Transmembrane polar interactions are required for signaling in the *Escherichia coli* sensor kinase PhoQ

Shalom D. Goldberg[a], Graham D. Clinthorne[b], Mark Goulian[c], and William F. DeGrado[a,1]

[a]Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; [b]Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; and [c]Department of Biology, University of Pennsylvania, 433 S University Avenue, Philadelphia, PA 19104

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PhoQ is the transmembrane sensor histidine kinase of the bacterial *phoPQ* two-component system, which detects and responds to divalent cations and to antimicrobial peptides, and can trigger virulence. Despite their ubiquitous importance in bacterial signaling, the structure and mechanism of the sensor kinases are not fully understood. In particular, the mechanism by which the signal is propagated through the transmembrane (TM) region remains unclear. We have identified a critical asparagine residue in the second TM helix of PhoQ. Replacement of this Asn202 with a variety of hydrophobic amino acids results in a protein that is blind to signal, fails to activate transcription of PhoQ-dependent genes, and abrogates transcription when coexpressed with wild-type PhoQ. Analysis of other two-component kinase sequences indicated that many such proteins contain similarly conserved polar residues, and the structure of one such domain shows a polar residue proximal to an extended cavity near the center of the TM bundle. We therefore examined the role of Asn202 in PhoQ. Our analysis indicated that its kinase function is dependent on the polarity of Asn202, rather than its precise structure or position in the TM region; it can be displaced up or down one turn of TM helix 2, or even moved to the adjacent TM helix 1. The presence of polar TM amino acids among many diverse sensor kinases suggest a widespread mechanism of two-component signal transduction; we speculate that they might stabilize underpacked water-containing cavities that can accommodate conformational changes required for switching from phosphatase to kinase-competent conformations.

histidine kinase | membrane proteins | signal transduction mechanism | two-component signaling

Two-component systems are prominent features of bacteria, used primarily to sense and respond to environmental stimuli. There are thousands of these systems spanning all species of bacteria (1), including about 25 in *Escherichia coli* alone (2). They detect and respond to varied stimuli, including general stresses such as pH (3), and specific signals such as concentration of a particular nutrient [such as citrate (4) or magnesium ions (5)]. In addition to their relevance to microbial ecology and physiology, two-component systems serve as simple examples of signal transduction across biological membranes and as such are a topic of great interest in biochemistry and biophysics.

The two protein components that comprise the typical system are a transmembrane (TM) sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) (1). The prototypical HK, represented by proteins such as EnvZ (6) and PhoQ (7), consists of three regions. The periplasmic region acts as a sensor, and detects changes in the surrounding medium. The TM region generally consists of two membrane-spanning antiparallel helices located at the N- and C-termini of the periplasmic region. The cytoplasmic region generally contains a small signaling domain at its N-terminus such as a HAMP, PAS, or GAF domain (1), that plays a role in transducing the signal from the periplasm into catalytic activity. The remainder of the cytoplasmic region is a well-conserved kinase domain that interacts with the RR. The RR is a soluble protein that typically promotes gene transcription.

The HK controls the phosphorylation state of the RR, which determines its ability to participate in the signaling cascade. HKs typically catalyze three distinct enzymatic reactions: autophosphorylation, RR phosphorylation, and RR dephosphorylation. Genetic and biochemical evidence suggests that prototypical HKs exist in two states, kinase-dominant (K^+P^-) and phosphatase dominant (K^-P^+), and that switching between these states is influenced by signal (8). In the K^+P^- state, the HK autophosphorylates a conserved histidine in the cytoplasmic region and subsequently transfers the phosphoryl group to an aspartate on the RR. In the K^-P^+ state, the HK dephosphorylates the RR. These properties produce a robust genetic circuit that shows a similar response to signal over a range of concentrations of the component proteins (9, 10). The mechanism by which HK proteins switch states in response to stimulus, though, is not entirely understood on a molecular level.

Elucidation of the structure and of the mechanism of signaling in the HK molecule has been the focus of a number of studies (1, 11, 12). The structure of a full-length HK has yet to be determined, though x-ray and NMR structures have been solved for portions of HK and HK-like proteins, most notably the Tar and Tsr chemotaxis receptors (11), but an atomic resolution structure of the TM region is not available. Those HKs that have been characterized are seen to form stable homodimers in vivo (13, 14), and x-ray and NMR structures of cytoplasmic and periplasmic portions indicate that the dimer interface extends the length the molecule (15–19). Based on these structures and on disulfide cross-linking studies, the two TM helices are thought to assemble into a 4-helix bundle in the membrane (11). This model has led to a proposed mechanism in which substrate binding causes a conformational change in the periplasmic domain, which is propagated along a continuous helix through the TM and into the HAMP domain, thereby altering the signaling state. A piston-shift model has been proposed for the chemoreceptor proteins (11), supported by structural and experimental data (20); and extended to include some families of HKs (21). Other proposals for conformational changes focus on rotations of helices (22) and domains (23). The HAMP domain has similarly been proposed to feature either a helical rotation (16) or bundle destabilization caused by lateral movement of helices (24). The role that the TM region may play in these transitions, however, is not entirely understood.

The x-ray structure of the HK-like protein, sensory rhodopsin transducer HtrII from *Natronomonas pharaonis*, provides the only detailed structural information available for the TM of a protein from this class (22). HtrII does indeed display a 4-helix bundle consisting of the two TMs from each monomer. This

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[1]To whom correspondence should be addressed. E-mail: wdegrado@mail.med.upenn.edu.

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protein has no periplasmic domain, however, and it is not clear if classic HKs that have sensor regions have similar TM arrangements. A wealth of experimental data, including x-ray crystallography (25) and EPR studies (26), indicate that displacement of the TM2 helix is involved in signal transduction; the precise dimensions of this movement and the role it plays in signal propagation into the cytoplasmic region are still being determined (27).

PhoQ is the HK of the *phoPQ* system, along with the RR, PhoP. The *phoPQ* system senses the divalent cations Mg^{2+} and Ca^{2+} (28). PhoQ is of particular interest because of its role in some species of bacteria such as *Salmonella enterica* serovar Typhimurium, where *phoPQ* orchestrates the expression of genes required for virulence in response to antimicrobial peptides of the innate immune system (29, 30). PhoQ is thus a potential antimicrobial target, as inhibition of signaling could prevent bacterial infection (30). PhoQ is a prototypical HK, with a periplasmic sensor domain that interacts with metal ions, a TM 4-helix bundle, cytoplasmic HAMP and DHp domains, and the well-conserved kinase (Fig. 1). Under physiological conditions, PhoQ is a homodimer with an interface that extends the length of the molecule (31). Because the TM region of the HKs is less understood, we chose to focus our efforts on determining its arrangement as we move toward developing a full picture of the structure and function of the HK proteins.

The TM2 helix of PhoQ contains an asparagine residue at position 202, which is predicted to lie in the center of the membrane bilayer. In the process of scanning mutagenesis of the TM region of PhoQ, we observed that most cysteine replacements in the TM helices produced functional proteins but the N202C variant did not appear to activate transcription of *phoPQ*-regulated genes. Asparagine is a very polar amino acid, and the amide side chain has a high propensity for association when in a membrane environment (32, 33). An Asn residue lies at a similar position in the TM domain of HtrII, where it helps relay information from the light-sensing subunit to the sensor kinase. We also noticed that this Asn sidechain lies along an elongated cavity that extends from near the bilayer center to the cytoplasmic end of HtrII (25) (see *Discussion*). Intrigued by these observations, we set out to analyze the role of Asn202 in PhoQ, and determined that it plays a critical role in the switching between kinase and phosphatase states. The function of Asn202 and the implications for the molecular mechanism of HK signal transduction are described herein.

Results

In Vivo Activity of PhoQ N202A. To determine the role of the Asn202 residue in the second TM helix of PhoQ, we constructed and analyzed the alanine variant. *E. coli* PhoQ N202A was expressed from an uninduced attenuated *trc* promoter (34) on the plasmid pSG262, in strain TIM206 [which has the native *phoQ* and *lacZ* genes deleted and contains a chromosomal *mgtA::lacZ* reporter for PhoQ activity (31)]. The basal expression from this plasmid is comparable to the steady-state level of expression from the chromosomal *phoQ* gene (35). In contrast to wild-type (wt) PhoQ, the N202A variant did not activate transcription of *lacZ* over baseline levels in either low- or high-magnesium conditions. The PhoQ N202A variant was also coexpressed with the wt PhoQ protein, using the pSG262 plasmid in the $phoQ^+$ TIM199 reporter strain. This strain failed to activate transcription of *lacZ* above baseline levels, indicating that the *phoQ N202A* gene has a dominant negative phenotype (Fig. 2). This suggests that the inability of PhoQ N202A to activate transcription is not due to changes in protein expression or localization [which appear to be similar to wt levels (Fig. S1)], but rather a direct effect of the Asn to Ala substitution on catalytic activity.

In Vitro Activity of PhoQ N202A. To assess the enzymatic activity of PhoQ N202A in vitro, *E. coli* membranes enriched in the protein were assayed for kinase and phosphatase function with

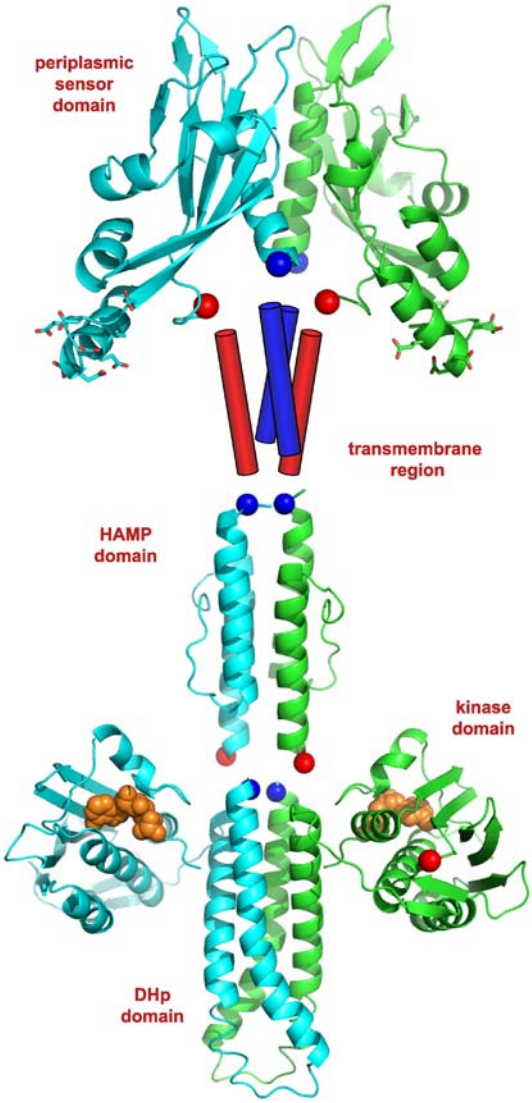

Fig. 1. PhoQ sensor kinase. Schematic of PhoQ structure—x-ray structures of the periplasmic domain of PhoQ (3BQ8) (15) and of the cytoplasmic domain of a *T. maritima* HK (2C2A) (17), and the NMR structure of a HAMP domain (2ASW) (16) are shown in the approximate orientation they would adopt in the full protein. Acidic residues that comprise the putative cation binding patch in the periplasmic domain are shown as sticks. ADP analog bound in the kinase domain is shown as orange spheres. N- and C-termini of the structures are indicated as blue and red spheres, respectively. TM helices are indicated as blue (TM1) and red (TM2) cylinders.

^{32}P-labeled ATP. In contrast to the wt PhoQ, the N202A variant showed no detectable autophosphorylation in vitro when incubated with labeled ATP (Fig. 3*A*). For the phosphatase assays, P-32 labeled phospho-PhoP was incubated with the PhoQ-enriched membranes. In these assays, the PhoQ N202A variant and the wt PhoQ protein showed similar dephosphorylation of the RR (Fig. 3*B*).

Full Range of Asn202 Replacements. To further explore the role of the asparagine, we constructed the full set of amino acid replacements at position 202. These variants were assayed for their ability to activate *lacZ* transcription in a Δ*phoQ* strain, and their ability to repress transcription in a $phoQ^+$ strain. We found that the PhoQ variants could be grouped into 3 categories. The first group consists of variants that have similar profiles to wt PhoQ, complementing the Δ*phoQ* strain and not interfering with the $phoQ^+$ strain: Arg, Asp, Gln, Glu, and His. Among the wt-like

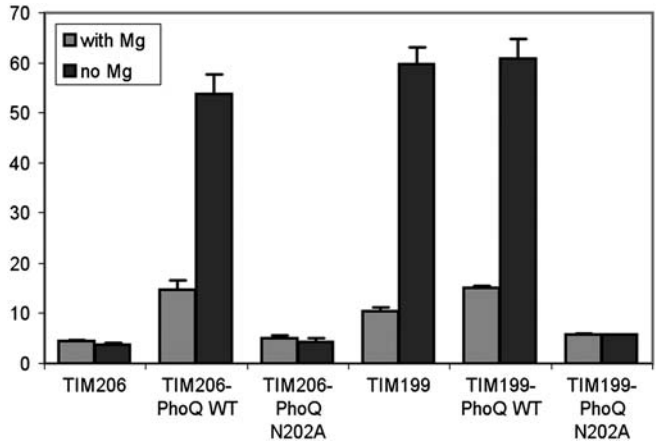

Fig. 2. In vivo activity of PhoQ N202A. Activity was determined with a β-galactosidase reporter for PhoQ activation consisting of a fusion of the *lacZ* gene to the PhoQ-regulated *mgtA* promoter. Activity was measured in both the TIM206 Δ*phoQ* reporter strain and the TIM199 *phoQ*$^+$ reporter strain, bearing either an empty plasmid, a plasmid encoding wt PhoQ, or a plasmid encoding PhoQ N202A. Data correspond to averages of at least 3 experiments; error bars show standard deviations.

variants, His and Arg show increased transcription of *lacZ* compared to wt *phoQ*. However, these variants are perhaps not as effective sensors as the wt protein, because they are not repressed to the same degree by divalent cations. Asp, Gln, and Glu show similar profiles to the wt, although Asp and Gln have lowered activity. The second group consists of variants that showed similar characteristics to N202A, failing to activate *lacZ* transcription and showing dominance over the wt gene. This group includes every hydrophobic amino acid (Ile, Leu, Met, Phe, Trp, Tyr, and Val) and the small residues Cys, Gly, and Pro. Because Gly and Pro show similar effects to Ala, it is likely that the phenotype is more related to the lack of polarity of their sidechains than it is to helix propensity. The third group of variants showed intermediate activities: the slightly polar residues Ser and Thr, as well as Lys. These variants activated transcription slightly in the *ΔphoQ* strain, and repressed transcription to some degree in the *phoQ*$^+$ strain (Fig. 4).

Asparagine Scanning of PhoQ TMs. To examine the effect of positioning of the critical asparagine residue, we used asparagine scanning mutagenesis. A series of variants was constructed in which the N202A substitution is combined with the replacement of another amino acid of TM2 with an asparagine. Variants were constructed

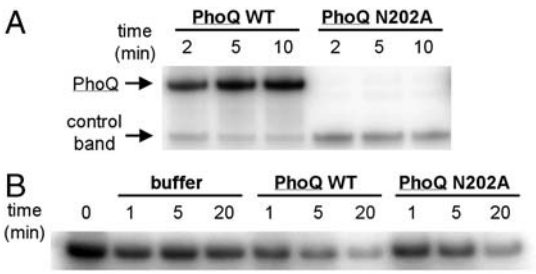

Fig. 3. In vitro activity of PhoQ N202A. (*A*) Autokinase activity of wt PhoQ and PhoQ N202A. Membranes enriched in PhoQ or PhoQ N202A were incubated with γ-^{32}P ATP. Aliquots were removed at different times, and the reactions stopped by removal of ATP and addition of SDS-PAGE buffer. (*B*) Phosphatase activity of wt PhoQ and PhoQ N202A. Phospho-PhoP was incubated with either buffer only, membranes enriched in wt PhoQ, or membranes enriched in PhoQ N202A. Aliquots were removed at different times, and the reactions stopped by the addition of SDS-PAGE buffer. All samples were subjected to SDS 10% PAGE, and the radiolabeled protein bands were visualized by phosphorimaging.

with asparagines at positions 198 to 206, and their ability to activate *lacZ* transcription was compared to wt PhoQ and to PhoQ N202A (Fig. 5). Though none of the asparagine-scan variants showed activity as high as the wt, some did recover *lacZ* transcription. The Asn203 protein in particular produced near-wt levels of *lacZ* transcription; Asn199 and Asn205 also showed moderate activity. These 4 positions are situated roughly along one face of TM2 (assuming that its structure resembles idealized α-helix geometry), and so provide a view of the patch on TM2 that can accommodate the necessary asparagine interaction.

TM1 Variants. We next examined whether there might be an interacting partner for Asn202 in the neighboring TM1 of PhoQ. Although there are no strongly polar residues in the central region of TM1, the hydroxyl-containing sidechains Ser29 and Tyr32 are predicted to lie at a similar depth in the membrane as Asn202. The S29A variant was found to produce *lacZ* transcription almost identical to the wt PhoQ (Fig. 6). The S29V variant, meanwhile, showed increased transcription of *lacZ*, especially under conditions of high Mg^{2+}. The Y32A protein showed a slight increase in *lacZ* transcription compared to wt, and Y32F showed a slight decrease. Thus, none of these variants produced a drastic change in transcription, with the largest being the 3-fold increase seen in high Mg^{2+} conditions with the S29V variant. These findings, together with the observation that a variety of strongly polar residues can substitute for Asn202 suggest that Asn202 does not engage in a specific interaction with sidechains on TM1; instead polarity seemed to be the key determinant.

We therefore asked whether the critical Asn sidechain could be moved from TM2 to TM1. A series of PhoQ variants were constructed combining N202A with the substitution of an Asn at positions between 27 and 31 (which are predicted to lie at a similar depth in the membrane as position 202). We found that the N202A/V27N and N202A/L30N variants both recovered Mg^{2+}-dependent signaling; V27N yielded less transcription than wt whereas the L30N was even more active than wt PhoQ, especially in high-Mg^{2+} media (Fig. 7). Residues 27 and 30 are predicted to lie on the same face of the TM1 helix, assuming ideal geometry.

Discussion

This manuscript demonstrates the functional importance of a polar residue in TM2 of PhoQ in vivo. Hydrophobic mutants of N202 abrogate signaling of coexpressed wt protein, indicating that they act as dominant negatives (DN). Cys cross-linking studies showed that this DN effect was not a result of the mutants preferentially forming heterodimers with wt PhoQ (Fig. S1), but is instead due to the phosphatase activity of mutant homodimers. Biochemical studies confirmed that the substitution of Asn202 with Ala eliminated kinase activity without affecting the intrinsic phosphatase activity. We therefore conclude that the DN effect reflects a disruption of the delicate balance of phosphatase versus kinase activity required for transcriptional activation via the PhoP protein (35).

Highly conserved polar residues are found in many TM helical bundles of HK and HK-like proteins. Asn202 is strongly conserved in PhoQ proteins of *E. coli* and related organisms, the only substitution being the functionally conservative His. Approximately half of the other approximately 25 HK sensor proteins in *E. coli* have a conserved TM Arg, Asp, Asn, Gln, Glu, or His residue (Table S1). Furthermore, the related chemoreceptor Tar has a conserved polar Gln residue at position 22 of its first TM helix (36), and the mutant Q22C is folded and membrane-embedded but no longer responsive to ligand (37).

Polar residues in membrane proteins often play essential roles by forming hydrogen bonds (38–40) that are dependent on the fine-grained physical properties and precise positioning of the interacting groups. Thus, it was initially surprising to discover

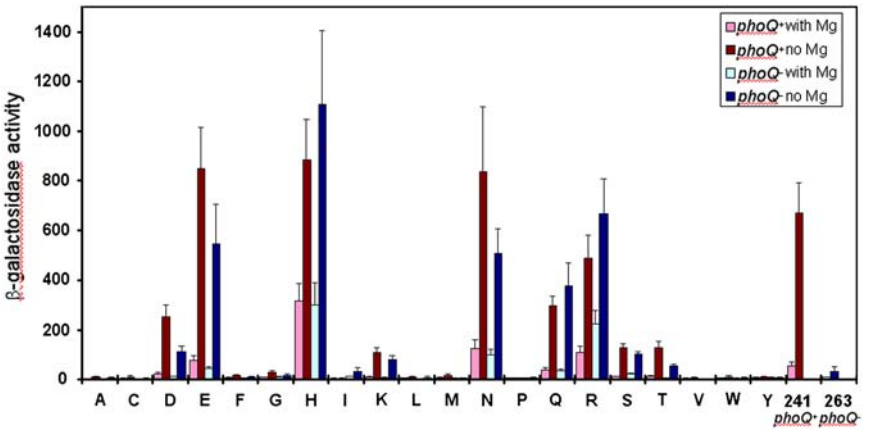

Fig. 4. In vivo activities of PhoQ variants at position 202. Activity was determined with a β-galactosidase reporter, consisting of a fusion of the *lacZ* gene to the PhoQ-regulated *mgtA* promoter, on the plasmid pSG238. Activity was measured in either the Δ*phoQ* strain SDG263 or the *phoQ*$^+$ strain SDG241, bearing the reporter plasmid and a second plasmid encoding the PhoQ variant. Data correspond to averages of at least 6 experiments; error bars show standard errors.

that hydrophilicity was the only commonality amongst the sidechains that functionally substitute for Asn202. Examples of allowed function-promoting polar sidechains that are otherwise diametric opposites include Arg and Asp, which differ greatly in (*i*) size (eight versus four heavy atoms, respectively); (*ii*) charge state at neutral pH in water; and (*iii*) hydrogen-bonding potential (Arg being replete with five hydrogen-bonded donors and Asp having three or four pairs of nonbonded electrons capable of accepting hydrogen bonds in its neutral and charged state, respectively). The function of Asn202 substitutions correlates with the hydrophilicity of the residue at 202. The correlation between PhoQ activity and quantitative measures of the hydrophilicity of the sidechain at 202 is approximately as high as the correlation between different hydrophilicity scales (Fig. S2). Lys is a singular major outlier in the correlation, but it is also an outlier between different hydrophilicity scales. Lys might be less effective than Arg because Lys has only a single polar heavy atom and its longer aliphatic chain allows for greater snorkeling.

The Asn sidechain can also be moved up or down a turn of TM2, corresponding to approximately one third the length of the hydrophobic region of a bilayer, or placed over a 100° arc over the cylindrical surface of the helix; remarkably, the Asn can even be transferred to the TM1 helix and retain PhoQ function. This indicates that the polar side chain only needs to be approximately placed in the TM bundle.

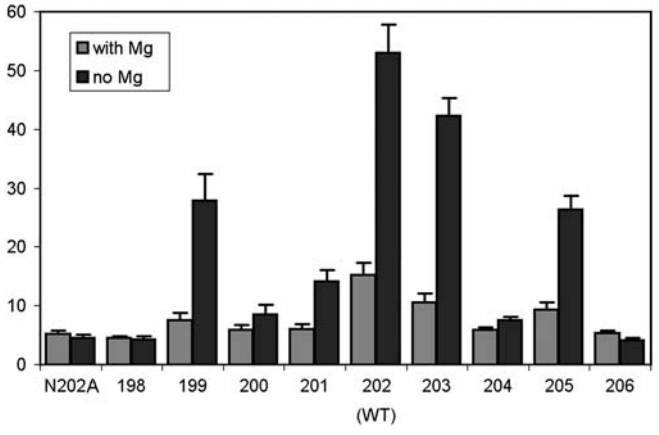

Fig. 5. Asparagine scanning of PhoQ TM2 helix. β-Galactosidase activity of the fusion of *lacZ* gene to the PhoQ-regulated *mgtA* promoter. Activity was measured in the TIM206 Δ*phoQ* reporter strain bearing a plasmid encoding wt PhoQ, PhoQ N202A, or an asparagine-shifted PhoQ TM2 variant. Data correspond to averages of at least 3 experiments; error bars show standard deviations.

In summary, the only stringent requirement for function is hydrophilicity, indicative of a strong affinity for water. Whereas this finding does not uniquely define a signaling mechanism, it provides a new rationale for previous measurements and should guide new experiments. Cys-scanning spin labeling experiments of the TM bundle of Tar indicate that the protein can accommodate the steric bulk of the spin label with greater flexibility than most transmembrane proteins (41)—a finding that is consistent with the relaxed size requirement for the polar residue in PhoQ. However, might the polarity of the residue facilitate signaling?

The only structurally characterized HK TM bundle is the HtrII transducer protein (22), which has a conserved Asn at position 74 in the TM2 helix that appears to play an important role in signaling (27). The x-ray structure reveals that the TM 4-helix bundle has a long open channel running two thirds the length of the bundle, from the cytoplasm to the center of the protein (Fig. 8*A*). Asn74 is the sole strongly polar sidechain projecting into the hemi-channel. Although luminal water molecules were not assigned in the deposited coordinates, an electron density map shows at least two well ordered water molecules in the hemi-channel, even at the intermediate 2.4 Å resolution of the structure (Fig. 8*B*). The dimer of water molecules is not immediately hydrogen-bonded to Asn, but could make a solvent-mediated H-bond. Thus, it is likely that the cavity is filled with solvent, which might be visible at higher resolution.

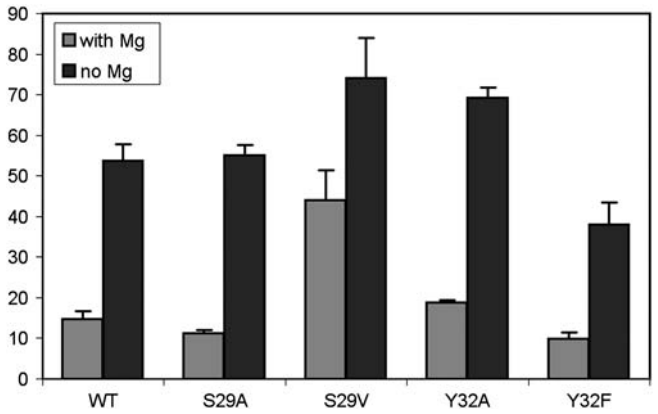

Fig. 6. In vivo activity of PhoQ variants at positions 29 and 32. β-Galactosidase activity of the fusion of *lacZ* gene to the PhoQ-regulated *mgtA* promoter. Activity was measured in the TIM206 Δ*phoQ* reporter strain bearing a plasmid encoding PhoQ S29A, S29V, Y32A, or Y32F. Data correspond to averages of at least 3 experiments; error bars show standard deviations.

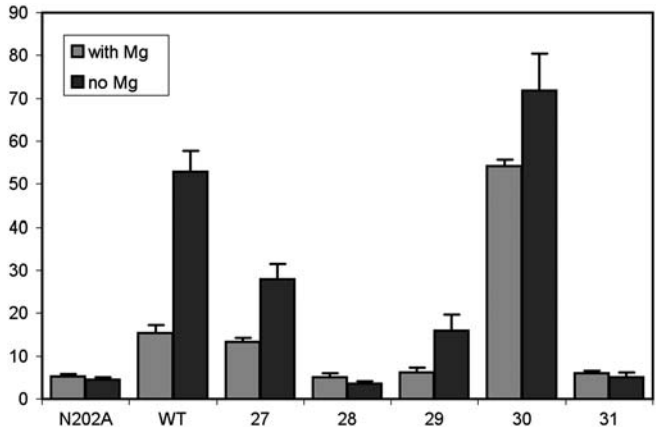

Fig. 7. Asparagine scanning of PhoQ TM1 helix. β-Galactosidase activity of the fusion of *lacZ* gene to the PhoQ-regulated *mgtA* promoter. Activity was measured in the TIM206 Δ*phoQ* reporter strain bearing a plasmid encoding wt PhoQ, PhoQ N202A, or an asparagine-shifted PhoQ TM1 variant. Data correspond to averages of at least 3 experiments; error bars show standard deviations.

HtrII is a sensory protein, which lacks an extracytoplasmic membrane domain. It instead has a rhodopsin-like subunit that interacts with a portion of the Asn74 sidechain, placing structural restraints that might not be present in PhoQ. Nevertheless, the presence of such a long cavity in HtrII, which is not involved in apparent substrate recognition or transport, is surprising and invites speculation that PhoQ might also have a water-filled cavity that can be stabilized by a variety of polar residues projecting from varying positions on one face of either TM1 or TM2. The inclusion of a central cavity would minimize complex tertiary packing interactions that might overly constrain the bundle to a single conformation or impede switching between states. Instead the bundle would be stabilized exclusively by pairwise, interhelical packing interactions that should be more permissive of the rotations and translations envisioned in various signaling mechanisms, and structurally characterized in gated ion channels (42). In closing, we note one intriguing feature of the HtrII structure, versus the inferred structure of Tar's TM bundle. Tar has been proposed to form a bundle with left-handed interhelical crossing angles, based on disulfide cross-linking studies that are capable of differentiating left-handed from right-handed crossings (43). Intriguingly, the "top" portion of the bundle of HtrII (Fig. S3), which faces the exterior of the cell, forms a left-handed bundle, but a bend in TM2 changes the interhelical packing from left- to right-handed near the beginning of the hemi-channel. Similarly, right- and left-handed packings have been seen in the structures of the water-soluble cytoplasmic four-helix bundles of other HK proteins, possibly reflecting static idiosyncratic differences in the helical rotational and interhelical packing between the different proteins under investigation (18, 19, 44). However, it is interesting to entertain the possibility that such changes might be a component in the signaling of HK proteins. Indeed, a similar switch was observed when comparing different states of the kinase domain of the DesK protein.

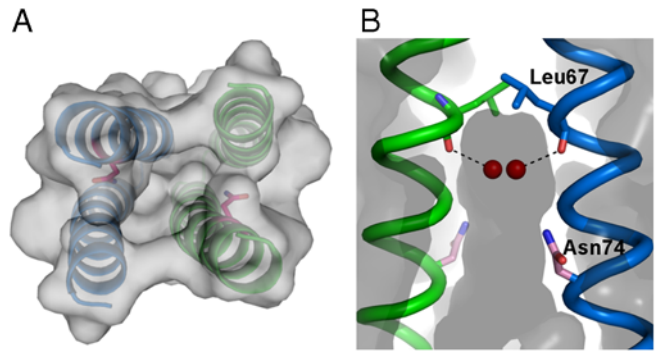

Fig. 8. Cavity in HtrII transmembrane region. (*A*) Structure of HtrII (PDB ID code 1H2S) (22) is shown from the "bottom" view, as it would be seen if looking into the membrane from the cytoplasm. The HtrII dimer is shown as cartoon, and colored by chain; Asn74 is shown in magenta, and sidechain is shown as sticks. Surface of HtrII is shown in gray. (*B*) Ordered water molecules in the TM bundle of HtrII. Examination of a $2F_o - F_c$ map contoured at 1.4σ (generated with the deposited structure factors) shows strong density from solvent near the end of the elongated cavity in the vicinity of the polar atoms. Protein is shown from the "side" view, seen from the plane of the membrane. TM2 helices are shown as cartoons and colored by chain; Leu67 and Asn74 sidechains are shown as sticks, water molecules are red spheres, and surface of HtrII, including the internal cavity, is shown in gray.

Materials and Methods

Strains and Plasmids. The strains and plasmids used in this work are summarized in Table S2. A detailed description of strain and plasmid construction is provided in *SI Methods*.

Media. Cells were grown in LB supplemented with the appropriate antibiotics (100 μg/mL of ampicillin, 34 μg/mL of chloramphenicol, 30 μg/mL of kanamycin) and with $MgCl_2$ to 10 mM where applicable.

β-Galactosidase Assays. Cells were grown overnight in LB and then subcultured in LB or LB supplemented with $MgCl_2$, and grown to an OD_{600} of approximately 0.2–0.4. For cells with the chromosomal *mgtA::lacZ* reporter, β-galactosidase activity was determined as described (45). For cells harboring the reporter plasmid pSG238, assays were performed in microtiter plates using a modified procedure (*SI Methods*).

Membrane Preparation. *E. coli* BL21(DE3) cells, transformed with either the pSG325 (wt PhoQ) or the pSG327 (PhoQ N202A) plasmid were grown at 37 °C in LB broth supplemented with antibiotics. Expression was induced at an OD_{600} of approximately 0.6 by adding 0.5 mM IPTG, and the culture was transferred to 25 °C. After 16 h of induction, cells were harvested by centrifugation and membranes were prepared essentially as described (46). Membranes were suspended in reaction buffer (50 mM Tris-HCl pH 7.5, 200 mM KCl, 0.1 mM EDTA, 5% glycerol) at 0.1 mg/μL, and stored at −80 °C.

Protein Purification. PhoP was expressed with a C-terminal His_6 fusion and purified by affinity chromatography, as described (35). The $EnvZ_{cyto}$ + MI+ loop5 chimeric protein was expressed as an N-terminal His_6-maltose binding protein fusion and purified as described (47).

In Vitro Autokinase Assay. Two mg of membranes enriched in PhoQ or PhoQ N202A were autophosphorylated with 40 μCi [γ^{32}P]ATP (3000 Ci/mmol) in 40 μL of reaction buffer supplemented with 0.1 mM $MgCl_2$. The reactions were continued at room temperature for various times before being stopped by application to a BioSpin 6 column (BioRad) to remove unincorporated ATP and addition of 4× lithium dodecyl sulfate sample buffer, and subjected to electrophoresis on a 10% SDS-PAGE gel. Radioactivity was detected using a phosphor screen and Storm imager.

In Vitro Phosphatase Assay. Phosphatase activity of PhoQ and PhoQ N202A was measured essentially as described (35). Briefly, 33 μg of His_6-MBP-$EnvZ_{cyto}$ + MI + loop5, a protein that efficiently phosphorylates PhoP but does not dephosphorylate it, was autophosphorylated with 40 μCi [γ^{32}P] ATP (3000 Ci/mmol) in 40 μL of reaction buffer supplemented with 1 mM $MgCl_2$. After 20 min. at room temperature, 56 μg PhoP in 40 μL reaction buffer (1 mM $MgCl_2$) was added. After 40 min, the mixture was applied to a BioSpin 6 column (BioRad) to remove unincorporated ATP. 20 μL of the flow-through was added to tubes with 20 μL reaction buffer containing either 100 μM ADP/1 mM $MgCl_2$ only, 1.2 mgs of wt PhoQ-enriched membranes + ADP/$MgCl_2$, or 1.2 mgs of PhoQ N202A-enriched membranes + ADP/$MgCl_2$. At each time point, samples were transferred to tubes containing 4× LDS sample buffer, and subjected to electrophoresis on a 10% SDS-PAGE gel. Radioactivity was detected using a phosphor screen and Storm imager.

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