Functional reconstitution of the *Salmonella typhimurium* PhoQ histidine kinase sensor in proteoliposomes

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Two-component signal-transduction systems are widespread in bacteria. They are usually composed of a transmembrane histidine kinase sensor and a cytoplasmic response regulator. The PhoP/PhoQ two-component system of *Salmonella typhimurium* contributes to virulence by co-ordinating the adaptation to low concentrations of environmental Mg^{2+} . Limiting concentrations of extracellular Mg^{2+} activate the PhoP/PhoQ phosphorylation cascade modulating the transcription of PhoP-regulated genes. In contrast, high concentrations of extracellular Mg^{2+} stimulate the dephosphorylation of the response regulator PhoP by the PhoQ kinase sensor. In the present study, we report the purification and functional reconstitution of PhoQ_{His}, a PhoQ variant with a C-terminal His tag, into *Escherichia coli* liposomes. The functionality of PhoQ_{His} was essentially similar to that of PhoQ as shown *in vivo* and *in vitro*. Purified PhoQ_{His} was inserted into lipo-

somes in a unidirectional orientation, with the sensory domain facing the lumen and the catalytic domain facing the extraluminal environment. Reconstituted PhoQ_{His} exhibited all the catalytic activities that have been described for histidine kinase sensors. Reconstituted PhoQ_{His} was capable of autokinase activity when incubated in the presence of Mg^{2+} -ATP. The phosphoryl group could be transferred from reconstituted PhoQ_{His} to PhoP. Reconstituted PhoQ_{His} catalysed the dephosphorylation of phospho-PhoP and this activity was stimulated by the addition of extraluminal ADP.

Key words: bacterial signal transduction, membrane receptor, phosphorylation, proteoliposome, *Salmonella typhimurium*, two-component system.

INTRODUCTION

Two-component regulatory systems are used by bacteria to sense and respond to a variety of environmental signals, usually by modulating the expression of specific genes. Prototypical two-component systems consist of a transmembrane histidine kinase sensor that detects specific environmental signals and a cytoplasmic response regulator that elicits the cellular response [1,2]. Signal transduction across the cytoplasmic membrane modulates an intracellular phosphorylation cascade [3,4]. The kinase sensor autophosphorylates in an ATP-dependent manner on a highly conserved histidine residue of its cytoplasmic catalytic domain. Subsequently, the phosphoryl group is transferred from the histidine residue to an invariant aspartic residue of the response regulator receiver domain. In turn, this affects the DNA-binding properties of the response regulator effector domain. Most kinase sensors also possess a phosphatase activity that allows the dephosphorylation of the response regulator. The balance between the autokinase and phosphatase activities of the kinase sensor controls the net phosphorylation of the response regulator [5–7].

In *Salmonella typhimurium*, the PhoP/PhoQ two-component system is composed of the PhoQ histidine kinase sensor and the PhoP response regulator. It controls the expression of more than 40 genes in response to changes in the extracellular concentrations of bivalent cations such as Mg^{2+} , Ca^{2+} and Mn^{2+} [8]. It has been proposed that Mg^{2+} acts as the physiologically relevant signal controlling the PhoP/PhoQ system [9]. Limiting concentrations of extracellular Mg^{2+} (μM range) activate the PhoP/PhoQ phosphorylation cascade promoting the transcriptional modulation of PhoP-regulated genes. High concentrations of extracellular Mg^{2+} (mM range) stimulate the phosphatase activity of the PhoQ

sensor kinase, leading to dephosphorylation of the PhoP response regulator. Although much research has addressed the details of the catalytic mechanisms for autokinase and phosphatase activities, many questions remain about how environmental signals regulate these activities [10–12]. In the case of the PhoQ kinase sensor, regulation of the catalytic activities occurs through Mg^{2+} binding to the extracellular PhoQ sensory domain [8]. Mg^{2+} recognition is believed to elicit a conformational change that controls the PhoQ enzymatic activities [8]. Recent studies, using the PhoQ protein overexpressed in membranes, have addressed whether one or both PhoQ catalytic activities are regulated by the Mg^{2+} -induced conformational change [10,11].

In the present study, we characterized the catalytic activities of the purified PhoQ_{His} protein reconstituted into *Escherichia coli* phospholipids. We showed that reconstituted PhoQ_{His} possesses autokinase activity and the ability to transfer the phosphoryl group to PhoP. In addition, we found that reconstituted PhoQ_{His} exhibits phosphatase activity that is stimulated by the presence of ADP.

EXPERIMENTAL

Plasmid constructs

Epicurean coli^{TM} strain XL-1 Blue cells (Stratagene, La Jolla, CA, U.S.A.) were used for all DNA manipulations. The molecular cloning techniques used were as described by Sambrook et al. [13] or as recommended by the manufacturer. Plasmids pET-Q and pET-P_{His} encoding the full-length PhoQ and PhoP_{His} proteins respectively have been described previously [10]. For construction of plasmid pET-Q_{His}, the *phoQ* gene of *S. typhimurium*

Abbreviations used: AmdIS, 4-acetamido-4'-maleidylstilbene-2,2'-disulphonic acid; DDM, decyl- β -D-maltopyranoside; DM, dodecyl- β -D-maltoside; GMP-PNP, guanosine 5'-[β , γ -imido]triphosphate; 5-IAF, 5-iodoacetamide fluorescein; LB, Luria-Bertani; LDAO, lauryldimethylamine-N-oxide; NEM, N-ethylmaleimide; ONPG, o-nitrophenyl β -D-galactopyranoside; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate.

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was amplified by PCR from plasmid pET-Q using the forward primer QS-5'-NDE (5'-GGGCCGCGCATATGAATAAATTTGCTCGCCATTTTCTG-3'), which contains an NdeI restriction site and the reverse primer QS-3'-XHO (5'-CGGCTCGAGTTCCTCTTTCTGTGTGGGATGCTG-3'), which contains an XhoI restriction site. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, U.S.A.), digested with NdeI and XhoI and inserted into the expression vector pET-20b(+) (Novagen, Madison, WI, U.S.A.) previously digested with the same restriction enzymes. To carry out *in vivo* analyses, we generated plasmid pPRO-Q by inserting the *phoQ* coding sequence into plasmid pPRO [14]. Plasmid pPRO-Q_{His} was described previously [14].

In vivo analysis

The *E. coli* strain MG1607 contains a chromosomal *mgtA::lacZ* transcriptional fusion and a disruption of the *phoQ* gene [15]. β -Galactosidase assays were conducted as described previously [14]. Briefly, MG1607 cells were transformed with the pPRO, pPRO-Q or pPRO-Q_{His} plasmids and grown overnight at 37 °C in LB (Luria-Bertani) broth supplemented with 0.2 % (w/v) glucose and 100 μ g/ml ampicillin. Cultures were diluted 1:100 in the above medium, in the absence or presence of 10 mM MgCl₂, and grown to late-exponential phase before inducing with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 2 h. Cell lysates were prepared by using the Reporter lysis buffer (Promega, Madison, WI, U.S.A.). β -Galactosidase activity was measured using ONPG (*o*-nitrophenyl β -D-galactopyranoside) as a substrate. One unit of β -galactosidase hydrolyses 1 μ mol of ONPG/min at pH 7.5 and 37 °C.

Overexpression of PhoQ_{His} and PhoP

The *E. coli* strain BL21(DE3)pLysE (Novagen) was used for protein expression. Cells were transformed with either the pET-20b(+), pET-Q or pET-Q_{His} plasmids and grown at 37 °C with aeration in LB supplemented with 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol. Cells grown to late-logarithmic phase were induced with 0.5 mM IPTG for 4 h. Cells were harvested by centrifugation (5000 g, 10 min and 4 °C) and membrane vesicles were prepared as described previously [14]. Overexpression and purification of the PhoP variant harbouring a C-terminal His tag was conducted as described previously [14].

Solubilization and purification of PhoQ_{His}

Membrane vesicles containing PhoQ_{His} were diluted at a final protein concentration of 2.5 mg/ml in a buffer consisting of 20 mM Tris/HCl (pH 7.9), 10 % (v/v) glycerol and detergent. Detergents used were 0.1 % (v/v) Triton X-100, 0.1 and 1 % (w/v) DM (dodecyl- β -D-maltoside), 0.1 and 1 % (w/v) DDM (decyl- β -D-maltopyranoside) or 0.1 % (v/v) LDAO (lauryldimethylamine-*N*-oxide). Samples were gently vortex-mixed and incubated on ice for 1 h. Insoluble material was removed by centrifugation (13 000 g, 30 min and 4 °C) and the supernatant was used for SDS/PAGE analysis and for *in vitro* global activity assays as described below. For purification of the PhoQ_{His} protein, PhoQ_{His}-containing membranes were solubilized with 1 % DM as described above. Following centrifugation, the supernatant was loaded on to a 1 ml HiTrap chelating column (Amersham Biosciences) equilibrated in loading buffer (20 mM Tris/HCl, pH 7.9, 500 mM NaCl, 50 mM imidazole and 0.1 % DM). The column was extensively washed with loading buffer and eluted by applying a 50 mM to 1 M imidazole gradient in loading buffer. Fractions containing PhoQ_{His} were pooled and dialysed extensively against 20 mM Tris/HCl

(pH 7.9), 50 mM NaCl, 10 % glycerol and 0.1 % DM. The concentration of purified PhoQ_{His} was measured using the BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, U.S.A.) with dilutions of BSA as standards.

Reconstitution of PhoQ_{His} into liposomes

E. coli phospholipids (*E. coli* polar lipid extract, acetone/ether washed) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Liposomes for reconstitution were prepared as described by Racher et al. [16] using a buffer consisting of 50 mM Tris/HCl (pH 8.0), 10 mM 2-mercaptoethanol and stored at -70 °C until use. Triton X-100, DDM, DM and LDAO at various concentrations were initially tested for their suitability to reconstitute PhoQ_{His}. PhoQ_{His} reconstitution was performed essentially as described for the *E. coli* ProP and PutP transporters [16, 17]. Liposomes were thawed at room temperature (22 °C), extruded 19 times through a 400 nm polycarbonate filter and diluted to 5 mg/ml with phosphorylation buffer (50 mM Tris/HCl, pH 7.5, 200 mM KCl, 0.1 mM EDTA and 5 % glycerol). Liposomes were destabilized by the addition of 0.2 % DDM and the sample was incubated for 30 min at 22 °C with agitation. The purified PhoQ_{His} protein was added at the lipid/protein ratio of lipid/protein (80:1, w/w), and the sample was incubated at 22 °C with agitation for another 30 min. Detergents were removed by adding Bio-Beads SM-2 (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at a wet weight bead/detergent ratio of 10:1 (w/w). The sample was incubated at 22 °C for 1 h with agitation, and a second aliquot of Bio-Beads SM-2 was added. The sample was then incubated with agitation at 4 °C overnight. The proteoliposome solution was removed from the Bio-Beads SM-2 and centrifuged at 470 000 g for 30 min at 4 °C. The pellet was resuspended in phosphorylation buffer and used immediately. The concentration of PhoQ_{His} in proteoliposomes was determined by SDS/PAGE using known concentrations of the purified protein. Urea extraction was performed as described previously [18], by incubating proteoliposomes with 6.0 M urea at 4 °C for 30 min. Following centrifugation (470 000 g, 30 min and 4 °C), the supernatant and the resuspended pellet were analysed by SDS/PAGE.

PhoQ_{His} orientation in proteoliposomes

Orientation of PhoQ_{His} was determined through the use of membrane-permeable and -impermeable thiol-reactive reagents. Fluorescent reagents were purchased from Molecular Probes (Eugene, OR, U.S.A.). Proteoliposomes (~1.5 μ M PhoQ_{His}) were subjected to one of the following treatments with an incubation of 10 min at 22 °C between reagent additions. For the determination of maximal labelling, proteoliposomes were solubilized with 1 % DM and subsequently labelled with 0.33 mM 5-IAF (5-iodoacetamide fluorescein). To determine the percentage of PhoQ_{His} molecules with the catalytic domain extraluminal, proteoliposomes were incubated with 0.33 mM 5-IAF to label externally exposed cysteine residues. To determine the percentage of PhoQ_{His} molecules with the catalytic domain intraluminal, proteoliposomes were incubated with 0.33 mM Amdis (4-acetamido-4'-maleidylstilbene-2,2'-disulphonic acid) to block externally exposed cysteine residues, solubilized with 1 % DM and then incubated with 0.33 mM 5-IAF to label solely the internally exposed cysteine residues. To control for unspecific labelling and fluorescence, proteoliposomes were incubated with 10 mM NEM (*N*-ethylmaleimide), a non-fluorescent membrane-permeable thiol-reactive reagent, and then incubated with 0.33 mM 5-IAF. All reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer (250 mM Tris/HCl, pH 6.8, 8 %, w/v, SDS,

40 % glycerol, 0.02 % Bromophenol Blue and 4 %, v/v, 2-mercaptoethanol) and analysed by SDS/PAGE. Fluorescence of proteins labelled with 5-IAF was visualized using an FX Scanner (Bio-Rad Laboratories) using the internal 532 nm laser for excitation and the 555 nm Longpass filter for emission.

Proteoliposome integrity

Proteoliposome integrity was assessed by determining the extent of leakage of β -galactosidase trapped within liposomes or proteoliposomes. Proteoliposomes were prepared as described above and preloaded with 7.5 units of β -galactosidase. Following centrifugation, proteoliposomes were resuspended in phosphorylation buffer or phosphorylation buffer supplemented with 10 mM MgCl_2 , CaCl_2 or MnCl_2 and incubated at 22 °C for up to 150 min. Samples were then centrifuged at 470 000 *g* for 30 min at 4 °C and the resulting supernatants and resuspended pellets were added to an equal volume of Reporter lysis buffer (Promega). β -Galactosidase activity was measured using ONPG as the substrate.

In vitro global activity assays

The net phosphorylation of PhoP was measured by incubating intact PhoQ- or PhoQ_{His}-containing membranes, solubilized PhoQ_{His}-containing membranes or proteoliposomes (all approx. 1.5 μM of PhoQ or PhoQ_{His}) with an 8-fold molar excess of PhoP in a 15 μl volume of phosphorylation buffer supplemented with 5 mM MgCl_2 . Reactions were initiated by the addition of 0.1 mM [γ - ^{32}P]ATP (10 Ci/mmol), followed by incubation at 22 °C for various time periods. Reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer. Reaction products were heated at 37 °C for 3 min and subjected to SDS/PAGE (10 % polyacrylamide). Gels were dried under vacuum and exposed to a phosphor screen. Phosphorylated proteins were visualized using an FX scanner and quantified by image analysis using the Quantity One software (Bio-Rad Laboratories). All arbitrary units of intensity were converted into concentrations of ^{32}P -phosphorylated protein based on a standard curve.

In vitro autophosphorylation assays

Proteoliposomes ($\sim 1.5 \mu\text{M}$ PhoQ_{His}) were incubated with 0.1 mM [γ - ^{32}P]ATP (10 Ci/mmol) in a 15 μl volume of phosphorylation buffer supplemented with 5 mM MgCl_2 . The phosphorylation reactions were continued at 22 °C for various time periods before being stopped by the addition of 4 $\times$ Laemmli loading buffer. Phosphorylation products were analysed by SDS/PAGE as described above.

In vitro phosphatase assays

Purified [^{32}P]phospho-PhoP was prepared as described previously [14]. Radiolabelled phospho-PhoP (12 μM) was mixed with proteoliposomes ($\sim 1.5 \mu\text{M}$ PhoQ_{His}) preloaded with 5 mM MgCl_2 in 15 μl of phosphorylation buffer supplemented with 5 mM MgCl_2 and various concentrations of ADP, GDP, p[NH]ppA (adenosine 5'-[β , γ -imido]triphosphate or AMP-PNP) or GMP-PNP (guanosine 5'-[β , γ -imido]triphosphate) where appropriate. Stability of [^{32}P]phospho-PhoP in the presence of ADP or p[NH]ppA was assessed as described above except that proteoliposomes were omitted from the reaction. After incubating at 22 °C for various time periods, reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer. Phosphorylated products were analysed by SDS/PAGE as described above.

Figure 1 *In vivo* and *in vitro* activities of the PhoQ and PhoQ_{His} kinase sensors

(A) *In vivo* assays. Transcription of the *mgtA* gene (a PhoP-activated gene) from the *E. coli* MG1607 strain carrying plasmids pPRO (control plasmid), pPRO-Q (PhoQ) or pPRO-Q_{His} (PhoQ_{His}). Cells were grown in LB medium or LB medium supplemented with 10 mM MgCl_2 . β -Galactosidase activity from the *mgtA::lacZ* transcriptional fusion was measured as described in the Experimental section. Results shown are the mean $\pm$ S.D. values for triplicate determinations. One unit of β -galactosidase hydrolyses 1 μmol of ONPG/min at pH 7.5 and 37 °C. (B) *In vitro* global activity of the PhoP/PhoQ and PhoP/PhoQ_{His} systems. The net phosphorylation of PhoP by PhoQ or PhoQ_{His} present in membrane fractions was measured. Membrane fractions from *E. coli* cells transformed with the empty plasmid or plasmids encoding either PhoQ or PhoQ_{His} ($\sim 1.5 \mu\text{M}$) were incubated with an 8-fold molar excess of PhoP in phosphorylation buffer supplemented with 0.1 mM [γ - ^{32}P]ATP and 5 mM MgCl_2 . After 20 min of incubation at 22 °C, reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer and analysed by SDS/PAGE.

RESULTS

Influence of the C-terminal His tag on PhoQ activity

To facilitate purification of PhoQ, the *phoQ* gene was cloned into pET-20b(+) to yield the plasmid pET-Q_{His}, which encodes the full-length PhoQ protein with six consecutive histidine residues at the C-terminus (PhoQ_{His}). We first examined the effect of the C-terminal His tag on PhoQ activity *in vivo* by measuring the expression of *mgtA* (a PhoP-activated gene that encodes an Mg^{2+} transporter) through the β -galactosidase activity of an *mgtA::lacZ* transcriptional fusion [15]. As shown in Figure 1(A), the resulting levels of β -galactosidase activity were essentially similar for cells producing PhoQ and PhoQ_{His} in the absence of MgCl_2 . In the presence of 10 mM MgCl_2 , β -galactosidase activity was reduced by 10- and 8-fold for cells producing PhoQ and PhoQ_{His} respectively (Figure 1A). Thus the C-terminal His tag does not grossly affect PhoQ activity and regulation by Mg^{2+} , *in vivo*.

To further compare PhoQ and PhoQ_{His}, we assessed the global activity of the PhoP/PhoQ system, *in vitro*, by measuring the net phosphorylation of PhoP resulting from both the autokinase and phosphatase activities of the PhoQ proteins. Both PhoQ and PhoQ_{His} were expressed in *E. coli* membranes at similar levels and represented approx. 10 % of the total membrane proteins

Figure 2 Solubilization and purification of PhoQ_{His}

(A) Solubilization of PhoQ_{His}. Membrane fractions containing the overexpressed PhoQ_{His} protein were solubilized in 20 mM Tris/HCl (pH 7.5), 10% glycerol and detergent. Lane 1, non-solubilized membranes; lane 2, 0.1% Triton X-100; lane 3, 0.1% LDAO; lane 4, 1% DDM; lane 5, 0.1% DDM; lane 6, 1% DM; lane 7, 0.1% DM. Following 1 h of incubation on ice, insoluble material was removed by centrifugation and the supernatants were analysed by SDS/PAGE. (B) Net phosphorylation of PhoP by solubilized PhoQ_{His}. *In vitro* global activity assays were conducted as indicated in the legend to Figure 1. Lanes are as in (A). (C) SDS/PAGE analysis of PhoQ_{His} purification. The gel was stained with Coomassie Blue. Lane 1, control membrane fractions from cells transformed with the empty plasmid; lane 2, membrane fractions from cells overexpressing PhoQ_{His}; lane 3, purified PhoQ_{His} (~4 µg). Molecular mass standards are shown on the left.

(results not shown). Membrane fractions containing either the PhoQ or PhoQ_{His} proteins were incubated with an 8-fold molar excess of PhoP in the presence of [γ -³²P]ATP and 5 mM MgCl₂. Figure 1(B) shows the amount of [³²P]phospho-PhoP resulting from the activity of PhoQ or PhoQ_{His} after a 20 min incubation period. Levels of radiolabelled phospho-PhoP were increased by 2-fold when incubated with PhoQ_{His} compared with PhoQ. Analysis of the individual activities showed that the phosphatase activity of PhoQ_{His} is slightly reduced compared with that of PhoQ, while the autokinase activities of both PhoQ proteins are similar (results not shown). These *in vitro* results, consistent with the *in vivo* results shown in Figure 1(A), show that the activity of PhoQ_{His} is not severely affected by the C-terminal His tag. Thus PhoQ_{His} was used for further experimentation.

Catalytic activity of the solubilized and purified PhoQ_{His}

To purify PhoQ_{His}, optimal solubilization conditions were first identified. The detergents Triton X-100, LDAO, DM and DDM were used to solubilize membrane vesicles containing the overexpressed PhoQ_{His} protein. All detergents tested were found to be 60–80% efficient in solubilizing PhoQ_{His} when compared with a non-solubilized control (Figure 2A). When the series of solubilized PhoQ_{His} was assayed for *in vitro* global activity by incubating for 20 min with an 8-fold molar excess of PhoP in the presence of [γ -³²P]ATP and 5 mM MgCl₂, PhoQ_{His} solubilized in 0.1% LDAO, DDM or DM retained more than 50% activity as compared with a non-solubilized control (Figure 2B). Although maximal activity was obtained with 0.1% DDM (Figure 2B, lane 5), 0.1% DM was deemed optimal for PhoQ_{His}

purification. PhoQ_{His} was purified by Ni-NTA (Ni²⁺-nitriloacetate) chromatography (Figure 2C) and the identity of the purified protein was confirmed by Western blotting using a monoclonal antibody directed against the His tag (results not shown). No activity of purified PhoQ_{His} was detected in the *in vitro* global activity assay regardless of the detergent used (results not shown). Approximately 2.5 mg of purified PhoQ_{His} was obtained from 50 mg of total membrane proteins.

Functional reconstitution of PhoQ_{His} in proteoliposomes

Purified PhoQ_{His} was reconstituted into detergent-destabilized liposomes prepared from *E. coli* phospholipids, based on the detergent-mediated method [19]. Purified PhoQ_{His} in 0.1% DM was added to liposomes destabilized with 0.2% DDM and detergents were removed by adsorption to Bio-Beads SM-2. The efficiency of PhoQ_{His} reconstitution was assessed by comparing solubilized proteoliposomes with an amount of purified PhoQ_{His} representing 100% incorporation on SDS/PAGE. The efficiency was determined to be approx. 86%. Urea extraction was performed to show that the PhoQ_{His} proteins are correctly inserted into liposomes. SDS/PAGE analysis of proteoliposomes treated with urea and subjected to centrifugation showed that approx. 80% of the PhoQ_{His} proteins are resistant to urea treatment (results not shown).

Orientation of PhoQ_{His} in proteoliposomes

PhoQ_{His} contains only two cysteine residues at positions 392 and 395 in the cytoplasmic catalytic domain. By exploiting the location of these cysteine residues, we determined the orientation of PhoQ_{His} in proteoliposomes through the differential use of thiol-reactive probes. A control reaction representing 100% labelling was determined by solubilizing proteoliposomes with 1% DM and labelling cysteine residues with 5-IAP (Figure 3A, lane 1). The percentage of PhoQ_{His} molecules with the catalytic domain facing the extraluminal environment was determined by labelling externally exposed cysteine residues with the membrane-impermeable probe 5-IAP (Figure 3A, lane 2). These results suggest that nearly all PhoQ_{His} proteins are orientated with the catalytic domain extraluminal. To confirm this, the percentage of PhoQ_{His} molecules with the catalytic domain facing the intraluminal environment was determined by labelling externally exposed cysteine residues with the membrane-impermeable thiol-reactive probe AmdS, solubilizing proteoliposomes with 1% DM and subsequently labelling the remaining (intraluminal) exposed cysteine residues with 5-IAP (Figure 3A, lane 3). Very little fluorescence attributable to 5-IAP was detected, suggesting that very few PhoQ_{His} proteins are orientated with the catalytic domain intraluminal. Proteoliposomes labelled with the membrane-impermeable thiol-reactive probe NEM followed by 5-IAP showed little fluorescence, indicating specific labelling of thiol groups (Figure 3A, lane 4). Following the fluorescence experiments, SDS/polyacrylamide gels were stained with Coomassie Brilliant Blue to confirm that similar amounts of protein were used in all reactions (Figure 3B). Thus the orientation of PhoQ_{His} in proteoliposomes was found to be essentially unidirectional, with the catalytic domain extraluminal (Figure 3C). To confirm that the sensory domain of reconstituted PhoQ_{His} is facing the lumen of proteoliposomes, we assessed the ability of PhoQ_{His} to sense the intraluminal environment. Proteoliposomes were preloaded with the various bivalent cations known to be sensed by the PhoQ sensory domain [8] and *in vitro* global activity assays were performed. The presence of 5 mM intraluminal MgCl₂, CaCl₂ or MnCl₂ inhibited the phosphorylation of PhoP compared with proteoliposomes preloaded with no bivalent cations

Figure 3 Orientation of PhoQ_{His} in proteoliposomes

(A) PhoQ_{His}-proteoliposomes (1.5 μ M PhoQ_{His}) were solubilized with 1% DM and/or incubated with various thiol-reactive probes. Reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer and analysed by SDS/PAGE. Fluorescence of PhoQ_{His} labelled with 5-IAF was visualized as described in the Experimental section. Lane 1, proteoliposomes were solubilized with 1% DM and labelled with 5-IAF; lane 2, proteoliposomes were labelled with 5-IAF; lane 3, proteoliposomes were treated with AmdIS, solubilized with 1% DM and labelled with 5-IAF; lane 4, proteoliposomes were treated with NEM and labelled with 5-IAF. (B) SDS/PAGE analysis of the PhoQ_{His}-proteoliposomes used in (A). (C) Schematic representation of the topology of PhoQ_{His} in proteoliposomes. The catalytic domain of PhoQ_{His} (Cat) is facing the extraluminal environment. The two cysteine residues, which are part of the catalytic domain, are indicated by 'C'.

(S. Sanowar and H. Le Moual, unpublished work). Altogether, these results provide strong evidence that PhoQ_{His} in proteoliposomes is able to sense the intraluminal environment through its sensory domain and signal to the extraluminal catalytic domain (Figure 3C).

Proteoliposome integrity

To assess proteoliposome leakage, liposomes or proteoliposomes were preloaded with β -galactosidase and incubated for up to 150 min in buffer or buffer supplemented with 10 mM MgCl₂, CaCl₂ or MnCl₂. The amount of β -galactosidase that leaked from the vesicles was measured using the substrate ONPG. There was no detectable leakage of β -galactosidase from liposomes or proteoliposomes exposed to these bivalent cations compared with a control exposed to buffer alone (results not shown). The amount of β -galactosidase activity released upon solubilization of the vesicles was similar for all samples. Although β -galactosidase is much larger than ionic solutes, these data indicate that MgCl₂, CaCl₂ or MnCl₂ at a concentration of up to 10 mM do not grossly disrupt the membrane permeability barrier.

In vitro autokinase activity of reconstituted PhoQ_{His}

In the *in vitro* autokinase assay, we measured the amount of phospho-PhoQ_{His} generated at various time points. To maximize the PhoQ_{His} autokinase activity, proteoliposomes were preloaded with phosphorylation buffer devoid of MgCl₂ and incubated in the presence of extraluminal [γ -³²P]ATP and 5 mM MgCl₂. Over time, we observed a slow and sustained net accumulation of

Figure 4 Time course of reconstituted PhoQ_{His} autophosphorylation

PhoQ_{His}-proteoliposomes (1.5 μ M PhoQ_{His}) preloaded with phosphorylation buffer devoid of MgCl₂ were incubated at 22 °C in phosphorylation buffer supplemented with 0.1 mM [γ -³²P]ATP and 5 mM MgCl₂. At the indicated time points, reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer and analysed by SDS/PAGE. The amounts of [³²P]phospho-PhoQ_{His} were determined with a phosphorimager.

Figure 5 Time course of net phosphorylation of PhoP by reconstituted PhoQ_{His}

PhoQ_{His}-proteoliposomes (1.5 μ M PhoQ_{His}) preloaded with phosphorylation buffer devoid of MgCl₂ were incubated at 22 °C with an 8-fold molar excess of PhoP in phosphorylation buffer supplemented with 0.1 mM [γ -³²P]ATP and 5 mM MgCl₂. At specific time points, reactions were stopped by the addition of 4 $\times$ Laemmli loading buffer and analysed by SDS/PAGE. The amounts of [³²P]phospho-PhoP were determined with a phosphorimager.

[³²P]phospho-PhoQ_{His} that reached an apparent steady-state level after 120 min (Figure 4). These results demonstrate that reconstituted PhoQ_{His} is capable of autokinase activity.

In vitro global activity of reconstituted PhoQ_{His}

To examine the kinetics of net phosphorylation of PhoP by reconstituted PhoQ_{His}, proteoliposomes preloaded with phosphorylation buffer devoid of MgCl₂ were incubated with an 8-fold excess of PhoP in the presence of [γ -³²P]ATP and 5 mM MgCl₂. Over time, we observed a slow and sustained net accumulation of [³²P]phospho-PhoP that reached an apparent steady state after a 120 min incubation (Figure 5). Thus the phosphoryl group can be transferred from reconstituted PhoQ_{His} to PhoP. As expected from the molar excess of PhoP used in the assay, higher levels of [³²P]phospho-PhoP were obtained compared with levels of [³²P]phospho-PhoQ_{His} (Figures 4 and 5).

In vitro phosphatase activity of reconstituted PhoQ_{His} is stimulated by nucleotides

To assess the phosphatase activity of reconstituted PhoQ_{His}, we incubated purified [³²P]phospho-PhoP with proteoliposomes

Figure 6 *In vitro* phosphatase activity of PhoQ_{His} in proteoliposomes

(A) Time course of reconstituted PhoQ_{His} phosphatase activity. PhoQ_{His}-proteoliposomes were preloaded with phosphorylation buffer supplemented with 5 mM MgCl₂. *In vitro* phosphatase assays were performed as described in the Experimental section with phosphorylation buffer supplemented with 5 mM MgCl₂ and nucleotides when indicated. Reaction products were analysed by SDS/PAGE. The amounts of [³²P]phospho-PhoP remaining were determined with a phosphorimager. Intrinsic dephosphorylation of [³²P]phospho-PhoP (○), dephosphorylation of [³²P]phospho-PhoP by reconstituted PhoQ_{His} in the absence (●) or in the presence of 1 mM extraluminal ADP (■). (B) Effect of extraluminal nucleotides on the dephosphorylation of [³²P]phospho-PhoP. *In vitro* phosphatase assays were performed for 20 min as described in (A) with phosphorylation buffer supplemented with 5 mM MgCl₂ and GDP, GMP-PNP, ADP or p[NH]ppA at the indicated concentrations. (C) Effect of ADP and p[NH]ppA on the stability of [³²P]phospho-PhoP. Intrinsic dephosphorylation of [³²P]phospho-PhoP in the presence of phosphorylation buffer (○), phosphorylation buffer supplemented with 1 mM ADP (■) or phosphorylation buffer supplemented with 1 mM p[NH]ppA (●).

preloaded with phosphorylation buffer supplemented with 5 mM MgCl₂. Aliquots were removed at different time points and the time course of the reaction was followed. A control reaction in which [³²P]phospho-PhoP was incubated with liposomes lacking PhoQ_{His} indicated the intrinsic stability of [³²P]phospho-PhoP. As shown in Figure 6(A), reconstituted PhoQ_{His} dephosphorylated only 20% of [³²P]phospho-PhoP after 60 min. Thus,

under these experimental conditions, reconstituted PhoQ_{His} had little phosphatase activity. ADP and the non-hydrolysable ATP analogue, p[NH]ppA have been shown to stimulate the phosphatase activity of the EnvZ and NRII (nitrogen regulator II) histidine kinase sensors [20–22]. To assess the effect of nucleotides on the phosphatase activity of reconstituted PhoQ_{His}, we conducted phosphatase assays in the presence of various concentrations of extraluminal ADP, p[NH]ppA, GDP or GMP-PNP. As shown in Figure 6(B), levels of [³²P]phospho-PhoP decreased when assays were performed in the presence of either ADP or p[NH]ppA. In contrast, the presence of GDP or GMP-PNP had no effect on the levels of [³²P]phospho-PhoP (Figure 6B). To assess whether ADP and p[NH]ppA act on reconstituted PhoQ_{His} or directly on phospho-PhoP, we tested the stability of [³²P]phospho-PhoP in the presence of 1 mM of these nucleotides. As shown in Figure 6(C), p[NH]ppA stimulated the dephosphorylation of [³²P]phospho-PhoP in a PhoQ_{His}-independent manner. In contrast, ADP had no major effect on the stability of [³²P]phospho-PhoP (Figure 6C), suggesting that it promotes the dephosphorylation of phospho-PhoP by stimulating the phosphatase activity of reconstituted PhoQ_{His}. As shown in Figure 6(A), ADP at a concentration of 1 mM increased [³²P]phospho-PhoP dephosphorylation to 70% after 45 min. These results suggest that ADP affects the conformation of the PhoQ_{His} cytoplasmic domain.

DISCUSSION

The PhoP/PhoQ two-component system of *S. typhimurium* controls the expression of many genes, including virulence factors, in response to depletion of environmental Mg²⁺ [8,23,24]. To date, the catalytic activities of the PhoQ kinase sensor have been characterized, *in vitro*, using membrane preparations in which PhoQ is overproduced [10,11,14]. In the present study, we developed a purified and reconstituted system to study trans-membrane signalling by PhoQ. PhoQ_{His} was purified and reconstituted into *E. coli* phospholipids. Under our experimental conditions, insertion of PhoQ_{His} into phospholipid vesicles was unidirectional with the sensory domain facing the lumen. We found that reconstituted PhoQ_{His} is functional and catalyses auto-phosphorylation, the transfer of the phosphoryl group to PhoP and the dephosphorylation of phospho-PhoP.

To facilitate purification, we used a PhoQ variant with a C-terminal His tag (PhoQ_{His}). The possibility that the tag severely affects the PhoQ catalytic activities is most unlikely, since both the tagged and non-tagged proteins show essentially similar activity *in vivo* and *in vitro* (Figure 1) as well as Mg²⁺ regulation, *in vivo* (Figure 1A). To date, the few kinase sensors that have been studied in a reconstituted system are KdpD, EnvZ and DcuS of *E. coli* [25–27]. In contrast with the solubilized KdpD and DcuS sensor kinases [25,27], PhoQ_{His} retained the ability to autophosphorylate and transfer its phosphoryl group to PhoP_{His} upon solubilization (Figure 2B). Results similar to those for PhoQ_{His} were obtained with solubilized EnvZ [26]. These differences may be due to the detergent used in the solubilization procedures. Following purification using Ni-NTA affinity chromatography, PhoQ_{His} was inactive (results not shown), the reason for which is not clear. It is possible that purified PhoQ_{His} lost its dimeric structure that is crucial for trans-autophosphorylation, which occurs between two PhoQ monomers [28,29]. Another possibility is that PhoQ_{His} necessitates lipid components for activity, which would be retained upon solubilization but washed away during purification. Importantly, PhoQ_{His} regained its ability to autophosphorylate and transfer its phosphoryl group to PhoP, once reconstituted into proteoliposomes (Figures 4 and 5). These results suggest

that a lipid environment is necessary to provide the appropriate structural arrangement required for PhoQ_{His} activity.

Although reconstituted PhoQ_{His} exhibited all the catalytic activities that are the hallmark of histidine kinase sensors, it showed significant differences compared with PhoQ or PhoQ_{His} overproduced in bacterial membranes [10,11,14]. Reconstituted PhoQ_{His} autophosphorylates slowly and the phospholinkage appears to be stable for at least 150 min (Figure 4). In contrast, the kinetics of autophosphorylation of PhoQ overproduced in *E. coli* membranes was strongly biphasic, with a rapid phosphorylation phase followed by a slower dephosphorylation phase [10]. This difference in the stability of phospho-PhoQ between the two systems can be explained in two ways. First, interaction of MgCl₂ (5 mM) with the periplasmic sensory domain of PhoQ in *E. coli* membranes may impose on PhoQ a conformation that promotes its dephosphorylation. The absence of MgCl₂ from the lumen of proteoliposomes would prevent reconstituted PhoQ_{His} from adopting such a conformation, leading to phospholinkage stability. Secondly, dephosphorylation of overproduced PhoQ may be due to a Mg²⁺-dependent phosphatase present in *E. coli* membranes but absent from the purified and reconstituted system. In cells, response regulator molecules appear to exist in excess over kinase sensor molecules [30]. By using an 8-fold molar excess of PhoP with respect to PhoQ_{His} in proteoliposomes, a 2-fold amplification of the signal was observed as shown by the difference between the levels of [³²P]phospho-PhoQ_{His} and [³²P]phospho-PhoP obtained at 150 min (Figures 4 and 5). Amplification of the signal to a higher extent may be limited by the intrinsic autophosphatase activity of PhoP observed *in vitro* [11]. Indeed, we found that the half-life of [³²P]phospho-PhoP is approx. 80 min in the presence of 5 mM MgCl₂ (Figure 6A). Kinetics obtained for PhoP phosphorylation (Figure 5) and PhoQ autophosphorylation (Figure 4) are consistent with the fact that transfer of the phosphoryl group to the response regulator is fast compared with the autophosphorylation of kinase sensors, which constitutes the rate-limiting step of the phosphorylation cascade [3].

We found that reconstituted PhoQ_{His} has little phosphatase activity in the absence of ADP (Figure 6A). In contrast, PhoQ or PhoQ_{His} overproduced in *E. coli* membranes were able to dephosphorylate [³²P]phospho-PhoP completely without additional ADP, keeping all other experimental conditions similar [10,14]. This highlights another major difference between the reconstituted system and membrane preparations in which PhoQ is overexpressed. One possibility is that PhoQ_{His} in proteoliposomes cannot adopt a phosphatase-dominant conformation in the absence of ADP, even though MgCl₂ is present at a concentration of 5 mM in the lumen of proteoliposomes. We found that ADP and p[NH]ppA, but not GDP and GMP-PNP, increased the dephosphorylation of phospho-PhoP possibly by stimulating the phosphatase activity of reconstituted PhoQ_{His} (Figure 6B). Strikingly, we found that p[NH]ppA directly affects the stability of phospho-PhoP (Figure 6C), the mechanism behind this being still unclear. In contrast, ADP did not affect phospho-PhoP stability (Figure 6C). Thus we conclude that ADP stimulates the phosphatase activity of reconstituted PhoQ_{His} by interacting with the catalytic domain. It has been proposed that signals transduced across the membrane alter the spatial arrangement between the ATP-binding domain and the central dimerization domain of sensor kinases [6,31]. Our data suggest that in the absence of ADP, these domains cannot adopt proper positioning for maximal phosphatase activity. Binding of ADP to the ATP-binding domain of reconstituted PhoQ_{His} appears to provide a conformation capable of enhanced phosphatase activity (Figure 6A). Altogether, these results indicate that reconstituted PhoQ_{His} is not locked in a

kinase-dominant conformation and can switch to a phosphatase-dominant conformation in the presence of ADP.

This *in vitro* reconstituted PhoP/PhoQ system will allow us to vary systematically the concentration of intraluminal bivalent cations acting as ligands (Mg²⁺, Ca²⁺ and Mn²⁺), while maintaining a constant concentration of extraluminal catalytic Mg²⁺. Future studies will clarify whether both the autokinase and phosphatase activities of PhoQ are targets for regulation by bivalent cations, as suggested previously for Mg²⁺ [10]. In addition, this system will aid in elucidating the molecular mechanism of signal transduction across the cell membrane.

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REFERENCES

- 1 Stock, J. B., Ninfa, A. J. and Stock, A. M. (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53**, 450–490
- 2 Parkinson, J. S. and Kofoid, E. C. (1992) Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**, 71–112
- 3 Stock, A. M., Robinson, V. L. and Goudreau, P. N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.* **69**, 183–215
- 4 Hoch, J. A. and Varghese, K. I. (2001) Keeping signals straight in phosphorelay signal transduction. *J. Bacteriol.* **183**, 4941–4949
- 5 Russo, F. D. and Silhavy, T. J. (1993) The essential tension: opposed reactions in bacterial two-component regulatory systems. *Trends Microbiol.* **1**, 306–310
- 6 Inouye, M., Dutta, R. and Zhu, Y. (2003) Regulation of porins in *Escherichia coli* by the osmosensing histidine kinase/phosphatase EnvZ. In *Histidine Kinases in Signal Transduction* (Inouye, M. and Dutta, R., eds.), pp. 25–46, Academic Press, New York
- 7 Jiang, P., Pioszak, A., Atkinson, M. R., Peliska, J. A. and Ninfa, A. J. (2003) New insights into the mechanism of the kinase and phosphatase activities of *Escherichia coli* NRII (NtrB) and their regulation by the PII protein. In *Histidine Kinases in Signal Transduction* (Inouye, M. and Dutta, R., eds.), pp. 143–164, Academic Press, New York
- 8 Garcia-Vescovi, E., Soncini, F. C. and Groisman, E. A. (1996) Mg²⁺ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* (Cambridge, Mass.) **84**, 165–174
- 9 Groisman, E. A. (1998) The ins and outs of virulence gene expression: Mg²⁺ as a regulatory signal. *BioEssays* **20**, 96–101
- 10 Montagne, M., Martel, A. and Le Moual, H. (2001) Characterization of the catalytic activities of the PhoQ histidine protein kinase of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **183**, 1787–1791
- 11 Castelli, M. E., Garcia Vescovi, E. and Soncini, F. C. (2000) The phosphatase activity is the target for Mg²⁺ regulation of the sensor protein PhoQ in *Salmonella*. *J. Biol. Chem.* **275**, 22948–22954
- 12 Chamnongpol, S., Cromie, M. and Groisman, E. A. (2003) Mg²⁺ sensing by the Mg²⁺ sensor PhoQ of *Salmonella enterica*. *J. Mol. Biol.* **325**, 795–807
- 13 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainville, NY
- 14 Sanowar, S., Martel, A. and Le Moual, H. (2003) Mutational analysis of the residue at position 48 in the *Salmonella enterica* serovar Typhimurium PhoQ sensor kinase. *J. Bacteriol.* **185**, 1935–1941
- 15 Kato, A., Tanabe, H. and Utsumi, R. (1999) Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: identification of extracellular Mg²⁺-responsive promoters. *J. Bacteriol.* **181**, 5516–5520
- 16 Racher, K. I., Voegelé, R. T., Marshall, E. V., Culham, D. E., Wood, J. M., Jung, H., Bacon, M., Cairns, M. T., Ferguson, S. M., Liang, W. J. et al. (1999) Purification and reconstitution of an osmosensor: transporter ProP of *Escherichia coli* senses and responds to osmotic shifts. *Biochemistry* **38**, 1676–1684
- 17 Jung, H., Tebbe, S., Schmid, R. and Jung, K. (1998) Unidirectional reconstitution and characterization of purified Na⁺/proline transporter of *Escherichia coli*. *Biochemistry* **37**, 11083–11088

- 18 Cunningham, K., Lill, R., Crooke, E., Rice, M., Moore, K., Wickner, W. and Oliver, D. (1989) SecA protein, a peripheral protein of the *Escherichia coli* plasma membrane, is essential for the functional binding and translocation of proOmpA. *EMBO J.* **8**, 955–959
- 19 Rigaud, J. L., Pitard, B. and Levy, D. (1995) Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta* **1231**, 223–246
- 20 Igo, M. M., Ninfa, A. J., Stock, J. B. and Silhavy, T. J. (1989) Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. *Genes Dev.* **3**, 1725–1734
- 21 Jiang, P., Atkinson, M. R., Srisawat, C., Sun, Q. and Ninfa, A. J. (2000) Functional dissection of the dimerization and enzymatic activities of *Escherichia coli* nitrogen regulator II and their regulation by the PII protein. *Biochemistry* **39**, 13433–13449
- 22 Zhu, Y., Qin, L., Yoshida, T. and Inouye, M. (2000) Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7808–7813
- 23 Groisman, E. A. (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol.* **183**, 1835–1842
- 24 Ernst, R. K., Guina, T. and Miller, S. I. (2001) *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect.* **3**, 1327–1334
- 25 Jung, K., Tjaden, B. and Altendorf, K. (1997) Purification, reconstitution, and characterization of KdpD, the turgor sensor of *Escherichia coli*. *J. Biol. Chem.* **272**, 10847–10852
- 26 Jung, K., Hamann, K. and Revermann, A. (2001) K⁺ stimulates specifically the autokinase activity of purified and reconstituted EnvZ of *Escherichia coli*. *J. Biol. Chem.* **276**, 40896–40902
- 27 Janausch, I. G., Garcia-Moreno, I. and Uuden, G. (2002) Function of DcuS from *Escherichia coli* as a fumarate-stimulated histidine protein kinase *in vitro*. *J. Biol. Chem.* **277**, 39809–39814
- 28 Yang, Y. and Inouye, M. (1991) Intermolecular complementation between two defective mutant signal-transducing receptors of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11057–11061
- 29 Ninfa, E. G., Atkinson, M. R., Kamberov, E. S. and Ninfa, A. J. (1993) Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): trans-phosphorylation between subunits. *J. Bacteriol.* **175**, 7024–7032
- 30 Cai, S. J. and Inouye, M. (2002) EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. *J. Biol. Chem.* **277**, 24155–24161
- 31 Jin, T. and Inouye, M. (1993) Ligand binding to the receptor domain regulates the ratio of kinase to phosphatase activities of the signaling domain of the hybrid *Escherichia coli* transmembrane receptor, Taz1. *J. Mol. Biol.* **232**, 484–492

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