The Phosphatase Activity Is the Target for Mg$^{2+}$ Regulation of the Sensor Protein PhoQ in Salmonella

Received for publication, November 23, 1999, and in revised form, March 21, 2000
Published, JBC Papers in Press, May 11, 2000, DOI 10.1074/jbc.M909335199

María E. Castellí, Eleonora García Véscovi, and Fernando C. Soncini

From the Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Argentina

The PhoP/PhoQ two-component system controls the expression of essential virulence traits in the pathogenic bacterium Salmonella enterica serovar Typhimurium. Environmental deprivation of Mg$^{2+}$ activates the PhoP/PhoQ signal transduction cascade, which results in an increased expression of genes necessary for survival inside the host. It was previously demonstrated that the interaction of Mg$^{2+}$ with the periplasmic domain of PhoQ promotes a conformational change in the sensor protein that leads to the down-regulation of PhoP-activated genes. We have now examined the regulatory effect of Mg$^{2+}$ on the putative activities of the membrane-bound PhoQ. We demonstrated that Mg$^{2+}$ promotes a phospho-PhoP phosphatase activity in the sensor protein. This activity depends on the intactness of the conserved His-277, suggesting that the phosphatase active site overlaps the H box. The integrity of the N-terminal domain of PhoQ was essential for the induction of the phosphatase activity, because Mg$^{2+}$ did not stimulate the release of inorganic phosphate from phospho-PhoP in a fusion protein that lacks this sensing domain. These findings reveal that the sensor PhoQ harbors a phospho-PhoP phosphatase activity, and that this phosphatase activity is the target of the extracellular Mg$^{2+}$-triggered regulation of the PhoP/PhoQ system.

Reversible protein phosphorylation is one of the most conspicuous mechanisms that regulate biological processes in cells, including modulation of enzymatic activities, protein-protein and protein-DNA interactions, and extracellular signal transduction. In prokaryotes and in lower eukaryotes and plants (1), the most widespread and efficient sensory-response devices rely on protein phosphotransfer. They are the so-called two-component regulatory systems that enable bacteria to monitor changes in their environment and adjust their structure and physiology accordingly to survive.

The prototypical architecture of the two-component regulatory systems consists of a sensor protein and an associated effector protein (2, 3). The sensor is generally a membrane protein whose C-terminal domain projects into the cytoplasm. This domain harbors a histidine autokinase activity and, in several sensors of this large family, a phosphatase activity (2, 4–9). The cognate response-regulator is most often a transcriptional regulator protein. Its N-terminal domain harbors a conserved aspartate that accepts the phosphate from the phospho-histidyl residue of the sensor protein. This modification is propagated to the C-terminal domain affecting its DNA binding properties (2).

Two major biochemical activities play an opposite role to balance the phosphorylation status of the response regulator: the autokinase activity of the sensor that defines the phosphotransfer availability and a specific phosphatase that dephosphorylates the response regulator. By modulating these reactions, the sensor component defines the phosphorylation status of the response regulator, which directs the expression of a specific set of target genes. This modulation results in the final adaptive response of the bacteria to its primary signal. Despite a considerable amount of research on different two-component systems, carried out to understand how the signal regulates the catalytic activities of the sensor to control the phosphorylation state in the effector molecule, a clear picture of this mechanism has not yet emerged.

In Salmonella, the PhoP/PhoQ two-component system governs the adaptation to environmental Mg$^{2+}$ deprivation and controls the expression of essential virulence factors (10–14). It was previously demonstrated that PhoQ is the sensor of the system that specifically recognizes extracellular Mg$^{2+}$. The interaction of the sensing periplasmic domain with the divalent cation promotes a conformational change in the protein that results in the repression of the transcription of at least 20 different PhoP-activated loci (10, 11, 15). However, it was not clear which activity or activities that reside in PhoQ were affected by the Mg$^{2+}$-induced conformational switch of the sensor.

In this work we examined how Mg$^{2+}$, as the specific regulatory signal, controls the phosphorylation state of PhoP. We demonstrated that the interaction of Mg$^{2+}$ with PhoQ activates a phosphatase activity of the sensor protein that promotes the dephosphorylation of phospho-PhoP. Stimulation by Mg$^{2+}$ of this phosphatase activity required the N-terminal domain of the sensor, because no phosphatase was detected in a fusion protein lacking this domain while it retained the reversible autokinase and phosphotransfer activities. These results reveal that the sensor PhoQ harbors a phosphatase activity that acts on PhoP and that this phosphatase activity is the target of the Mg$^{2+}$-triggered regulation of the PhoP/PhoQ system.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Nitrocellulose membranes were from Bio-Rad. [γ-32P]ATP was obtained from NEN Life Science Products. The oligonucleotides were purchased from Bio-Synthesis, Inc. (Lewisville, TX). Cell culture media reagents were from Difco, and chemicals were from Sigma.

Bacterial Strains, Plasmids, and Growth Conditions—Salmonella enterica serovar Typhimurium (S. typhimurium) strain EGS172 (phoQ-MudJ) was transformed with plasmid pEG0950 (16) or with the vector pUHE21-2acP (16). These strains were used for the isolation of membranes harboring PhoQ or the control membranes, respectively. Escherichia coli strain BL21(DE3) carrying plasmid pMB1020. pMB1020 harbors a phoP His-tag fusion gene (full-length phoP fused to 6 His codons in its C terminus) under the control of the T7 -10 promoter of the phoP-7 protein. This fusion gene was generated by polymerase chain reaction, using forward primer PhoP-NTP (5'-GAG-GATCATATGATGGCCGTACTG-3') and reverse primer PhoP-H6-NTF (5'-TCCAACCTTATGTTGGTGTTGGTGTTGGCGCAAATC-C-AAGATAC-3') and cloned between the NdeI and HindIII sites of plasmid pBluescript II KS1 (Stratagene). Bacteria were grown at 37 °C in Luria-Bertani (LB) broth with shaking, and with the addition of 0.7 mM isopropyl-β-n-thiogalactopyranoside (IPTG) when indicated. Ampicillin was used at a final concentration of 50 μg/ml.

Genetic and Molecular Biology Techniques—Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus as recommended by the manufacturer. Recombinant DNA techniques were performed according to standard protocols (17). To replace his-277 with Val in PhoQ, and Asp-55 with Ala in PhoP, we used the site-directed mutagenesis protocol described by Deng et al. (18). Polymerase chain reaction-derived constructions and site-directed mutagenesis were all confirmed by DNA sequence analysis performed using the M13 end-sequencing kit (Amersham) performed according to manufacturer's instructions (NEB, Inc.). All procedures were carried out at 4°C.

The protein profile of the purified proteins was determined by SDS-polyacrylamide gel electrophoresis (PAGE). In Vitro Phosphorylation, Dephosphorylation, and TLC Analysis—For the autokinase assay, membranes (50 μg of total protein) harboring PhoQ were incubated with 50 μM [γ-32P]ATP (7500 cpm/pmol, NEN Life Science Products), in a 30-μl reaction mixture containing 20 μg Tris-HCl (pH 8.0), 50 mM KCl (buffer TK). Unless stated otherwise, 1 μg MgCl2 was added to the reaction medium. Reactions were started by addition of the membrane fraction, incubated 5 min at 37 °C, and stopped by addition of 6 μl of 5 × SDS-PAGE sample buffer (2.5% β-mercaptoethanol, 9% glycerol, 10% SDS, 600 mM Tris-HCl (pH 6.8), 0.006% bromophenol blue). The amount of radiolabeled PhoQ increased proportionally to the amount of membrane protein used in the range from 0.16 to 2.6 μg/μl; this indicated that PhoQ was not in excess at 1.66 μg/μl, the membrane concentration used in the autophosphorylation assay. To remove remnant Mg2+, ATP, and free inorganic phosphate, the membranes containing autophosphorylated PhoQ were washed once with buffer TK with 5 mM EDTA, followed by two washes with buffer TK. This fraction was analyzed by TLC to confirm absence of residual ATP and inorganic phosphate and used to test the stability of phosphorylated PhoQ and for the PhoQ → PhoP phosphotransfer assay. The phosphotransfer assay was carried out as follows: typically, membranes (50 μg of total protein) harboring phosphorylated PhoQ were reincubated in a 30-μl total volume with 10 μg of purified PhoP-H6 in a reaction buffer consisting of 50 mM Tris-HCl (pH 8.0), 50 mM KCl for different periods of time. MgCl2 was added to the reaction as indicated in each individual assay. Reactions were stopped by addition of 5 × SDS-PAGE loading buffer. To obtain isolated phosphorylated PhoP-H6, 10 μg of purified PhoP-H6 was added to a 30-μl final volume containing membranes (50 μg of total protein) harboring PhoQ, 50 μM [γ-32P]ATP (7500 cpm/pmol), and 1 μg MgCl2 in buffer TK, and incubated for 5 min at 37 °C. This reaction mixture was centrifuged at 24,000 × g for 45 min at 4°C to remove the membrane fraction. The supernatant containing phosphorylated PhoP-H6 was recovered, purified using a Ni2+-NTA agarose affinity column to eliminate contaminating ATP, applied to a G-50 gel filtration column equilibrated with buffer TK to remove imidazole, and immediately used to test phosphorylated PhoP stability or for the phosphatase assay. For the phosphatase assay, phosphorylated PhoP-H6 (5 μg of protein) was incubated with control membranes, membranes enriched in PhoQ or in PhoQΔDTTV (50 μg), or with purified MBP-Δc fusion protein (5 μg) in a 30-μl reaction mixture containing 20 μg Tris-HCl (pH 8.0), 50 mM KCl, at 37°C in the presence or absence of 1 mM MgCl2 for different periods of time as indicated in each assay. The reaction was stopped by adding 5 μl SDS-PAGE loading buffer. All reactions were analyzed by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and then subjected to autoradiography. When TLC analysis was performed, reactions were stopped by addition of 1% SDS, applied to a polyethyleneimine (PEI)-cellulose plate (J. T. Baker), and developed in 0.8 M LiCl, 0.8 M acetic acid as described (19). The plates were air-dried and then exposed to x-ray film. Additionally, the stability of phosphorylated PhoP-H6 or phospho-PhoQ was determined by incubating each phosphoprotein in a 30-μl reaction mixture with addition of 5 μl of 50× SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1.25% SDS, 40% glycerol, 0.025% bromophenol blue). Aliquots were withdrawn at different time points, and the reaction was stopped by adding 5 μl SDS-PAGE loading buffer. The samples were analyzed by SDS-PAGE and transferred to nitrocellulose membranes, followed by autoradiography. Autoradiographs from SDS-PAGE analysis or TLC assays were densitometrically scanned to perform quantitative determinations. For the stability assays, the PhoP or PhoQ bands were cut from the gel and the incorporation of 32P was determined using a Wallac 1209 Rackbeta liquid scintillation counter. Chemical stability assays of the phosphoproteins (alkali and acid liability) were performed as described (9).

1 M. E. Castelli, E. García Vescovi, and F. C. Soncini, unpublished results.
2 The abbreviations used are: LB broth, Luria-Bertani broth; IPTG, isopropyl-β-n-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PEI, polyethyleneimine; MBP, maltose binding protein.

Mg2+-dependent Control of the Phosphorylation State of PhoP 22949
**RESULTS**

**PhoQ Autokinase Activity**—Binding of Mg\(^{2+}\) to the sensor protein PhoQ was identified as the primary event in the signal transduction cascade that results in the down-regulation of the PhoP-activated genes (10, 15). To determine the activities of PhoQ that are affected in response to the signal, we sought to analyze sequentially the steps that are involved in the transduction mechanism.

To examine *in vitro* the biochemical activities of the Mg\(^{2+}\)-sensor protein PhoQ, this protein was expressed from the pEG9050 plasmid. Cellular fractionation was performed to isolate the membranes that harbor the sensor protein. The electrophoretic analysis of the membrane fraction revealed a major protein band (estimated to be 5–7% of the total protein by densitometric analysis) that corresponded to the predicted mobility of PhoQ. The identity of the protein was assessed also by Western blot analysis using polyclonal antibodies raised against the protein. This autokinase activity was dependent on the presence of different concentrations of Mg\(^{2+}\), as shown in the time course assays performed in the presence of micromolar concentrations of Mg\(^{2+}\) (Fig. 1A). When Mg\(^{2+}\) concentrations above 200 \(\mu M\) were added to the reaction medium containing \(\gamma\)-ATP, as demonstrated under “Experimental Procedures,” without (–) or with addition of 0.01, 0.05, 0.2, 1.0, 5.0, or 20.0 \(mM\) MgCl\(_2\), as indicated. The autophosphorylation reactions were analyzed by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, followed by autoradiography. The total amount of phosphorylated PhoQ (PhoQ-P, arbitrary units) present in each well was determined by densitometry and plotted against the concentration of Mg\(^{2+}\) present in the autophosphorylation assay. Data shown represent results from three independent experiments.

**Phosphotransfer from PhoQ to PhoP**—We decided to investigate the next step in the activation by phosphorylation of the response regulator PhoP. To uncouple the autokinase from the phosphotransfer reaction and to analyze the effect of Mg\(^{2+}\) exclusively on the latter, the membrane-bound PhoQ was first subjected to autophosphorylation. Immediately after, the membranes were exhaustively washed with EDTA to deplete the membranes of remnant Mg\(^{2+}\) and ATP. The membranes harboring phospho-PhoQ were incubated with purified PhoP in the phosphotransfer medium, as described under “Experimental Procedures,” with addition of 1 \(mM\) EDTA or with addition of 0.05, 0.25, 0.5, 1.0, 2.0, 5.0, 10.0, or 20 \(mM\) MgCl\(_2\). Aliquots were withdrawn at 30 s, 1, 5, and 30 min, and the reaction was stopped by the addition of 5 \(\times\) SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE, followed by transfer to nitrocellulose and autoradiography.

**Mg\(^{2+}\)-ATP** is the true substrate of the autokinase. Additionally, we determined that the acid lability and alkali resistance of the phosphorylated moiety corresponded to the chemical characteristics of a phosphohistidine residue (not shown). Moreover, the PhoQ\(_{11277V}\) mutant protein, where the conserved His-277 is replaced by Val, was unable to undergo autophosphorylation, irrespective of the presence of Mg\(^{2+}\) in the phosphorylation reaction (data not shown). This indicates that the phosphorylation takes place in the predicted conserved histidine residue.

**Phosphotransfer from PhoQ to PhoP**—We decided to investigate the next step in the activation by phosphorylation of the response regulator PhoP. To uncouple the autokinase from the phosphotransfer reaction and to analyze the effect of Mg\(^{2+}\) exclusively on the latter, the membrane-bound PhoQ was first subjected to autophosphorylation. Immediately after, the membranes were exhaustively washed with EDTA to deplete the membranes of remnant Mg\(^{2+}\) and ATP. The membranes harboring phospho-PhoQ were incubated with purified PhoP resulting in the phosphorylation of the response regulator. Chemical stability tests and site-specific mutagenesis identified amino acid Asp-55 of PhoP as the phosphorylated residue (not shown).

As it is shown in the time course assays performed in the presence of different concentrations of Mg\(^{2+}\) (Figs. 2 and 3), the initial rate of phosphotransfer is favored by increasing the concentration of the divalent cation. Strikingly, Mg\(^{2+}\) concentrations higher than 250 \(\mu M\) stimulated the dephosphorylation of phospho-PhoP along the time course (Figs. 2 and 3B). A net loss of the protein-bound radiolabeled phosphate becomes evident when we compare the radioactivity associated to PhoQ (Figs. 2 and 3A) and to PhoP (Figs. 2 and 3B) as a function of time, for each Mg\(^{2+}\) concentration used. Moreover, the total protein-bound phosphate decreased up to 60% when concentrations of MgCl\(_2\) higher than 250 \(\mu M\) were added to the phospho-
transfer reaction and up to 90% at concentrations higher than 10 mM MgCl₂ (Figs. 2 and 3C). On the other hand, when the stability of each individual phosphorylated protein was assayed, phospho-PhoQ retained a 72 ± 13% of the initial label over the 120-min period tested (irrespective of the MgCl₂ concentration used), whereas phospho-PhoP showed a half-life of 64 min in the presence of 1 mM EDTA and of 60 min in the presence of 20 mM MgCl₂ (Fig. 4).

The above results indicate that Mg²⁺ neither activates an autophosphatase activity of the response regulator nor promotes PhoQ dephosphorylation and strongly suggest that a Mg²⁺-induced phosphatase is present and acts on phosphorylated PhoP.

**PhoQ Harbors a Mg²⁺-controlled Phosphatase Activity**—The status of the environment as detected by the sensor is reflected by the phosphorylation state of the effector molecule. The phosphorylation status of PhoP is predicted to be due to the balance of three fundamental biochemical reactions: PhoQ autophosphorylation, the phosphotransfer from PhoQ to PhoP, and the dephosphorylation of phospho-PhoP that restores the effector protein to its original state.

To examine if PhoQ was responsible for the Mg²⁺-induced dephosphorylation of PhoP, radiolabeled phospho-PhoP was incubated with membranes harboring unlabeled PhoQ. Surprisingly, in the absence of added Mg²⁺ we observed dephosphorylation of PhoP with the simultaneous phosphorylation of PhoQ, indicating that a reverse phosphotransfer from PhoP to PhoQ took place (Fig. 5A). The reverse phosphotransfer was partially inhibited by EDTA, showing that a low concentration of the divalent cation favors the reaction. However, when we added 1 mM MgCl₂ to the reaction medium, dephosphorylation of phospho-PhoP took place but no phosphorylated PhoQ was detected, resulting in a net loss of the total label associated to proteins (Fig. 5A). This effect was not due to a PhoQ-unrelated phosphatase activity present in the membrane fraction, because membranes lacking the sensor protein did not show Mg²⁺-stimulated phosphate release from phospho-PhoP (Fig. 5A). Moreover, this result was consistent with the PhoQ-mediated dephosphorylation of PhoP triggered in the presence of high concentrations of Mg²⁺ previously observed in the direct phosphotransfer assay (Figs. 2 and 3).

Although controlled proteolysis of native PhoQ and binding assays performed using its purified sensing domain demonstrated that extracellular Mg²⁺ interacts with the periplasmic domain of the sensor, thereby triggering a conformational change in the protein (10, 15), it was still unclear how this change was transduced in the modulation of the biochemical activities of PhoQ. To assess the role of the periplasmic sensing domain of PhoQ in the Mg²⁺-induced phosphatase activity, we constructed a fusion protein containing its cytoplasmic domain fused to the maltose binding protein (MBP-Qc). MBP-Qc was able to undergo autophosphorylation (not shown) and to receive the phosphate from PhoP analogously to wild-type PhoQ (Fig. 5A). However, the addition of Mg²⁺ did not promote the dephosphorylation of PhoP. A comparative densitometric analysis of a reverse phosphotransfer time course from phospho-PhoP to either PhoQ or to MBP-Qc in the presence of 1 mM MgCl₂ is shown in Fig. 5B. A net loss of label associated to the proteins was evident only when wild-type PhoQ was used (Fig. 5C).

When we analyzed by TLC the dephosphorylation of phospho-PhoP in the presence of control membranes, membranes harboring PhoQ, or MBP-Qc, we confirmed that the detected loss of label shown in Fig. 5 corresponded to the release of inorganic phosphate (Fig. 6). This release was significantly enhanced only when wild-type PhoQ and 1 mM MgCl₂ were present in the reaction (Fig. 6A). The densitometric analysis shows the relative distribution of the total label between the phosphate bound to the proteins and the inorganic phosphate.
released in each sample (Fig. 6B). More than 80% of the total label was released as inorganic phosphate by membrane-bound PhoQ in the presence of MgCl₂, whereas less than 15% of the label was detected as inorganic phosphate in the absence of the cation. On the other hand, regardless of the concentration of Mg²⁺ used in the assay, more than 80% of the total radioactivity remained protein-bound when phospho-PhoP was incubated with either control membranes or MBP-Qc.

Cumulatively, these results demonstrate that Mg²⁺ triggers a specific PhoP-phosphatase activity in PhoQ that renders a dephosphorylated response regulator. Moreover, they show that the N-terminal sensing domain of PhoQ plays a fundamental role in the Mg²⁺-activated PhoP-phosphatase activity of PhoQ.

To assess the involvement of the conserved autoprophosphorylation site, His-277, in both the Mg²⁺-induced phosphatase activity and the reverse phosphotransfer, we used membranes harboring the mutant PhoQH₂₇⁷V instead of the wild-type PhoQ. As expected, there was no reverse transfer of the label in any of the conditions tested, indicating that the conserved His-277 is the target residue that accepts the phosphate from phospho-PhoP (Fig. 5A). In addition, the mutant protein was unable to promote the dephosphorylation of PhoP even in the presence of added Mg²⁺. This points out that His-277 plays an essential role in the phosphatase activity of PhoQ.

Our experimental data showed that two processes are involved in the dephosphorylation of PhoP: the Mg²⁺-triggered phosphatase activity present in PhoQ and the reversion of the phosphotransfer between PhoP and PhoQ. To gain an insight into the latter, ADP was added to the reaction mixture. ADP stimulated the reverse phosphotransfer with subsequent dephosphorylation of PhoQ and regeneration of labeled ATP, as it is shown in the TLC analysis (Fig. 7). Formation of ATP was also achieved by addition of ADP to isolated phospho-PhoQ, and Mg²⁺ did not stimulate the release of inorganic phosphate in this reaction (not shown). These results suggest that the PhoP phosphorylation state depends, at least partially, on the balance of a totally reversible phosphotransfer reaction between PhoP and PhoQ. Additionally, when Mg²⁺ and ADP were simultaneously added to the reaction, there was dephosphorylation of phospho-PhoP with production of both ATP and inorganic phosphate (Fig. 7). This result shows the additive effect of the phosphatase activity and the reverse phosphotransfer taking place at the same time, implying that they are independent events.

**DISCUSSION**

According to the paradigm of the “two-component” regulatory systems, upon interaction with the environmental signal the sensor protein defines the phosphorylation state of its cognate effector protein. However, there is not a unique model that comprehensively depicts how this process is achieved, and different strategies arise from the analysis of individual systems.
with the \( \text{Mg}^{2+} \) phosphorylation in micromolar concentrations. This is consistent with the Mg\(^{2+}\)-ATP chelate complex being the actual phosphodonor for the autokinase reaction. Also, the cation was shown for other two-component systems (28).

Phospho-PhoP was incubated with membranes harboring PhoQ without (-) or with the addition of 1 mM MgCl\(_2\) (Mg\(^{2+}\)), in the presence or absence (-) of 1 mM ADP. The samples were spotted onto a PEI-cellulose TLC plate. Inorganic \([\gamma^{32}P]\) phosphate and \([\gamma^{32}P]ATP\) were used as standards. Reactions were stopped by addition of 1% SDS, applied to a PEI-cellulose plate, and developed with 0.8 M LiCl, 0.8 M acetic acid, followed by autoradiography.

For example, in systems that include OmpR/EnvZ (6, 7, 20), the Bacillus subtilis PhoP/PhoR (9), and CpxR/CpxA (21) the sensor protein simultaneously harbors the autokinase and the phosphatase activities. On the other hand, some response regulators exhibit an intrinsic phosphatase activity, and the sensor protein acts as a cophosphatase or enhancer of its autodephosphorylation (20, 22, 23). Additionally, in other systems accessory proteins either stimulate the phosphatase reaction provided by the sensor (i.e. PII proteins (8)) or act independently and dephosphorylate the phosphoaspartate in the response regulator (24–26).

The PhoP/PhoQ signal transduction mechanism, extracellular Mg\(^{2+}\) controls the activity of the system acting as the specific ligand of the sensor protein PhoQ. It was demonstrated that Mg\(^{2+}\) interacts with the periplasmic domain of PhoQ changing the conformation of the protein (10, 15, 27). However, it was not known how this conformational change affects the function of PhoQ, which is finally reflected in the down-regulation of the PhoP-activated genes. In this work, we demonstrate that Mg\(^{2+}\) induces a specific phospho-PhoP phosphatase in the membrane-bound PhoQ sensor protein.

To analyze the effect of Mg\(^{2+}\) on the putative activities of PhoQ, we first set up the conditions for the autokinase, PhoQ → PhoP phosphotransfer, and PhoP-phosphatase assays, using the membrane-bound sensor protein. We decided not to use detergents for the extraction of PhoQ, or to purify it as a fusion protein harboring the N-terminal region of EnvZ fused to the cytoplasmic domain of PhoQ (10). Considering the evidence of both, it becomes clear that the interaction of Mg\(^{2+}\) with the N-terminal sensing domain of PhoQ is essential for triggering its phospho-PhoP phosphatase activity but does not affect the autokinase activity of the sensor. Additionally, the mutant protein PhoQ\(_{HV277V}\) was ineffective as a phosphatase irrespective of the Mg\(^{2+}\) concentration used, indicating that this activity structurally or functionally requires the intactness of the conserved His residue that is also the target for the autophosphorylation reaction.

Autophosphorylated PhoQ serves as a phosphodonor of the effector protein PhoP, which becomes phosphorylated in the predicted Asp-55 residue. Mg\(^{2+}\) is required for PhoQ autophosphorylation in micromolar concentrations. This is consistent with the Mg\(^{2+}\)-ATP chelate complex being the actual phosphodonor for the autokinase reaction. Also, the cation was required for the subsequent phosphotransfer to PhoP, as was shown for other two-component systems (28).

Interestingly, in the course of the phosphotransfer reaction, PhoP became phosphorylated and it rapidly lost the label when concentrations of MgCl\(_2\) higher than 250 \(\mu M\) were present in the reaction, pointing out that the divalent cation was somehow stimulating such dephosphorylation. We determined that two independent mechanisms of dephosphorylation of phospho-PhoP occur. One involves the reversion of the reaction that takes place to phosphorylate the response regulator, and the other is a specific phospho-PhoP phosphatase induced by high concentrations of Mg\(^{2+}\) that renders the release of inorganic phosphate. When phospho-PhoP was incubated with PhoQ, a reverse phosphotransfer took place in the absence of added Mg\(^{2+}\). This transfer was further stimulated by ADP, showing that the PhoQ autokinase activity can be reversed, transferring the phosphate to ADP. The His-277 residue in the sensor protein was key to this mechanism, because the mutant PhoQ\(_{HV277V}\) was unable to receive the phosphate from phosphorylated PhoP. It is not clear whether this reverse phosphotransfer is a common mechanism shared by all two-component systems (see Refs. 28–31). In the PhoP/PhoQ system, the reversion of the kinase activity may operate as one of the mechanisms that control the phosphorylated state of PhoP. Because the steady state of this reaction can be altered by the ATP/ADP ratio, we can speculate that the energetic charge could be an ancillary cellular mechanism that controls this system. Although from our in vitro model we cannot assess the physiological conditions in which the reverse reaction might become relevant, it is clear that Mg\(^{2+}\) is not promoting this dephosphorylation mechanism.

Only when high concentrations of Mg\(^{2+}\) were added simultaneously with phospho-PhoP and -PhoQ, we detected dephosphorylation of PhoP with release of inorganic phosphate. Additionally, we showed that no Mg\(^{2+}\)-activated phospho-PhoP phosphatase was associated to membranes lacking PhoQ and that each individual phosphorylated protein (phospho-PhoP and phospho-Pho-Q) was stable for more than 30 min regardless of the Mg\(^{2+}\) concentration present in the assay. Taking also into account that PhoQ autokinase activity remained maximal and constant while increasing the MgCl\(_2\) concentration over 200 \(\mu M\), we conclude that Mg\(^{2+}\) induces a phosphatase activity in the membrane-bound PhoQ that dephosphorylates PhoP.

The fusion protein MBP-Qc retained the autokinase activity as well as its capability to accept the phosphate from phospho-PhoP, while it was unable to exert the phosphatase activity on phospho-PhoP at all Mg\(^{2+}\) concentrations tested. This result is consistent with the loss of the capacity to be down-regulated by the signal previously demonstrated for a chimeric sensor protein harboring the N-terminal region of EnvZ fused to the cytoplasmic domain of PhoQ (10). Considering the evidence of both, it becomes clear that the interaction of Mg\(^{2+}\) with the N-terminal sensing domain of PhoQ is essential for triggering its phospho-PhoP phosphatase activity but does not affect the autokinase activity of the sensor. Additionally, the mutant protein PhoQ\(_{HV277V}\) was ineffective as a phosphatase irrespective of the Mg\(^{2+}\) concentration used, indicating that this activity structurally or functionally requires the intactness of the conserved His residue that is also the target for the autophosphorylation reaction.

Whereas these data are consistent with the fact that PhoQ harbors the described phosphatase activity, we cannot rule out the possibility that Mg\(^{2+}\) induces in PhoQ a conformation that, upon interaction with phosphorylated PhoP, activates an autophosphatase in the latter. However, we favor the first hypothesis, because in the presence of Mg\(^{2+}\) phospho-PhoP showed a half-life of approximately 60 min under nondenaturing conditions and the half-life of the regulators that exhibit autophosphatase activity ranges from seconds in CheY to a few minutes in NtrC and PhoB (31–33).

According to the data presented in this work, the PhoP/PhoQ
system adjusts to the “single-regulation model” proposed by Ninfa (5). In this case the kinase is the activity that remains essentially constant while the stimuli simply elicits the phosphatase activity, and the conformational requirements for the two activities of the bifunctional state would not be mutually exclusive.

Under Mg\(^{2+}\)-limiting conditions PhoQ would be in the kinase-dominant state. Because the system is transcriptionally autoregulated, this would increase the amount of both PhoP and PhoQ and result in the subsequent increased level of phospho-PhoP. This will, in turn, induce the expression of a set of genes whose products are known to be necessary for survival in Mg\(^{2+}\)-limiting environments such as the Salmonella-containing vacuole inside macrophages (34). On the other hand, when the bacterium encounters an environment with a concentration of Mg\(^{2+}\) in the millimolar range, the divalent cation would interact with the periplasmic domain of PhoQ shifting the balance toward a phosphatase-dominant state of the sensor protein. Under this condition PhoP would become dephosphorylated, and the expression of the PhoP-activated genes would be shut down. Maintenance of a basal level of expression of PhoP and PhoQ is driven by a PhoP-independent promoter in the phoPQ operon (16).

Although it is clear that more than one pathway may exist for the dephosphorylation of PhoP by PhoQ, this work explains the molecular basis of the modulation of PhoQ by Mg\(^{2+}\) and places the regulatory key of the PhoP/PhoQ system in the control of a phosphatase activity in the sensor protein PhoQ.

Finally, from this model we envision that a detailed structural knowledge of the protein domain involved in the phosphatase activity of PhoQ and the characterization of the interaction of phospho-PhoP with the active site will provide new clues for understanding the signal transduction mechanism of two-component systems.

Acknowledgments—We thank Dr. A. M. Viale and Dr. E. C. Serra for critical reading and helpful suggestions during the preparation of this manuscript.

REFERENCES

1. Perraud, A.-L., Weiss, V., and Gross, R. (1999) Trends Microbiol. 7, 115–120
2. Stock, J. B., Surette, M. G., Levit, M., and Park, P. (1995) in Two-component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 25–51, ASM Press, Washington, DC
3. Parkinson, J. S. (1993) Cell 73, 857–871
4. Cavicchioli, R., Schroder, I., Constantini, M., and Gunsalus, R. P. (1995) J. Bacteriol. 177, 2416–2424
5. Ninfa, A. J. (1996) in Escherichia Coli and Salmonella: Cellular and Molecular Biology (Neidhart, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) Vol. 1, 2nd Ed., pp. 1246–1262, ASM Press, Washington, DC
6. Hsing, W., and Silhavy, T. J. (1997) J. Bacteriol. 179, 3729–3735
7. Hsing, W., Russo, F. D., Bernd, K. K., and Silhavy, T. J. (1998) J. Bacteriol. 180, 4538–4546
8. Jiang, P., and Ninfa, A. F. (1999) J. Bacteriol. 181, 1906–1911
9. Shi, L., Liu, W., and Hulet, F. M. (1999) Biochemistry 38, 10119–10125
10. Garcia Vescovi, E., Soncini, F. C., and Groisman, E. A. (1996) Cell 84, 165–174
11. Soncini, F. C., Garcia Vescovi, E., Solomon, F., and Groisman, E. A. (1996) J. Bacteriol. 178, 5092–5099
12. Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) Science 276, 250–253
13. Guo, L., Lim, K. B., Pudjie, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) Cell 95, 189–198
14. Blanc-Potard, A.-B., and Groisman, E. A. (1997) EMBO J. 16, 5376–5385
15. Vescovi, E. G., Ayala, Y. M., DiCera, E., and Groisman, E. A. (1997) J. Biol. Chem. 272, 1440–1443
16. Soncini, F. C., Garcia Vescovi, E., and Groisman, E. A. (1995) J. Bacteriol. 177, 4364–4371
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Deng, W. P., and Nickoloff, J. A. (1992) Anal. Biochem. 200, 81–90
19. Hess, J. F., Bourret, R. B., and Simon, M. I. (1991) Methods Enzymol. 200, 188–204
20. Igo, M. M., Ninfa, A. J., Stock, J. B., and Silhavy, T. J. (1989) Genes Dev. 3, 1725–1734
21. Raivo, T. L., and Silhavy, T. J. (1997) J. Bacteriol. 179, 7724–7733
22. Aiba, H., Mizuno, T., and Mizushima, S. (1989) J. Biol. Chem. 264, 8563–8567
23. Aiba, H., Nakasai, S., Mizushima, S., and Mizuno, T. (1989) J. Biol. Chem. 264, 14090–14094
24. Blat, Y., Gillespie, B., Boren, A., Dahlgquist, F. W., and Eisenbach, M. (1998) J. Biol. Chem. 273, 1191–1199
25. Ohlen, K. L., Grimesley, J. K., and Hoch, J. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1756–1760
26. Perego, M., Hanstein, C. G., Welsh, K. M., Djavakhishvili, T., Glaser, P., and Hoch, J. A. (1994) Cell 79, 1047–1055
27. Walderburger, C. D., and Sauer, R. T. (1996) J. Biol. Chem. 271, 26630–26636
28. Grimesley, J. K., Huang, S., Hanstein, C. G., Strouch, M. A., Burbulyss, D., Wang, L., Hoch, J. A., and Whiteley, J. M. (1998) Biochemistry 37, 1365–1375
29. Stewart, R. C. (1997) Biochemistry 36, 2030–2040
30. Dutta, R., and Inouye, M. (1996) J. Biol. Chem. 271, 1424–1429
31. Weiss, V., and Magasanik, B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8919–8923
32. Hess, J. F., Oosawa, K., Kaplan, K., and Simon, M. I. (1988) Cell 53, 79–87
33. Makino, K., Shinagawa, M., Amemura, M., Kawamoto, T., Yamada, M., and Nakata, K. (1989) J. Mol. Biol. 210, 551–559
34. Garcia del Portillo, F., Foster, J. W., Maguire, M. E., and Finlay, B. B. (1992) Microbiology 306, 3289–3297