Characterization of the Bacterial Sensor Protein PhoQ

EVIDENCE FOR DISTINCT BINDING SITES FOR Mg$^{2+}$ AND Ca$^{2+}$

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The PhoP/PhoQ two-component regulatory system governs several virulence properties in the Gram-negative bacterium Salmonella typhimurium. The PhoQ protein is a Mg$^{2+}$ and Ca$^{2+}$ sensor that modulates transcription of PhoP-regulated genes in response to the extracellular concentrations of these divalent cations. We have purified a 146-amino acid polypeptide corresponding to the periplasmic (i.e. sensing) domain of the PhoQ protein. Mg$^{2+}$ altered the tryptophan intrinsic fluorescence of this polypeptide whereas Ba$^{2+}$, which is unable to modulate transcription of PhoP-regulated genes, did not. Mg$^{2+}$ was more effective than Ca$^{2+}$ at repressing transcription of PhoP-activated genes in vivo. However, maximal repression was achieved when both cations were present. An avirulent mutant harboring a single amino acid substitution in the sensing domain of PhoQ exhibited lower affinity for Ca$^{2+}$ but similar affinity for Mg$^{2+}$. Cumulatively, these experiments demonstrate that Mg$^{2+}$ can bind to the sensing domain of PhoQ and establish the presence of distinct binding sites for Mg$^{2+}$ and Ca$^{2+}$ in the PhoQ protein.

Two-component regulatory systems often mediate the adaptive response of bacteria to new environmental conditions (1–4). These systems generally consist of a sensor protein that, in response to specific chemical or physical signals, modifies the transcription factor whose affinity for DNA is modulated by phosphorylation. Sensor proteins are usually conserved in their C-terminal, cytoplasmic domain, which mediates the phosphorylation/dephosphorylation of the cognate regulatory proteins (5). On the other hand, the N-terminal, periplasmic domain of sensors is often involved in signal sensing and, therefore, confers specificity to each system.

The PhoP/PhoQ two-component system governs several virulence properties in the Gram-negative bacterium Salmonella typhimurium (6, 7). The PhoQ protein is a Mg$^{2+}$ sensor that, in the presence of millimolar concentrations of this divalent cation, represses transcription of some 25 different PhoP-regulated loci (8, 9). Several of these genes are essential for growth in low Mg$^{2+}$ environments, consistent with Mg$^{2+}$ deprivation being the regulatory signal that activates the PhoP/PhoQ system (9). Ca$^{2+}$ and Mn$^{2+}$ can replace Mg$^{2+}$ to repress transcription of PhoP-activated genes, but other divalent cations, including Ni$^{2+}$, Cu$^{2+}$, and Ba$^{2+}$, have no effect (8). The regulatory role of the PhoP/PhoQ system is not limited to Salmonella pathogenesis, because several PhoP-regulated loci are not essential for virulence in mice (10, 11), and phoP-PhoQ hybridizing sequences have been detected in a wide variety of non-pathogenic Gram-negative species (12).

The PhoQ protein features two transmembrane regions, a long cytoplasmic tail, and a large periplasmic domain rich in acidic residues that could be involved in binding divalent cations (Ref. 13; Fig. 1). Several lines of experimental evidence suggest that the regulatory effect of Mg$^{2+}$ results from direct conformational changes provoked in the periplasmic domain of the PhoQ protein. First, a protein chimera in which the periplasmic domain of PhoQ was replaced by the corresponding region of the osmolarity sensor EnvZ lost the capacity to respond to Mg$^{2+}$ (8). Second, Mg$^{2+}$ modifies the trypsin susceptibility of the PhoQ protein in vitro at the same concentrations that are required to repress transcription of PhoP-activated genes in vivo (8). Finally, the changes in trypsin susceptibility in the PhoQ protein were detected in spheroplasts, which indicates that soluble components from the periplasmic space are not necessary for the Mg$^{2+}$-mediated effect (8).

In this paper, we demonstrate that the sensing domain of the PhoQ protein specifically binds Mg$^{2+}$. Furthermore, we establish that the PhoQ protein has distinct binding sites for Ca$^{2+}$ and Mg$^{2+}$, and we identify a mutant PhoQ protein that is differentially altered in its response to Ca$^{2+}$. Our data indicate that the PhoP/PhoQ system mediates the response to changes in the environmental levels of Ca$^{2+}$ and Mg$^{2+}$ and suggest a model in which binding of these divalent cations to the periplasmic domain of the PhoQ protein promotes a conformation that is unfavorable for the activation of the PhoP protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—S. typhimurium strains EG9065 (psiD::MudJ) and EG9564 (psiD::MudJ pho24) have been described (8). Escherichia coli strain EG9649 is BL21(DE3) carrying plasmid pT7-7Qp. Plasmid pT7-7Qp, which harbors the DNA region encoding the sensor domain of PhoQ under the control of the T7 $\alpha$ promoter, was constructed in two steps. First, a DNA fragment comprising the region encoding the sensing domain of PhoQ (codons 45–190) was generated by the polymerase chain reaction using primers F05, 5'-AGGAATTCCGACATGATAAAAACCCATTTTC-3', and F06 5'-TGGCAAGGTATCAGGACGCGTTTAG-3', and plasmid pEG9721 (14) as template. The polymerase chain reaction product was digested with NcoI and HindIII and cloned between the NcoI and HindIII sites of plasmid pMON5907 (a generous gift of Stephen Lee (15)) to form pEG7242. The primary sequence of phoQ in plasmid pEG7242 was confirmed by DNA sequence analysis. Second, pEG7242 plasmid DNA was digested with NcoI, the 5'-protruding ends were filled in with the Klenow fragment of DNA polymerase and dNTPs and then digested with HindIII. The resulting 440-base pair fragment was purified and cloned between the XclI (filled in) and HindIII sites of plasmid pT7-7 to form pT7-7Qp. Plasmid pT7-7Qp harbors an ATG initiation codon 11 base pairs downstream of the ribosome binding site in pT7-7. The ATG is followed by the DNA sequence corresponding to codons 45–190 of phoQ and by the translation termination codon TAA.
Bacteria were grown at 37 °C in Luria broth (LB (16)) or in N-minimal medium containing 0.1% casamino acids, 38 mM glycerol, and the indicated concentrations of MgCl₂ and CaCl₂ (17). Ampicillin and isopropyl-β-D-thiogalactopyranoside were used at 50 μg/ml and 0.7 mM, respectively.

Purification of the Sensor Domain of PhoQ—The sensor domain of the S. typhimurium PhoQ protein (residues 45–190 preceded by an N-terminal Met; PhoQp) was purified from E. coli strain EG9649. Expression of PhoQp was achieved by addition of 0.7 mM isopropyl-β-D-thiogalactopyranoside to induce the DE3-encoded T7 RNA polymerase. Cells were pelleted, resuspended in 10 mM Tris (pH 8.0), and subjected to sonication. Cell debris was removed by centrifugation, and ammonium sulfate was added (55% saturation) to the supernatant. Following centrifugation, the pellet was resuspended in 10 mM Tris (pH 8.0), and the supernatant was combined with ammonium sulfate (90% saturation), and following centrifugation, the pellet was resuspended in 10 mM Tris (pH 8.0). Both resuspended pellets were passed through an Econo-Pac 10DG desalting column (Bio-Rad) in 100 mM NaCl, 10 mM Tris (pH 8.0) and then combined. The PhoQp fragment was further purified on a Waters high performance liquid chromatography system (flow rate 0.3 ml/min) and protein monitoring at 280 nm) using several chromatographic columns. First, we used Waters Protein Pak 300 SW 7.5 mm x 30-cm gel filtration column. Fractions containing PhoQp (as determined by Coomassie Blue staining of SDS-polyacrylamide gel electrophoresis) were pooled and passed through an Econo-Pac 10DG desalting column (Bio-Rad) in 20 mM NaCl, 10 mM Tris (pH 8.0). These fractions were loaded onto an ion exchange column (Waters advanced purification glass column AP-1), and PhoQp was eluted using a 20 mM to 1.0 M NaCl linear gradient in 10 mM Tris (pH 8.0). The PhoQp-containing fractions, which eluted at 510 mM NaCl, were pooled and passed through an Econo-Pac 10DG desalting column (Bio-Rad) in 10 mM KH₂PO₄, 10 mM Tris (pH 6.9). These fractions were loaded onto a Bio-Rad Bio-Scale ceramic hydroxyapatite CHT-5 type I column. PhoQp was eluted with a 10–500 mM KH₂PO₄ linear gradient in 10 mM Tris (pH 6.9). The PhoQp-containing fractions, which eluted at 410 mM KH₂PO₄, were pooled and passed through an Econo-Pac 10DG desalting column (Bio-Rad) in 10 mM Tris (pH 8.0).

The identity, purity, and concentration of the fragment were analyzed by electrospray mass spectrometry, quantitative amino acid analysis, and N-terminal amino acid sequencing. The predicted molecular mass for PhoQp was 17,042.2 daltons and that determined by electrospray mass spectrometry was 17,040 daltons; the extinction coefficient determined from the amino acid analysis was ε₂₈₀ = 1.94 cm² mg⁻¹. Electrospray mass spectrometry, quantitative amino acid analysis, and N-terminal amino acid sequencing were performed by the Protein Chemistry Laboratory of the Washington University School of Medicine.

Fluorescence and Circular Dichroism Measurements—Circular dichroism studies of the PhoQp fragment were carried out in a Jasco J600A spectropolarimeter. The PhoQp sample was analyzed at a concentration of 0.25 mg/ml in 50 mM choline chloride, 10 mM Tris (pH 7.0) at 25 °C from 195 to 250 nm. Intrinsic fluorescence was measured using a PTI (Alphascan) spectrophotometer at a constant excitation wavelength of 280 nm, and emission spectra were collected from 300 to 400 nm. These experiments were carried out with the PhoQp fragment at a concentration of 0.026 mg/ml in 10 mM Tris (pH 7.0) and varying concentrations of monovalent (choline chloride, KCl, and NaCl) and divalent salts (MgCl₂, CaCl₂, and BaCl₂). The solution containing choline chloride was titrated with aliquots of the solution containing MgCl₂, and the change in intrinsic fluorescence was monitored as a function of MgCl₂ concentration. At each titration step the concentration of PhoQp and the ionic strength remained constant, thus the fluorescence changes were not due to changes in the ionic strength of the medium. A buffer blank was subtracted from the spectrum under all conditions.

β-Galactosidase Assays—β-Galactosidase activity was determined in overnight cultures of strains EG9065 and EG9564 as described (16).
revealed a composition that was consistent with that predicted from the nucleotide sequence of this portion of the phoQ gene with an additional N-terminal Met, which had been incorporated into the phoQ gene in the expression vector. Furthermore, electrospray mass spectrometry revealed a mass of 17,040 daltons for the purified polypeptide, which is consistent with a predicted mass of 17,042.2 daltons.

**Mg**^{2+} and Ca^{2+}—*Alter the Trp Fluorescence Pattern of the Sensing Domain of PhoQ*—To investigate the ability of the periplasmic domain of PhoQ to bind divalent cations, we examined the circular dichroism (CD) and fluorescence spectra of this polypeptide, which harbors four Trp residues (Fig. 1). The CD spectra of PhoQp suggested the presence of both α-helix and β-sheet structures in this polypeptide. However, no changes were detected in the CD spectra in the presence of MgCl_2 or NaCl. On the other hand, the intrinsic fluorescence at 335.0 nm of PhoQp was significantly modified in the presence of MgCl_2 (Fig. 2A). Changes could be observed with as little as 7 mM MgCl_2. CaCl_2 could also modify the fluorescence spectra of PhoQp (data not shown) but BaCl_2, which is unable to repress transcription of PhoP-regulated genes in *vivo*, hardly modified the intrinsic fluorescence of this polypeptide when tested at concentrations up to 200 mM (Fig. 2B).

These results provide a direct demonstration that the periplasmic region of PhoQ specifically binds Mg^{2+} and Ca^{2+}. Moreover, they are consistent with our previous findings that Mg^{2+} could alter the tryptophanization pattern of the periplasmic domain of the PhoQ protein presented in spheroplasts. Furthermore, they are in agreement with those reported by Waldburger and Sauer (18), who found that divalent cations promoted stabilization to urea denaturation of a similar PhoQ fragment derived from *E. coli*. Waldburger and Sauer (18), however, could not detect changes in the fluorescence spectra of their PhoQ-derived fragment when MgCl_2 (10 mM) was added. The differences in the fluorescence results may reflect the presence of different salts in the buffers used to fix the ionic strength; 0.2 mM KCl in the Waldburger and Sauer’s experiments and choline chloride in those reported here. Consistent with this hypothesis, we established that higher concentrations of MgCl_2 were required to promote fluorescence changes in PhoQp when KCl was present at 100 mM (data not shown). On the other hand, choline, which is not predicted to compete with Mg^{2+} for binding to PhoQp due to its large size, did not change the intrinsic fluorescence of PhoQp when added up to 500 mM (data not shown). Alternatively or in addition, the differences in the fluorescence results could be due to the 28 amino acid differences that exist between the 146 residues that comprise the periplasmic regions of the *Salmonella* and *E. coli* PhoQ proteins.

**The PhoQ Protein Has Distinct Binding Sites for Ca^{2+} and Mg^{2+}**—We had established previously that Ca^{2+} could repress expression of the PhoP-activated gene psiD with half-maximal repression attained at lower concentrations than those achieved with Mg^{2+} (8). However, the Ca^{2+}-mediated effect could have resulted from the combined actions of Ca^{2+} and Mg^{2+}, since the repressing role of Ca^{2+} had only been investigated in the presence of 8 µM Mg^{2+}.

To establish the importance of Ca^{2+} and Mg^{2+} in the regulation of the PhoP/PhoQ system, we examined the expression of

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**FIG. 3. Regulation of the PhoP/PhoQ system by Ca^{2+} and Mg^{2+} in *Salmonella* harboring a wild-type PhoQ protein and in an avirulent mutant harboring a mutation in the sensing domain of the PhoQ protein.** A, β-galactosidase activity from a psiD-lac transcriptional fusion expressed by a wild-type *phoQ* bacterium grown in N-minimal medium with MgCl_2, CaCl_2, or a mixture of both salts present at the same concentration as indicated on the abscissa. B, β-galactosidase activity from a psiD-lac transcriptional fusion expressed by mutant *pho*-24 bacterium grown in N-minimal medium with MgCl_2, CaCl_2, or a mixture of both salts present at the same concentration as indicated on the abscissa. β-Galactosidase activity is in Miller units (16). The data correspond to mean values of three independent sets of experiments done by duplicate. The data can be fit to a model where binding of Mg^{2+} and Ca^{2+} occurs at distinct sites that do not interact with each other. Binding of a divalent cation also brings about a reduction in β-galactosidase activity. The specific expression used to fit the data in the presence of MgCl_2 or CaCl_2 was $v = v_0 + v_1k_1x(1 + k_2x)$, where $v_0$ and $v_1$ are the activities in the absence and under saturating concentrations ($x$), respectively, of Mg^{2+} or Ca^{2+}, while $k_1$ is the binding affinity. The expression in the presence of both cations was $v = (v_0 + v_1k_1x + v_2k_2y + v_3k_4xy/(1 + k_1x)(1 + k_2y))$, where $v_0$, $v_1$, $v_2$, and $v_3$ are the velocities in the absence (0) or presence (1) of Mg^{2+} ($i$) or Ca^{2+} ($j$), $k_1$ and $k_2$ are the binding affinities of Mg^{2+} and Ca^{2+}, and $x$ and $y$ are the Mg^{2+} and Ca^{2+} concentrations. These expressions were derived from application of linkage thermodynamics (24). A global analysis of the data in Fig. 3 gives the following best fit parameter values: for the wild-type strain, $k_1 = 1.4 \times 10^9$ M^{-1}; $k_2 = 4 \times 10^8$ M^{-1}; $v_0 = 965 \pm 98$; $v_1 = 106 \pm 11$; $v_2 = 0$; and $v_3 = 0$; and for the *pho*-24 mutant, $k_1 = 1.5 \times 10^8$ M^{-1}; $k_2 = 6 \times 10^8$ M^{-1}; $v_0 = 909 \pm 91$; $v_1 = 273 \pm 23$; $v_2 = 0$; and $v_3 = 0$. The possibility of Ca^{2+} and Mg^{2+} competing for the same site was examined and ruled out from the fit of the data with both cations present. On the other hand, we cannot presently rule out more complicated models in which Ca^{2+} and Mg^{2+} bind with low affinity to the Mg^{2+}- and Ca^{2+}-binding sites, respectively.
of MgCl₂, CaCl₂ (with no added Mg²⁺), or a mixture of these two salts. Repression of psiD transcription was achieved at lower concentrations of Mg²⁺ than Ca²⁺, and maximal repression was attained when both cations were present (Fig. 3A). An analysis of these data is consistent with the existence of distinct binding sites for Mg²⁺ and Ca²⁺ in the PhoQ protein (see legend to Fig. 3). The apparent affinities of the PhoQ protein for Mg²⁺ and Ca²⁺ were 1.4 ± 0.2 10⁵ M⁻¹ and 4 ± 1 10⁸ M⁻¹, respectively. These two sites do not appear to interact with one another because the repressing effect in the presence of both cations was equivalent to the sum of the individual repressing effects.

A Mutant PhoQ Protein That Is Less Responsive to Ca²⁺—A S. typhimurium strain harboring the pho-24 allele expresses several PhoP-activated genes and is attenuated for mouse virulence (19). We have established previously that this mutant harbors a single amino acid substitution, Thr⁴⁸ → Ile, in the periplasmic domain of the PhoQ protein (8). To establish the sensitivity of the mutant PhoQ protein to Mg²⁺ and Ca²⁺, we examined the transcriptional activity of the psiD gene in a strain harboring a chromosomal pho-24 allele. The mutant PhoQ protein had a lower affinity for Ca²⁺ than the wild-type (Kₐ₃Ca²⁺: 6 ± 1 10⁵ M⁻¹ versus 4 ± 1 10³ M⁻¹; Fig. 3B). On the other hand, the affinity for Mg²⁺ was virtually identical in the wild-type and mutant PhoQ proteins (Kₐ₃Mg²⁺: 1.5 ± 0.2 10⁵ M⁻¹ versus 1.4 ± 0.2 10⁸ M⁻¹); yet, the transcriptional activity of the psiD gene at repressing concentrations of Mg²⁺ was higher in the pho-24 mutant than in the wild-type strain (Fig. 3B). Cumulatively, these results indicate that Thr⁴⁸ is required for normal Ca²⁺ sensing and support a model in which the PhoQ protein has distinct sites for Mg²⁺ and Ca²⁺.

Conclusions—The PhoQ protein represents the first and only example of a receptor that senses extracellular Mg²⁺ and Ca²⁺. In the PhoP/PhoQ signal transduction cascade, these cations act as primary signaling molecules rather than their familiar roles as cofactors (20) and second messengers (21). We have now provided direct evidence that Mg²⁺ and Ca²⁺ specifically bind to the sensing domain of the PhoQ protein. We would like to suggest that binding of divalent cations promotes a conformation in the PhoQ protein that is unfavorable for the activation of the PhoP protein, resulting in transcriptional repression of PhoP-activated genes.

The demonstration that the PhoQ protein harbors distinct sites for Mg²⁺ and Ca²⁺ and that these cations act independently to modulate PhoQ activity imply that both, Mg²⁺ and Ca²⁺, are physiologically relevant in the control of PhoP-regulated loci. This is further substantiated by the virulence attenuation that results from a single amino acid substitution in the periplasmic domain of the PhoQ protein that alters its response to Ca²⁺. Finally, the PhoQ protein may represent a new paradigm for divalent cation-binding proteins, since it does not have an EF-hand, a motif that is common among a large number of Ca²⁺-binding proteins (22), and shows no sequence similarity with the Ca²⁺-sensing receptor from mammalian parathyroid cells (23). Solving the crystal structure of the sensing domain of the PhoQ protein may reveal features of the divalent cation-binding sites that are not apparent from homology analysis and structural predictions of its primary amino acid sequence.

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