic region, which in many other histidine kinases is the site where signals from the environment are recognized (23). Immediately downstream of the hydrophobic membrane region is a sequence of about 35 residues that is predicted to have a high helical content and which contains a central span of six positively charged residues. We have termed this segment the charged region. The function of the charged region is unknown, but its high positive-charge density may allow it to play a role in stabilizing membrane interactions.

Genetic evidence suggests that in addition to being a phosphodonor to PhoB, PhoR may also function as a phospho-PhoB phosphatase under conditions of phosphate sufficiency (16, 23, 24). In the absence of PhoR and under high-phosphate growth conditions, PhoB is activated by CreC (24). If PhoR possesses phosphatase activity, in which domain is this activity localized and how is it regulated? Other histidine kinases also function as phosphatases, and in EnvZ, the DHp domain has been identified as the site of this activity (25).

To further investigate the phospho-PhoB phosphatase activity of PhoR, we chose to genetically and biochemically dissect PhoR. We hypothesized that the functions of individual PhoR domains can be studied by comparing the activities of precise deletions in which one or more domains have been ablated to the activity of the wild-type protein. Four *phoR* deletion mutants were constructed to initiate our investigation: KH500, KH550, KH570, and KH580 (Fig. 1). KH500 contains a complete *phoR* deletion, whereas KH550 is missing the charged region through the CA domain, KH570 is missing the DHp and CA domains, and KH580 is missing only the CA domain. These deletion mutations were made by using a technique recently developed by Datsenko and Wanner (3). In short, linear PCR products containing short homologous sequences to *phoR* flanking a Kan^r gene were recombined into the *Escherichia coli* chromosome by using plasmid-encoded λ Red recombinase gene products. The Kan^r gene was then excised from the *phoR* deletion strains by introducing the temperature-sensitive plasmid pCP20, which expressed the FLP recombinase, allowing it to catalyze the site-directed recombination

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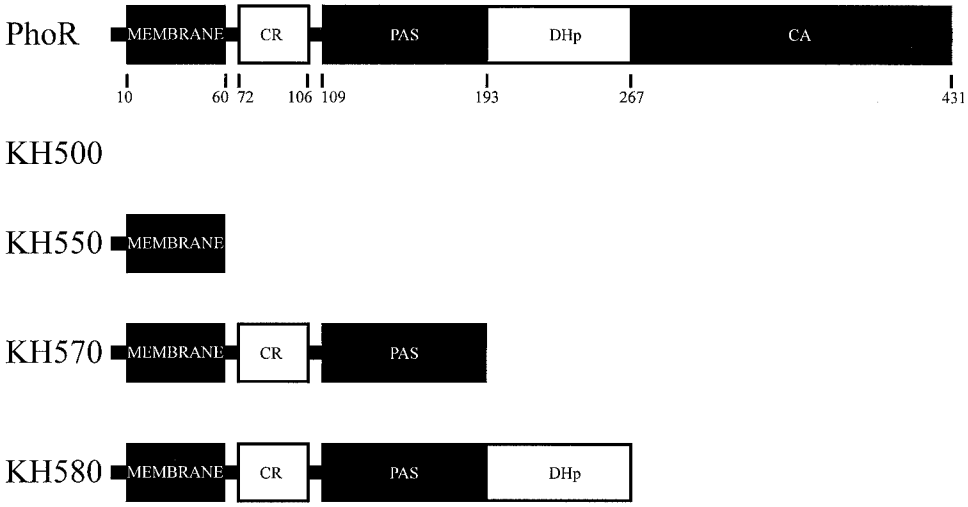

FIG. 1. Domain structure of PhoR. The diagram at the top indicates the predicted protein domains of PhoR, including the membrane-spanning region, the charged region (CR), and the PAS, DHp, and CA domains. The *phoR* deletion mutations presented in this paper are shown below. The diagrams depict the PhoR coding capacity of each of the mutants.

between the two FLP recombinase target sites flanking the Kan^r gene.

Alkaline phosphatase assays, performed as described previously (26), were used to determine the effects of these deletions on the Pho regulon (Table 1). *E. coli* strains BW25113, which is wild type at the *phoBR* locus, and BW25141, a Δ*phoBR* strain, were used as positive and negative controls, respectively. BW25113 showed normal alkaline phosphatase expression when grown under both high-phosphate (4 ± 1.5 [arbitrary units]) and low-phosphate (519 ± 41.2) conditions. BW25141 showed very low alkaline phosphatase expression under both high-phosphate (2 ± 0.6) and low-phosphate (1 ± 0.6) conditions.

To focus on the phosphatase activity of PhoR, alkaline phosphatase assays were performed on deletion strains grown in high-phosphate medium—the growth conditions in which this activity is normally evident. As was previously documented (24), certain *phoR* mutants showed moderate alkaline phosphatase levels when grown in high-phosphate medium. This alkaline phosphatase production was attributed to phosphorylation of PhoB by CreC in the absence of the normally expressed phosphatase activity from PhoR. The *E. coli* strains KH500, KH550, and KH570 each produced moderate levels of alkaline phosphatase. However, strain KH580 showed a marked decrease in alkaline phosphatase levels compared to strains KH550 and KH570 (Table 1). Two possible explanations for this observation are that the truncated PhoR protein expressed in the KH580 strain displayed some phospho-PhoB phosphatase activity within its DHp domain and that the DHp domain in this protein participated in a reverse phosphotransfer reaction of unknown biological relevance.

To further investigate the biochemical activities of the DHp domain, this domain of PhoR as well as the CA domain and a PAS-DHp-CA fragment comprising residues 83 to 431 of PhoR (known hereafter as PhoR*) were cloned into the pBAD/Thio-Topo vector (Invitrogen, Carlsbad, Calif.) for overexpression and purification. DNA that encoded each of these regions of PhoR was amplified from the chromosome by using *Taq* polymerase. The pBAD/Thio-Topo plasmid contained a modified thioredoxin gene upstream of the inserted sequence and a V5 epitope tag and a six-His tag downstream of the inserted sequence that enabled us to purify the fusion protein on an Ni^+ column. Transcription from this plasmid was under the control of the P_{BAD} promoter. The plasmids pDOC1, pDOC2, and pDOC9 encode the CA domain, the DHp domain, and PhoR*, respectively. pDOC1, pDOC2, and pDOC9 were introduced into *E. coli* strain TOP10 cells (Invitrogen) to create strains DC1, DC2 and DC9, respectively. The PhoR fragments were purified from 1-liter overnight batch cultures of each strain grown in LB broth containing 0.02% arabinose.

Preliminary results showed that the CA and DHp protein domains were expressed as inclusion bodies, whereas PhoR* was expressed in a soluble form. Protein purifications were performed with an Ni-nitrilotriacetic acid column (Qiagen, Valencia, Calif.) as directed by the manufacturer. The proteins were identified according to size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and by Western blotting with anti-V5 serum (Invitrogen) to confirm the presence of the V5 epitope.

Since these were novel fusion proteins, we tested their autophosphorylation and phosphotransfer activities before pro-

TABLE 1. Alkaline phosphatase activities in *E. coli* cells harboring different *phoR* deletion mutations

Strain (reference)	Deletion	Alkaline phosphatase activity (arbitrary units ± SD)
BW25113 (3)	None (wild type)	4.0 ± 1.5
BW25141 (3)	Δ*phoBR*	1.0 ± 0.6
KH500	Δ*phoR*, entire gene	56 ± 4
KH550	ΔPAS, DHp, CA	83 ± 5
KH570	ΔDHp, CA	82 ± 9
KH580	ΔCA	21 ± 1

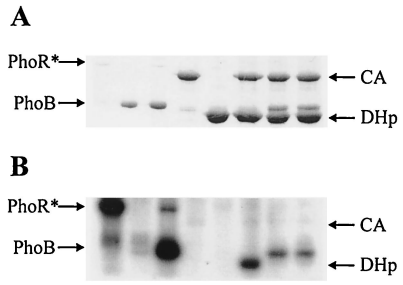

FIG. 2. PhoR*, DHp, and CA domains form functional fusions. The various proteins were incubated in the presence of [γ-^{32}P]ATP and separated by SDS-PAGE. Lane 1 contains PhoR*, lane 2 contains PhoB, lane 3 contains PhoR* and PhoB, lane 4 contains the CA domain, lane 5 contains the DHp domain, lane 6 contains the CA and DHp domains, and lanes 7 and 8 are the CA and DHp domains with PhoB added after a 1-min and a 5-min incubation, respectively. Panel A shows the Coomassie-stained gel, and panel B shows the autoradiogram. The concentration of PhoR* in the reaction was 0.01 mg/ml, whereas the CA and DHp protein concentrations were 0.24 mg/ml and the PhoB concentration was 0.15 mg/ml. The volume of each reaction mix was 10 μl, and the incubation temperature was 25°C. Incubations lasted from 5 min for reactions with PhoR* to 90 min for reactions with the CA and/or DHp fragment. When PhoB was added to a reaction mix, the reaction mixes were incubated for an additional 1 to 5 min. All reactions were stopped by adding 5 μl of loading buffer and placing the reaction mixes on ice; 5-μl aliquots were then loaded and run on an SDS–10% polyacrylamide gel. The gel was dried, exposed to X-ray film for 16 h, and developed.

ceeding with phosphatase experiments. Proteins were incubated in a reaction buffer (20 mM Tris-HCl, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.6) containing 4 μM ATP (10 μCi of [γ-^{32}P]ATP) at room temperature. Following the reactions, products were separated by SDS-PAGE, and the gel was dried and exposed to X-ray film. The PhoR* protein was shown to autophosphorylate in the presence of ATP (Fig. 2, lane 1). This phosphate could then be transferred to PhoB (lane 3), which was unable to autophosphorylate in the presence of ATP (lane 2). Both the CA and the DHp protein domains were incubated separately and together in the presence of ATP. Neither the CA domain (lane 4) nor the DHp domain (lane 5) could autophosphorylate. However, when incubated together with ATP, phosphorylation occurred on the DHp protein domain (lane 6). When PhoB was added to the reaction mix, all the phosphate on the DHp fragment was transferred to PhoB within 1 min (lane 6). These results are similar to those from experiments done with individual domains of EnvZ (12). These results also demonstrate that the individual domains fused to the thioredoxin/six-His tag maintain biochemical activity and suggest that they are folded correctly.

DHp domain of PhoR has phosphatase activity. To test whether the DHp domain of PhoR harbors a phospho-PhoB phosphatase activity or if it participates in a simple reverse phosphotransfer reaction, it was incubated in the presence of phospho-PhoB. A prediction of the reverse phosphotransfer model is that the DHp domain would become phosphorylated as phospho-PhoB lost its phosphate. PhoB was phosphorylated by acetyl-[^{32}P]phosphate as described previously (11). Then 15 μM phospho-PhoB was incubated by itself or with DHp (10 μM) or CA (7.5 μM) fusion proteins, and samples were removed at various times thereafter. The reaction mixes were separated by SDS-PAGE, and the gels were dried. A Bio-Rad (Hercules, Calif.) Molecular Imager was used to estimate the amount of phosphorylated PhoB in each reaction by exposing the dried gel to a phosphor screen. The data were analyzed by regression analysis to an equation for first-order exponential decay.

It was observed that the half-life of phospho-PhoB was 13.4 min (Fig. 3B), which is very similar to previously published values (11). When phospho-PhoB was incubated with the DHp domain, there was a decrease in the half-life of phospho-PhoB to 7.4 min. As a control to determine if other fusion proteins also decreased the half-life of phospho-PhoB, phospho-PhoB was incubated with the CA domain fusion protein, which showed no decrease in the half-life of the phospho-PhoB. Figure 3A shows that as phospho-PhoB was dephosphorylated in the presence of the DHp domain, the DHp domain did not become phosphorylated. This result provides evidence that the DHp domain was not a substrate for a reverse phosphotransfer reaction. However, these experiments cannot rule out a transient DHp phosphorylation. Taken together with the phosphotransfer experiments reported above, in which the DHp fusion protein was stably phosphorylated, we believe that the simplest explanation of these results is that this domain possesses a phospho-PhoB phosphatase activity and is not functioning as a substrate for reverse phosphotransfer.

This is the first report describing this biochemical activity in PhoR. Our conclusions are consistent with previous results from other laboratories in which several *phoR* mutations within the proposed DHp domain were defective in their inhibitory activity (phosphatase activity) but not in their ability to activate

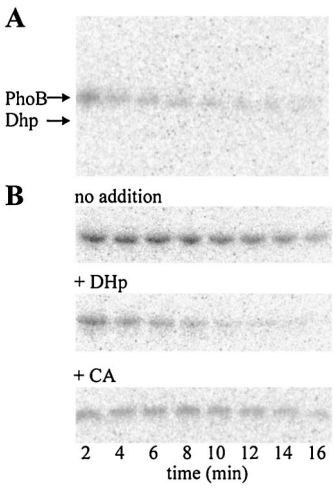

FIG. 3. Phosphatase assay. Phospho-PhoB was prepared as previously described (11). In each reaction, 35 μg of [^{32}P]phospho-PhoB was incubated with the CA or DHp fusion protein at 37°C in 20 mM Tris-HCl (pH 7.0)–50 mM NaCl–10 mM $MgCl_2$. At 2, 4, 6, 8, 10, 12, 14, and 16 min, 10 μl was removed from each reaction mix, mixed with 5 μl of loading buffer, and placed on ice. When all reactions were completed, 8 μl of each reaction mix was separated on an SDS–10% polyacrylamide gel. The dried gel was analyzed with a Molecular Imager FX system from Bio-Rad. (A) The full-length gel is shown for a reaction with the DHp domain. (B) Only the phospho-PhoB band is shown for reactions with PhoB by itself, with the DHp domain plus PhoB, and with the CA domain plus PhoB.

PhoB (16, 23). It is noted that the phosphatase activity observed in the in vitro experiments was quite modest, which is consistent with the modest effects observed in vivo (Table 1). This may reflect that this activity is normally expressed as part of a full-length protein and that other domain contacts are involved in optimizing this activity or that the fusion protein prevents full activity.

How phosphatase activity is regulated is unclear. It is interesting that the PhoR* construct, consisting of the PAS, DHp, and CA domains, did not display phosphatase activity (data not shown). This kinase-only phenotype has previously been observed in PhoR and other histidine kinases that had their periplasmic domains removed (2, 6, 16). These results suggest that other domains of histidine kinases may interact with their DHp domains to control the opposing kinase and phosphatase activities.

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D.O.C. and K.H. contributed equally to this work.

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