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Analysis of the conserved acidic residues in the regulatory domain of PhoB

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Abstract The PhoB protein from *Escherichia coli* is a member of the two-component signal transduction pathway that controls an adaptive response to limiting phosphate. Activation involves its phosphorylation on a conserved aspartate. Site-directed mutations were introduced at conserved acidic residues. The E9D, D10E, D10N, E11A, E11D and E11Q mutants were each able to induce alkaline phosphatase under low phosphate growth conditions whereas the E9A, D10A, D53A, D53E and D53N could not. The E9Q mutant was constitutively active. Phosphorylation assays showed that only the E9D, E11A, E11Q and E11D mutants were phosphorylated by acetyl phosphate. Most mutants also displayed defects in magnesium binding.

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Key words: PhoB; Response regulator; Aspartate phosphorylation; Magnesium binding; Signal transduction; *Escherichia coli*

1. Introduction

Two-component regulatory systems are integral parts of numerous signaling pathways in many organisms. For example, there are over 20 two-component systems in *Escherichia coli* alone [1–4]. These systems operate to transduce extracellular signals into the cell and also to propagate signals within the cell. In their most basic forms, two-component regulatory systems consist of a histidine kinase and a response regulator [5]. The histidine kinase is generally a transmembrane protein that has extracellular and intracellular domains. This structural organization allows the kinase to act simultaneously as an environmental sensor and as a signal transducer. The response regulator is generally a cytoplasmic transcription factor [2]. In most cases, the mechanism of signal transduction involves the autophosphorylation of a histidine kinase in response to an extracellular signal and the subsequent donation of this phosphate to a response regulator protein which becomes phosphorylated on a conserved aspartate residue [3–5]. The catalytic residues for phosphotransfer reside entirely within the response regulator protein [6]. Phosphorylation activates the response regulator enabling it to carry out its intended function in the cell.

Bacterial response to changes in extracellular phosphate levels is mediated by the Pho system, a two-component system comprised of PhoR and PhoB [7–10]. PhoR is the histidine kinase and it senses extracellular phosphate levels through the phosphate-specific ABC transporter, encoded by the *PstSCAB* genes, and an ancillary protein, PhoU [11]. When phosphate becomes limiting, PhoR autophosphorylates on a histidine residue [12]. Under phosphate sufficient conditions, PhoR most likely dephosphorylates phospho-PhoB [13]. In addition to phospho-PhoR, phospho-CreC and acetyl phosphate also donate phosphoryl groups to PhoB [13]. PhoB is the response regulator of the Pho system. It is a 26.3-kDa DNA-binding protein comprised of two domains [14,15]. The N-terminal domain contains the catalytic site which is comprised of residues that are highly conserved throughout the response regulator family. A prominent feature of these proteins is an acidic pocket where phosphorylation occurs [5]. The structure of this domain can be modeled upon the structure of CheY, a single-domain chemotaxis protein [16–19]. The C-terminal domain of PhoB is similar in structure to the C-terminal DNA-binding domain of OmpR, a member of the subfamily of response regulators to which PhoB belongs [20–22]. In order for PhoB to be activated, Asp-53 must be phosphorylated. A divalent metal cation is essential for phosphorylation [23–25]. In CheY, coordination of the divalent metal cation configures the acidic pocket for catalysis [26]. Physiologically, an Mg^{2+} ion is bound in CheY, although other metals have been shown to bind in vitro [23,27]. The crystal structure of the CheY:Mg^{2+} complex suggests that the Mg^{2+} ion is involved in anionic charge shielding and in stabilizing a transition state intermediate during phosphorylation [26]. In PhoB, activation induces a conformational change that allows it to form a dimer which binds to DNA at specific sequences termed the *pho*-box, interact with the σ70-subunit of the RNA polymerase and induce transcription of Pho regulon genes [22,28,29]. This regulon is comprised of over 30 genes including *phoA*, which encodes alkaline phosphatase [7].

In this paper we have examined the nature of the acidic pocket by creating and analyzing site-directed mutants of the conserved acidic residues within the pocket. We have investigated the physiological response of these mutants to phosphate limitation. We have also examined these mutants in vitro, testing for phosphorylation and magnesium binding.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

DH5α (Promega) or XL1-Blue (Stratagene) were the *E. coli* strains used for routine cloning procedures. DWE1 is a *recA* derivative of ANCL1 and was constructed by P1 generalized transduction from CSH126 [36,37]. Antibiotics, when required, were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol and tetracycline at 40 μg/ml and kanamycin at 50 μg/ml. DWE1 derivatives were grown at 30°C in modified MOPS minimal media containing either 5 mM (high phosphate) or 0.2 mM (low phosphate) [30].

pMP1 was constructed by amplifying the *phoB* gene plus 750 basepairs of flanking DNA using the polymerase chain reaction with primers containing a 5′ *Sac*I site and a 3′ *Hin*dIII site. These restriction sites were used to clone the gene into pIB307, a chloramphenicol resistant, low copy number, temperature sensitive vector [31].

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2.2. Site-directed mutagenesis

Using the QuikChange kit from Stratagene, point mutations were introduced into the pT7PhoB plasmid [29]. Mutagenic primers for each reaction were synthesized by Genemed Synthesis (Table 1). Mutagenesis was verified by cycle sequencing using a LI-COR 4000L automated DNA sequencer.

2.3. Alkaline phosphatase assay

Cells were grown in MOPS minimal media and after sufficient growth was observed, 1 ml of culture was removed and the cells were harvested by centrifugation. The cells were resuspended in 1 ml 1 M Tris-HCl, pH 8.2. Two drops of chloroform and 1 drop of 0.1% SDS were added to each sample which was then vortexed for 1 min. 500 μl of cells were mixed with 500 μl of 4 mM *para*-nitrophenylphosphate in 1 M Tris-HCl, pH 8.2. The reaction was incubated at 37°C until it turned yellow at which time 400 μl of 1 M KH_2PO_4 were added to stop the reaction. Arbitrary alkaline phosphatase units were calculated using the following equation: AP units = $(OD_{420} \times 1000)/(OD_{600} \times \text{time})$.

2.4. Overexpression, purification and phosphorylation of PhoB and mutant derivatives

pT7PhoB or its mutated derivatives were transformed into BL21(DE3)pLysS cells (Stratagene) and grown overnight at 37°C in 50 ml of LB broth with ampicillin. This overnight culture was used to inoculate 4 l of fresh LB ampicillin broth to an initial OD_{600} of 0.05. The cultures were incubated in baffled flasks with aeration until the OD_{600} reached 0.1. Overexpression was induced by the addition of 0.2 mM IPTG and the cells were harvested after three additional hours of growth. Cell pellets were resuspended in 25 ml of Buffer A (25 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 100 μM DTT, pH 7.2) and cells were disrupted by sonication for 3–5 min followed by centrifugation to remove cell debris. PhoB was precipitated from the supernatant with 70% ammonium sulfate at 0°C following which the sample was dialysed overnight against Buffer A. The protein was purified from the dialysed sample using a Bio-Rad Biologic System by loading the sample onto a phosphocellulose column and eluting the protein with a linear gradient of Buffer B (25 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 100 μM DTT, pH 7.2). A sufficient volume of 25 mM Tris-HCl, pH 7.2 was added to the pooled PhoB fractions to bring the conductivity of the sample to that of Buffer A. PhoB was further purified on a Bio-Rad Q column equilibrated in Buffer A and eluted with a linear gradient of Buffer B. The purity of PhoB was verified by SDS-PAGE. Protein samples were quantified with the Bio-Rad Protein Assay. Acetyl [^{32}P]phosphate was prepared and proteins were phosphorylated as described elsewhere [29].

2.5. Fluorescence quenching assay

A 3-ml sample containing 0.05 mg of purified protein in 50 mM Tris, pH 7.0, was assayed for changes in fluorescence intensity with a Photon Technology International QM1 fluorescence spectrophotometer. Assays were carried out at 20°C in a temperature-controlled, continuously stirred chamber. Fluorescence emission was monitored at 348 nm following excitation at 295 nm. $MgCl_2$ was added stepwise in aliquots to a maximum dilution of 3% and emission readings were corrected for dilution.

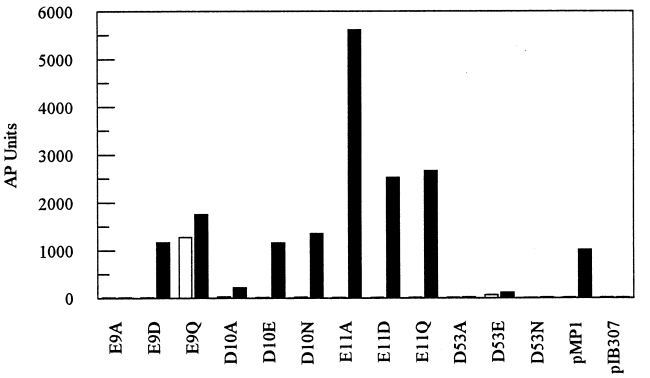

Fig. 1. Alkaline phosphatase assays of PhoB and mutant derivatives. High phosphate indicated by black bars and low phosphate indicated by white bars.

3. Results

To probe the role of each acidic residue in the active site, mutations were introduced by site-directed mutagenesis at residues Glu-9, Asp-10, Glu-11 and Asp-53. Each amino acid was mutated to an alanine, to its corresponding amide residue (Asn for Asp or Gln for Glu) and to its complementary acidic residue (Glu for Asp or Asp for Glu).

3.1. Physiological responses of PhoB mutants

To determine the effects of the mutations upon the activity of the PhoB protein, each mutation was introduced into the low copy number plasmid pMP1 and transformed into the *recA phoB* strain, DWE1. Cells were grown overnight in minimal media containing either high or low phosphate and were assayed for their ability to induce alkaline phosphatase (Fig. 1). A functional PhoB protein was indicated by the appropriate induction of alkaline phosphatase (low levels in high phosphate and high levels in low phosphate). Cells containing pMP1 showed proper induction of alkaline phosphatase whereas the parent vector, pIB307, did not. Nearly wild-type induction was observed with plasmids carrying the E9D, D10E and D10N mutations. An elevated level of induction was observed with each of the mutations at position 11 whereas negligible induction was seen with the E9A, D10A, D53A, D53E and the D53N mutations. The E9Q mutation produced a constitutive phenotype for alkaline phosphatase expression. The simplest explanation for the above results is that those mutants that allowed normal induction of alkaline

Table 1
Primer sequences and plasmid names of site-directed mutants

Mutant	Primer sequence	pT7PhoB plasmid	pMP1 plasmid
E9A	5′-TTCTGGTCGTAGCAGATGAAGCTCC-3′	pCZ3	pDC1
E9D	5′-TTCTGGTCGTAGACGATGAAGCTCC-3′	pBM5	pDC9
E9Q	5′-ATTCTGGTCGTACAAGATGAAGCTC-3′	pCZ4	pDC11
D10A	5′-TGGTCGTAGAAGCTGAAGCTCCAAT-3′	pCZ9	pDC2
D10E	5′-GGTCGTAGAAGAGGAAGCTCCAATT-3′	pCZ5	pDC3
D10N	5′-CTGGGTCGTAGAAAATGAAGCTCCAA-3′	pCZ10	pDC4
E11A	5′-TCGTAGAAGATGCAGCTCCAATTCG-3′	pCZ11	pDC5
E11D	5′-CGTAGAAGATGACGCTCCAATTCGC-3′	pCZ6	pDC6
E11Q	5′-GTCGTAGAAGATCAAGCTCCAATTC-3′	pCZ7	pDC7
D53A	5′-TAATTCTCCTCGCCTGGATGTTACC-3′	pCZ12	pDC8
D53E	5′-AATTCTCCTCGAATGGATGTTACCT-3′	pCZ8	pDC10
D53N	5′-TTAATTCTCCTCAACTGGATGTTAC-3′	pCZ2	pDC12

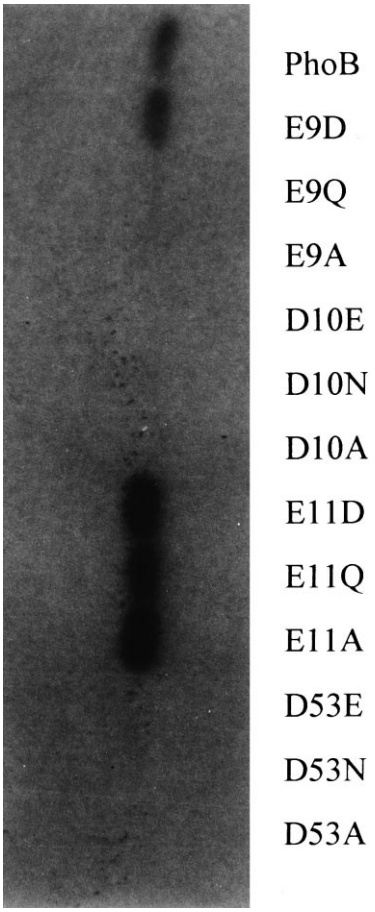

Fig. 2. Phosphorylation of wild-type and mutant proteins with acetyl [^{32}P]phosphate. Equal molar amounts of wild-type and mutant PhoB incubated in the presence of acetyl [^{32}P]phosphate. Samples were separated on a 12.5% SDS-polyacrylamide gel and exposed to X-ray film.

phosphatase are normal in their phosphorylation activities whereas those that did not induce are defective.

3.2. Phosphorylation of mutant proteins

To further study the effects of these mutations on PhoB function, they were introduced into a *phoB* expression vector for over-expression and purification [29]. Phosphorylation assays, using acetyl [^{32}P]phosphate as phospho-donor, were carried out as outlined previously [25,29]. The proteins were incubated in the presence of 10 mM acetyl [^{32}P]phosphate and 10 mM $MgCl_2$ for 60 min. The reaction mixtures were then separated by SDS-PAGE and subsequently exposed to X-ray film. Fig. 2 shows that PhoB, the E9D and each of the mutant proteins at position 11 were phosphorylated by acetyl phosphate. These reactions each required magnesium as phosphorylation was not observed in the presence of 5 mM EDTA (data not shown). Each of the Glu-11 mutants showed higher steady-state levels of phosphorylation than PhoB. This high level of phosphorylation is consistent with the elevated alkaline phosphatase induction levels observed with these same mutations.

3.3. Metal binding

To examine the basis for the phosphorylation defects observed with the mutant proteins, we developed a fluorescence

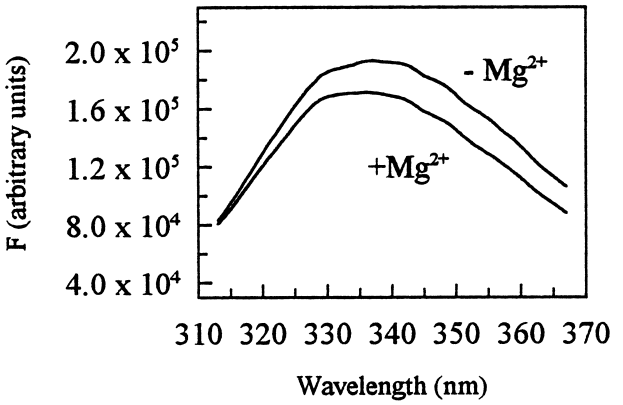

Fig. 3. Fluorescence quenching experiments. The upper line is the fluorescence of purified PhoB before the addition of Mg^{2+} and the lower line is the fluorescence of PhoB after the addition of Mg^{2+} to a final concentration of 30 mM.

assay for metal ion binding. This assay is similar to that used by Lukat et al. to measure metal binding in the CheY protein [23]. For both proteins, the assay is made possible by the presence of a tryptophan residue immediately adjacent to the site of phosphorylation [15,32]. In PhoB, Trp-54 serves as a reporter for events at the active site [29]. The fluorescence emission spectrum of PhoB is presented in Fig. 3. The addition of Mg^{2+} to purified PhoB caused significant quenching of the fluorescence signal. We used this fluorescence quenching to determine a K_D value for metal binding by fitting the fluorescence data from the step-wise additions of magnesium to the following equation [33]:

$$\frac{F_0 - F}{F_0} = \frac{f_a \times K_{SV} \times [Q]}{1 + (K_{SV} \times [Q])}$$

where F_0 and F are the fluorescence in the absence and presence of metal, respectively. f_a is the fraction of the initial fluorescence that is accessible to the metal ion and K_{SV} is the Stern-Volmer constant in mM^{-1}. In these experiments, K_{SV} equals the reciprocal of the dissociation constant, K_D. [Q] is the metal ion concentration in mM. These data indicate

Table 2
Fluorescence analysis of Mg^{2+} binding for PhoB and its mutant derivatives

Protein	Change in fluorescence	K_D (mM Mg^{2+})
PhoB	Q[a]	2.0 ± 0.5
E9A	N.C.[b]	N.D.[c]
E9D	Q	0.1 ± 0.01
E9Q	E[d]	N.D.
D10A	E	N.D.
D10E	E	N.D.
D10N	E	N.D.
E11A	Q	10.5 ± 3.3
E11D	N.C.	N.D.
E11Q	N.C.	N.D.
D53A	E	N.D.
D53E	E	N.D.
D53N	E	N.D.

[a]Q: quenching of fluorescence.
[b]N.C.: no change in fluorescence.
[c]N.D.: K_D values not determined.
[d]E: enhancing of fluorescence.

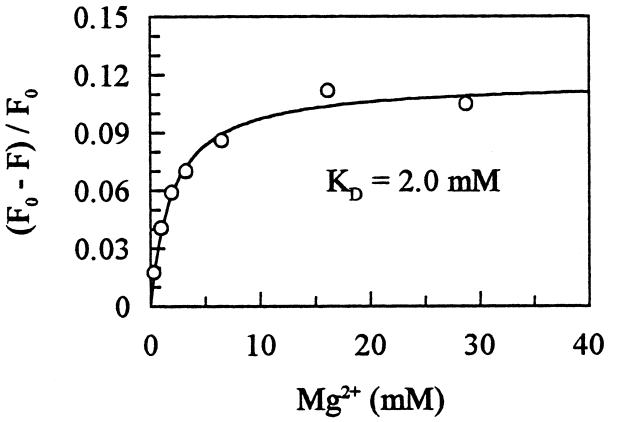

Fig. 4. Stern-Volmer plot for PhoB.

that PhoB has a K_D for Mg^{2+} of 2.0 ± 0.5 mM (Fig. 4 and Table 2).

The fluorescence data for the mutant proteins is presented in Table 2. Only two of the mutant proteins, E9D and E11A, quenched fluorescence upon the addition of metal. The fluorescence of the E9A, E11D and the E11Q proteins did not change. Increased fluorescence was observed with the E9Q protein and each of the mutants at positions 10 and 53. Dissociation constants for Mg^{2+} binding were only determined for the two mutant proteins that quenched fluorescence (Table 2). The E9D mutant bound metal 20 times more tightly than PhoB whereas the E11A mutant bound metal five-fold less tightly.

4. Discussion

Activation of the PhoB protein involves first its phosphorylation at Asp-53 and subsequently conformational changes that permit dimerization and enhanced DNA binding [28,29]. In this paper, we have studied the role in activation of the conserved acidic residues within the acidic pocket of PhoB by generating and analyzing site-specific mutants.

4.1. Classes of mutants

Four classes of mutants were observed. The first class consists of those mutants that showed at least a normal induction of alkaline phosphatase activity upon phosphate starvation and that were phosphorylated by acetyl phosphate (E9D, E11A, E11D and E11Q). Each of the E11 mutations caused a hyper-induction of alkaline phosphatase and an elevated steady-state level of phosphorylation. The phenotypes of the E11 mutations may reflect an increased rate of phosphorylation and/or a decreased rate of dephosphorylation. The second class of mutants showed normal induction but were not phosphorylated by acetyl phosphate. This class includes the D10E and D10N mutants. The most likely explanation for this class of mutants is that they become phosphorylated in the presence of phospho-PhoR (PhoB's primary phospho-donor) but not with acetyl phosphate. Correspondingly, residue D10 may play an important role in substrate discrimination. The third class consists of a single mutant, E9Q, that showed constitutive alkaline phosphatase activity and was not phosphorylated by acetyl phosphate. Mutations in the acidic pocket leading to constitutively active proteins have been observed in other response regulators. For example, the introduction of a lysine residue in place of Asp-13 of CheY causes a tumbly phenotype in *E. coli* [34]. The E9Q mutant may show phosphorylation-independent activation or perhaps may be phosphorylated by PhoR but is a poor substrate for PhoR-mediated dephosphorylation. The final class of mutants did not express alkaline phosphatase and were not phosphorylated. This group consists of the E9A, D10A, D53A, D53E and D53N mutants.

4.2. Metal binding

It has been previously demonstrated that PhoB requires a divalent cation for phosphorylation [25]. PhoB binds Mg^{2+} with a K_D of 2.0 ± 0.5 mM. This value is approximately two- to five-fold greater than that determined for CheY [23,27]. This difference probably reflects the alternate amino acid residues in PhoB at positions that have been shown in CheY to coordinate the divalent cation. In CheY, the octahedral coordination sphere for Mg^{2+} is comprised of side chain carboxyl atoms from Asp-13 and Asp-57 and the backbone carbonyl of Asn-59 [26,35]. The three additional coordination sites arise from water molecules bound within the acidic pocket. One of these water molecules is bound by Asp-12. A comparison of the amino acid side chains involved in metal binding between CheY and PhoB reveals that they are identical except for the substitution of a glutamate residue at position 9 of PhoB which corresponds to Asp-12 of CheY [15,32]. The E9D PhoB mutant differs from the wild-type protein by having one less methylene group in the side chain and therefore more closely reflects the CheY structure. This mutant bound metal much more tightly than PhoB and may reflect that the metal was coordinated through a water molecule, similarly to CheY. In the wild-type protein, the additional methylene group at position nine may cause the metal ion to be complexed directly with the carboxylate group. This coordination may result in weaker binding because of stearic constraints. It should be noted that a CheY mutant that changed Asp-12 to a glutamate residue, making it identical to PhoB at this site, bound metal less well than the wild-type protein [26].

The metal binding characteristics of the other mutant proteins were also investigated. The E11A protein was the only other protein that showed fluorescence quenching. This protein had a binding constant about five times greater than PhoB. Although this position is not directly involved in the Mg^{2+} coordination sphere, it did influence metal binding. The remainder of the mutant proteins did not quench fluorescence upon the addition of magnesium. We did not determine binding constants from this data because the fluorescence enhancement may be due to anomalous metal binding at alternate sites. However, we assume that at least the E11D and E11Q proteins bound magnesium because they were phosphorylated by acetyl phosphate in an Mg^{2+}-dependent manner. Most of the mutations that result in either fluorescence enhancement or in no change in fluorescence alter the metal binding parameters of PhoB and affect its phosphorylation. However, not all of the mutant proteins that display aberrant fluorescence upon interaction with Mg^{2+} show defects in metal binding as determined by phosphorylation in the presence of acetyl phosphate. This observation underlines the caution that must be exercised when testing mutant proteins with techniques that depend upon an intrinsic characteristic (such as tryptophan fluorescence) of the wild-type protein.

In summary, the conserved acidic residues in the response

regulator domain of PhoB have important functions in the activation of PhoB. Glu-9, Asp-10 and Asp-53 are directly involved in metal binding and consequently mutations in these residues affect phosphorylation. Alanine substitutions at each of these positions inactivate the protein. Asp-10 may play an additional role in substrate recognition. Asp-53 is the site of phosphorylation and must therefore function as a nucleophile on an incoming phosphorus atom. Glu-11 influences metal binding, but mutations at this site do not preclude it. It seems likely, however, that Glu-11 influences catalysis because mutations at this site affect rates of phosphorylation and/or dephosphorylation.

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