Cardiolipin Controls the Osmotic Stress Response and the Subcellular Location of Transporter ProP in *Escherichia coli*

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The phospholipid composition of the membrane and transporter structure control the subcellular location and function of osmosensory transporter ProP in *Escherichia coli*. Growth in media of increasing osmolality increases, and entry to stationary phase decreases, the proportion of phosphatidate in anionic lipids (phosphatidylglycerol (PG) plus cardiolipin (CL)). Both treatments increase the CL:PG ratio. Transporters ProP and LacY are concentrated with CL (and not PG) near cell poles and septa. The polar concentration of ProP is CL-dependent. Here we show that the polar concentration of LacY is CL-independent. The osmotic activation threshold of ProP was directly proportional to the CL content of wild type bacteria, the PG content of CL-deficient bacteria, and the anionic lipid content of cells and proteoliposomes. CL was effective at a lower concentration in cells than in proteoliposomes, and at a much lower concentration than PG in either system. Thus, in wild type bacteria, osmotic induction of CL synthesis and concentration of ProP with CL at the cell poles adjust the osmotic activation threshold of ProP to match ambient conditions. ProP proteins linked by homodimeric, C-terminal coiled-coils are known to activate at lower osmalilities than those without such structures and coiled-coil disrupting mutations raise the osmotic activation threshold. Here we show that these mutations also prevent polar concentration of ProP. Stabilization of the C-terminal coiled-coil by covalent cross-linking of introduced Cys reverses the impact of increasing CL on the osmotic activation of ProP. Association of ProP C termini with the CL-rich membrane at cell poles may raise the osmotic activation threshold by blocking coiled-coil formation. Mutations that block coiled-coil formation may also block association of the C termini with the CL-rich membrane.

Osmotic pressure changes elicit transmembrane water fluxes that concentrate or dilute the cytoplasmic volume of living cells, disrupting their structure and function. Cells respond by actively adjusting the distributions of selected solutes across the cytoplasmic membrane and water follows, restoring cellular hydration and volume (1). Bacteria can use K⁺ as an osmoregulatory solute but they prefer protein-stabilizing organic osmolytes like proline, glycine, glycine betaine, and ectoine (1, 2). These compounds are also called osmoprotectants because, when provided exogenously, they stimulate bacterial growth in high (but not low) osmotic pressure media. Well characterized, functionally redundant transporters, enzymes, and channels modulate the osmolyte composition of Gram-negative bacterium *Escherichia coli* (3–5). We are exploiting that system to learn how osmotic pressure is sensed, how resulting signals are transduced, and how cells respond by modulating their own structure, growth, and division.

The mole fractions of the major phospholipids in the cytoplasmic membrane of *E. coli* are usually cited as 0.75 for phosphatidylethanolamine (PE), 0.20 for phosphatidylglycerol (PG), and 0.05 for cardiolipin (CL, also known as diphosphatidylglycerol) (6). CL and PG are anionic, whereas PE is zwitterionic, and each CL molecule contains 2 phosphates, whereas each PE or PG contains only 1. The proportions of these lipids vary with growth medium, osmolality, and growth phase. The proportion of CL increases ~2-fold at the expense of PE, and PG remains constant, as *E. coli* is cultivated to exponential phase in minimal media of increasing osmolality ([II/RT] (7). The CL synthase encoded by the osmotically inducible *cls* gene is responsible for most CL synthesis but an alternate pathway for CL synthesis is evident in CL synthase-deficient (*cls*) bacteria (6, 8). The proportion of PG increases at the expense of PE as *cls*− bacteria are cultivated to exponential phase in minimal media of increasing osmolality (8). Thus PG synthesis is also osmotically induced and/or PE synthesis is osmotically repressed, and the *cls* defect does not significantly alter the proportion of phosphate in anionic phospholipid head groups. The proportion of anionic lipid decreases 2-fold on entry to stationary phase, but the proportion of phosphate in anionic lipid head groups decreases only 1.5-fold because CL rises as PG falls (8).

*CL* is enriched in regions of the cytoplasmic membrane near the nucleoid-free poles and septa of growing *E. coli* (9) and in *E. coli* minicells (spherical, nucleoid-free cells resulting from polar cell division) (10). Some researchers suggest that CL spontaneously concentrates at cell poles because membrane

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*3* The abbreviations used are: PE, phosphatidylethanolamine; II, osmotic pressure; II/RT, the osmolality; IIₙ/RT, the osmolality at which ProP activity is half-maximal; CL, cardiolipin; DAPI, 4',6'-diamidino-2-phenylindole; DTME, dithio-bis(maleimidoethane); FISH–EDT, fluorosein arsenical helix binder bis-EDT adduct; MOPS, 4-morpholinopropanesulfonic acid; NAO, 10-N-nonyl-3,6-bis(dimethylamino)acridine; PG, phosphatidylglycerol; PRL, proteoliposome; TM, transmembrane.

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*2* The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and S2.

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curvature is more acute there than elsewhere on the cell surface (9, 11). Some Escherichia coli proteins are also concentrated at the cell poles (12, 13) but neither the mechanism nor the purpose of that localization is understood (14). Proteins could be directed to the poles by specific interactions with phospholipids like CL (14) or with the cytoskeleton. Alternatively, coupled transcription, translation, and insertion of new proteins (denoted transinsertion (15, 16)) may force existing membrane proteins away from the nucleoid, toward the poles and septa, and cause them to accumulate in minicells. Polar localization of proteins could influence their functions, it could potentiate the distribution of cellular constituents during cell division, or the poles could serve as receptacles for superstructures with properties that are location-independent but nevertheless transcend the functions of individual constituents (e.g. chemoreceptor arrays) (17, 18).

Recently we showed that the osmosensory transporter ProP co-localizes with CL at E. coli cell poles and that this polar localization is less pronounced in cls/H11002 than in cls/H11001 bacteria (8). Thus CL-ProP interactions may promote polar localization of ProP and, although CL comprises a small proportion of total cellular lipid, ProP at cell poles is embedded in a CL-enriched region of the cytoplasmic membrane. Here we compare the behavior of ProP with that of the well characterized lactose transporter LacY (19). LacY and ProP are both H⁺-symporters and members of the major facilitator superfamily comprised of 12 transmembrane segments with cytoplasmic N and C termini (19–21) (Fig. 1). ProP is denoted an “osmosensory transporter” because it can sense increasing osmolality and respond by mediating organic osmolyte accumulation, both in cells and after purification and reconstitution into proteoliposomes (PRLs) (22, 23). In contrast, LacY inactivates as ProP activates in response to osmotic stress (24). Nagamori et al. (12) reported polar localization of a LacY-green fluorescent protein (LacY-GFP) fusion protein in E. coli. Here we confirm that LacY is located at the poles of E. coli cells and show that, unlike ProP, LacY remains polar in cls/H11002 bacteria. Thus the CL dependence of ProP localization may be related to the osmoregulatory function of ProP.

Indeed, data suggest that both membrane phospholipid composition and a regulatory protein domain influence ProP function. Recently we showed that the osmolality yielding half-maximal ProP activity (and hence the osmotic activation “threshold”) increased in parallel with the proportion of CL when E. coli was cultivated in media of increasing osmolality. This suggested that CL modulates ProP function, adapting the osmolality range over which ProP activity is regulated to match ambient (growth) conditions (7). The C termini of E. coli ProP and its orthologues are longer than those of its paralogues (like LacY) (3, 7). The C terminus of E. coli ProP and some orthologues includes heptad repeats characteristic of proteins that form α-helical coiled-coils (Fig. 1). Indeed, antiparallel, homodimeric α-helical coiled-coils link the C termini of E. coli ProP dimers (25–29). ProP orthologues with and without C-terminal coiled-coils are osmosensory transporters but orthologues and variants with the coiled-coil activate at lower osmolalities than those without (7). Mutations that disrupt or eliminate the coiled-coil raise the activation threshold (7, 25, 26).
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Thus the coiled-coils present on some ProP orthologues tune them to osmoregulate at low osmolalities (7).

In this work we tested the hypotheses that CL. adjusts the osmolality range over which ProP activity is regulated and that the C-terminal, coiled-coil contributes to the polar localization of ProP. We report that the activation threshold of ProP correlates with mole percent of CL in wild type bacteria, with mole percent of PG in CL-deficient bacteria, and with percent of phosphate in anionic lipid (% PG + 2% CL) in cells and PRLs. Thus the activation threshold of ProP rises with the anionic lipid content of the membrane. A CL-rich environment is attained in vivo by enhancing CL synthesis and co-localizing ProP with CL at the cell poles. Coiled-coil disrupting mutations that increase the osmotic activation threshold also impaired the polar localization of ProP. Stabilization of the ProP coiled-coil by covalent cross-linking of introduced Cys reversed the impact of high CL on the osmotic activation threshold. These data support a functional model in which coiled-coil formation lowers the osmotic activation threshold of E. coli ProP (7). The osmotic activation threshold rises when the ProP C termini associate with the CL-rich membrane at cell poles, blocking coiled-coil formation. Mutations that block coiled-coil formation also block association of individual ProP C termini with the CL-rich membrane.

EXPERIMENTAL PROCEDURES

Culture Media and Growth Conditions—E. coli strains were cultivated at 37 °C in LB medium (30) or in NaCl-free MOPS medium, a variant of the MOPS medium described by Neidhardt et al. (31) from which all NaCl was omitted. MOPS medium was supplemented with NH₄Cl (9.5 mM) as a nitrogen source and glycerol (0.4% v/v) as a carbon source. 1-Tryptophan (245 μM) and thiamine hydrochloride (1 μg/ml) were added to meet auxotrophic requirements, ampicillin (100 μg/ml) to maintain plasmids, and arabinose as specified to adjust ProP expression. NaCl was added to adjust the osmolality as indicated and osmolalities were measured with a Wescor vapor pressure osmometer (Wescor, Logan, UT).

Unless otherwise stated, bacteria were grown in LB medium at 37 °C for ~7 h, subcultured into MOPS medium (25 ml in a 125-ml Erlenmeyer flask), and incubated for 16 to 18 h with rotary shaking (200 rpm) at 37 °C. Bacteria were harvested by centrifugation and subcultured in the same medium to achieve an optical density (OD) at 600 nm of 0.4 as determined with a Bausch and Lomb Spectronic 70 spectrophotometer. Cultures were grown to an OD of 1 (exponential phase) or for 24 h (stationary phase), harvested by centrifugation, and resuspended in un-supplemented MOPS medium (NaCl-free MOPS medium devoid of organic supplements). Low osmolality MOPS medium contained no NaCl, high osmolality medium contained 0.3 M NaCl, and these media had osmolalities of 0.15 and 0.7 mol/kg, respectively.

Bacteria, Plasmids, and Molecular Biological Manipulations—Basic molecular biological techniques were as described by Sambrook and Russell (32). The polymerase chain reaction was carried out as described by Brown and Wood (33). Site-directed mutagenesis was performed as described by Culham et al. (34). Oligonucleotides were purchased from CorteC DNA Services (Kingston, ON, Canada). Each recombinant plasmid was recovered from a ligation mixture by transformation of E. coli DH5α (35) and the entire sequence of the encoded proP or lacY variant was confirmed (GenAlEyTiC, Guelph, ON, Canada). Genes encoding ProP, LacY, and their variants were expressed from the AraC-controlled pBAD promoter in plasmid-bearing derivatives of E. coli W35G30 (F− trp lacZ rpsL thi Δ(putPA)101 Δ(proU)600 Δ(proP-melAB)212) (20) or W9G980 (W5350 cls:Tn10ΔTet3) (8). Each strain contained pBAD24 (36), or one of the pBAD24 derivatives listed in supplemental Table S1. E. coli ECK1164 (rrnB3 ΔlacZ4787 hsdR514 Δ(arabBAD)567 Δ(rhaBAD)568 rph-1 minC::kan (37)) was obtained from E. D. Brown (McMaster University).

Fluorescence Microscopy—Bacteria were cultivated as described above and labeled with fluorescent probes as described previously (8). Where specified, chloramphenicol (100 μg/ml) was added when cultures reached an optical density of 0.8 and they were further incubated for 1 h at 37 °C with rotary shaking. CL was stained with NAO (10- N-nonyl-3,6-bis (dimethylamino)acridine), nucleoids were stained with DAPI (4′,6′-diamidino-2-phenylindole), appropriately tagged proteins (MVCCPGC- ProP, MCCPGC-LacY, and their derivatives) were stained with FHsH-EDT₂ (Fluorescein arsenical helix binder, bis-EDT adduct) in the presence of 2,3-dimercapto-1-propanol, and cells were viewed with an Imaging RetigaEX CCD camera mounted on an Axiovert 200M inverted fluorescence microscope (Carl Zeiss Microimaging Inc.) equipped with a Zeiss Plan Neofluor ×100 oil NA1.3 objective as described (8).

Separation of Rod-shaped Cells from Minicells—Minicells and rod-shaped cells were prepared from the minC mutant strain E. coli ECK1164 as described by Koppelman et al. (10) with the following modifications. Cultures were prepared as described above except that 0.5-liter cultures were prepared in 4-liter Fernbach flasks. Cells were harvested, resuspended in unsupplemented MOPS medium, and kept in the same medium as minicells were separated from rod-shaped cells by sucrose gradient centrifugation as described (10). The purity of the preparations was checked by brightfield and fluorescence microscopies after 4′,6′-diamidino-2-phenylindole staining. The sucrose gradient centrifugation and wash steps were repeated if the preparation contained more than 5% rod-shaped, DNA-containing cells. The protein content of each cell suspension was determined and the suspensions were stored at −40 °C.

Phospholipid Analysis—To determine phospholipid compositions, samples of lipid extracts, liposomes, or PRLs and of standards containing ~20–100 μg of lipid were separated by thin layer chromatography using Merck Kieselgel 60 plates (0.25 mm, from EMD Chemicals, Inc., Darmstadt, Germany) developed with a solvent comprised of chloroform, methanol, water, and ammonia in proportions 60:37.5:3:1 by volume (38). Phospholipid spots were identified with reference to standards by exposing the plates to iodine vapor for 5 min. The phosphorus content of each spot was measured as described (39, 40). Lipid samples scraped from chromatography plates and phosphorus standards (up to 200 μl of a 20 μg/ml solution (Sigma) were transferred into clean glass tubes (15 × 100 mm) and
mixed with 225 μl of 8.9 N H₂SO₄. The tubes were heated in sand at 215 °C until the solutions had completely evaporated (30 min to 1 h). After cooling, 75 μl of hydrogen peroxide (30%) was added and tubes were heated at 215 °C for 30 min. After cooling, 1.95 ml of water, 0.25 ml of ammonium molybdate (2.5%, w/v), and 0.25 ml of ascorbic acid (10%, w/v) were added with vortex mixing after each step. The tubes were then heated for 7 min at 100 °C and the absorbance of the cooled samples and standards was measured at 820 nm.

Preparation and Characterization of Proteoliposomes—The phospholipids used for this study, including E. coli polar lipid extract (catalog number 100600) and synthetic lipids 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholinolamine (catalog number 850757), 1-palmitoyl-2-oleoyl-sn-glycerol-3-[phospho-rac-(1-glycerol)] (catalog number 840457), and 1,1′,2,2′-tetraoleoyl cardiolipin (catalog number 710335) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). ProP-His₆ was purified, liposomes and PRLs were prepared as described by Racher et al. (22) except that the liposomes were comprised of either the polar lipid extract or a PE:PG:polar lipid extract mixture (8:1:1). The compositions of the PRLs were further adjusted by mixing them with an appropriate quantity of CL or PG liposomes and extruding 21 times through polycarbonate filters with 0.4-μm pores (Avanti Polar Lipids, Inc.) as described by van der Heide et al. (41). To determine their size distributions, extruded (proteoliposomes were diluted to achieve a concentration of ~0.1 mg lipid/ml buffer, transferred to square cuvettes, and analyzed with a Zetasizer Nano Series Dynamic Light Scattering Spectrometer (Malvern Instruments, Inc., Westborough, MA).

Transport Assays—Bacteria were cultivated and transport assays performed on them as described by Culham et al. (42, 43). Transport assays were performed with PRLs as described by Racher et al. (22). Initial rates of proline uptake were measured using [3-¹⁴C]proline or [d-glucose-1-14C]lactose (cells) and [2,3,4,5-3H]proline (PRLs) (Amersham Biosciences) at 0.2 mM. All assays were done in triplicate, and all experiments were performed at least twice. Rates are cited as mean ± S.E. For data relating rates of proline uptake to assay medium osmolality, regression lines were obtained by fitting the data to empirical Equation 1 (42) using non-linear regression performed by Sigma Plot 8.0,

$$a_0 = A_{\text{max}} [1 + \exp(-(\Pi - \Pi_{1/2})/(RT))]^{-1} \quad \text{(Eq. 1)}$$

where $\Pi$ is the osmotic pressure of the transport assay medium, $a_0$ is the initial rate of proline uptake measured with medium osmolality $\Pi/RT$, $A_{\text{max}}$ is the uptake rate that would be observed at infinite medium osmolality, $R$ is the gas constant, $T$ is the temperature, $\Pi_{1/2}/RT$ is the medium osmolality yielding half-maximal activity, and $B$ is a constant inversely proportional to the slope of the response curve. This process yielded estimates for parameters $A_{\text{max}}$, $\Pi_{1/2}/RT$, and $B$.

In Vivo Cross-linking of ProP—Bacteria expressing pertinent ProP variants were cultivated as described above. ProP was cross-linked in vivo with dithio-bis(maleimidoethane) (DTME) as described by Hillar et al. (28).

Protein Assays—The protein concentrations of cell suspensions and lysates were determined by the bicinchoninic acid assay (44) using the BCA Kit from Pierce (Rockford, IL) with bovine serum albumin as standard.

RESULTS

Subcellular Locations of CL, LacY, and ProP—Fluorescence microscopy has been used to visualize the proportion and location of CL in E. coli cells. 4′,6′-Diamidino-2-phenylindole-stained nucleoids (blue fluorescence) are readily distinguished from NAO-stained membranes (8, 9) (also see Fig. 2A, left panel). NAO binds weakly to anionic lipid (giving green fluorescence), whereas it aggregates while associating tightly with CL-rich membranes (giving red excimer fluorescence) (45). The red NAO fluorescence observed on the poles and at the septa of living cells indicates that CL is concentrated in those areas. In contrast green NAO fluorescence is more uniformly distributed over the surfaces of clans bacteria, reflecting weak association of NAO with residual anionic lipid (predominantly PG).

FlAsH-EDT₂ is a biarsenical fluorescein derivative that yields green fluorescence only after reacting with a helical CXXC motif, introduced to target proteins (46). Green fluorescence appeared near the poles and septa when cells expressing low or high levels of MVCCPGCC-tagged ProP were FlAsH-labeled (8). Analogous images were obtained by FlAsH labeling cells that expressed low levels of either MVCCPGCC-ProP or MCCPGGC-LacY (Fig. 2A, middle and right panels, respectively). Surprisingly the CL deficiency of clans bacteria did not alter the subcellular location of LacY (Fig. 2A, right panel), even though it reduced the concentration of ProP at the cell poles (Ref. 8 and Fig. 2A, middle panel).

Fig. 2 shows typical images, whereas Table 1 summarizes the frequencies with which ProP or LacY fluorescence was clearly concentrated at the cell poles. They may only be concentrated at the cell poles in a fraction of the bacteria or they may have appeared polar in only a subset of the images because the zones of localization were out of the focal plane in others. The proportion of cells with ProP concentrated at the cell poles correlated with membrane CL content in clans but not clans bacteria (Ref. 8 and Table 1). The data suggest that 2–4% CL is sufficient to promote the creation of polar, ProP-containing patches detectable with this microscopic technique. Neither the tested growth conditions nor the clans defect markedly altered the polar localization of LacY (Table 1).

The mechanisms that locate CL and some membrane proteins at E. coli cell poles are unknown (14). Chloramphenicol inhibits translation, causing nascent RNAs to detach from the DNA template and the nucleoid to become compact (47) (Fig. 2B). Thus chloramphenicol treatment would be expected to eliminate polar localization caused by exclusion of existing proteins from the nucleoid-proximal transcretion zone (the area of the membrane where transcription, translation, and insertion of new proteins are coupled (15)). Red NAO fluorescence, indicating high CL concentration, remained at the poles of chloramphenicol-treated clans cells (compare Fig. 2, C with A, left panels). However, the green fluorescence associated with FlAsH-labeled ProP or LacY became more diffuse and uniformly distributed over the cell surface (compare Fig. 2, C with A, middle and right panels). Thus passive exclusion from nucle-
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A. Polar Localization of Cardiolipin, ProP and LacY

| RT | NAO-Cardiolipin | FlAsH-ProP | FlAsH-LacY |
|----|----------------|------------|------------|
| Low | cls<sup>+</sup> | cls<sup>-</sup> | cls<sup>-</sup> |
| High | cls<sup>+</sup> | cls<sup>-</sup> | cls<sup>-</sup> |

B. Impact of Chloramphenicol on the Nucleoid & ProP (cls<sup>-</sup> bacteria, high osmolality)

| Treatment | FlAsH-ProP | DAPI-Nucleoid | Merge |
|----------|------------|---------------|-------|
| CAM | - | - | - |
| +CAM | - | - | - |

C. Impact of Chloramphenicol on Polar Localization

| RT | NAO-Cardiolipin | FlAsH-ProP | FlAsH-LacY |
|----|----------------|------------|------------|
| Low | cls<sup>+</sup> | cls<sup>-</sup> | cls<sup>-</sup> |
| High | cls<sup>+</sup> | cls<sup>-</sup> | cls<sup>-</sup> |

FIGURE 2. Co-localization of CL, ProP, and LacY at the bacterial poles and septa. Bacteria expressing MVCCPGCC-ProP (strains WG350 pDC232 (cls<sup>-</sup>) or WG980 pDC232 (cls<sup>-</sup>)) or MCCPGCC-LacY (strains WG350 pDC245 (cls<sup>-</sup>) or WG980 pDC245 (cls<sup>-</sup>)) were grown in low or high osmolality MOPS media to exponential phase. They were prepared for microscopy and micrographs were obtained as described in Ref. 8. Cells were stained with DAPI (nucleoids, blue fluorescence), NAO (CL, phase. They were prepared for microscopy and micrographs were obtained as described in Ref. 8. Cells were treated bacteria as described under “Experimental Procedures.” As for ProP (8), tagged LacY retained transport activity. Representative uptake rates for LacY and MCCPGCC-LacY were 23 ± 2 and 7.1 ± 0.9 nmol/min/mg of protein, respectively, when bacteria were cultivated in low osmolality medium with 1.33 mM arabinose, and lactose uptake was assayed at low osmolality.

The Osmotic Activation of ProP Is Phospholipid Dependent—The initial rate of proline uptake via ProP is a sigmoid function of assay medium osmolality that can be described by Equation 1 (see “Experimental Procedures”) (42). The osmolality at which ProP activity is half-maximal (\(\Pi_{1/2}/RT\)) depends on the structure of phospholipid compositions that can occur at E. coli cell poles was estimated using minicells. Cultures of E. coli min<sup>C::kan</sup> mutants include both minicells and rod-shaped cells. Koppelman et al. (10) found that, although the proportions of phosphate in anionic lipid head groups were similar in both cell types, minicells were higher in CL and lower in PG than rod-shaped cells from the same exponential phase cultures. As expected, the phospholipid compositions of rod-shaped cells from cultures of E. coli ECK1164 (minC::kan) were similar to those previously...
TABLE 1
Frequency with which transporters are concentrated at cell poles and CL content

Bacteria were prepared and microscopy was performed as described under “Experimental Procedures” to determine the localization of FlAsH-ProP or FlAsH-LacY in 100 cells. Each protein included the N-terminal FlAsH tag MVCCPGCC (ProP) or MCCPGCC (LacY).

| Growth phase   | Osmolality | ProP cls+ | ProP cls− | LacY cls+ | LacY cls− | Bacterial CL contenta |
|----------------|------------|-----------|-----------|-----------|-----------|-----------------------|
|                |            | % of cells with transporters concentrated at cell poles |          |           |           |                       |
| Exponential    | Low        | 46        | 4         | 54        | 49        | 3.8                   |
|                | High       | 51        | 11        | 59        | 58        | 6.4 ± 1.8             |
| Stationary     | Low        | 48        | 21        | 61        | 53        | 8.3                   |
|                | High       | 50        | 37        | 67        | 62        | 8.6 ± 3.0             |

a For comparison, these data are reproduced from Romantsov et al. (8).

FIGURE 3. Representative osmotic activation profiles for ProP in cells and PRLs. A. E. coli WG980 pDC79 (cls−) was cultivated in MOPS medium containing no NaCl (circles, 0.14 mol/kg), 0.15 M NaCl (triangles, 0.44 mol/kg), or 0.30 M NaCl (squares, 0.71 mol/kg). Initial rates of proline uptake were determined in assay media with the indicated osmolalities and lines were created by fitting the data to Equation 1 (see “Experimental Procedures”). Uptake activities are plotted relative to Amax (70, 71, and 37 nmol/min/mg of protein for cells cultivated in low osmolality medium to 13 mol % in minicells cultivated to stationary phase in high osmolality medium, the proportion of PG ranging from ∼21 to 4 mol %, and the proportion of PE remaining essentially constant at ∼80 mol % (Table 2). ProP-His6 was purified and reconstituted with a polar lipid extract from E. coli as described (Ref. 22 and “Experimental Procedures”). To adjust their phospholipid compositions, the PRLs were co-extruded with liposomes comprised of appropriate phospholipid mixtures (see “Experimental Procedures”) or the same polar lipid extract (as a control). Preparations were designed so that CL content varied at a constant PG:PE ratio (0.2) or PG content varied at a constant CL:PE ratio (0.05). The phospholipid compositions of the resulting PRLs were as expected and their diameters remained uniform (supplemental Table S2). As in cells (Fig. 3A), the osmotic activation profile of ProP in PRLs varied with phospholipid composition (Fig. 3B). Π1/2/RT varied directly with CL or PG content (Fig. 4C). Combining the two data sets, Π1/2/RT varied directly with the percent of head group phosphate in anionic lipid (Fig. 4D). Thus Π1/2/RT for ProP correlated with CL content in wild type bacteria, with PG content in CL-deficient bacteria, and with anionic lipid content (CL plus PG) in both bacteria and PRLs. A much lower proportion of CL than of PG was required to attain the same Π1/2/RT value, in vivo and in vitro (Fig. 4, A and C). These data suggest that an anionic lipid environment, attained by enhancing CL synthesis and co-localizing ProP with CL, modulates the osmotic activation threshold of ProP in vivo.

The Coiled-coil Domain and ProP Localization—Intermolecular, antiparallel coiled-coils link the C termini in dimers formed by E. coli ProP and some of its orthologues (Fig. 1). Absence or disruption of this coiled-coil elevates the osmotic activation threshold (and Π1/2/RT) (7, 25, 29). Neither ProP-R488I nor ProP-I474P can form a stable coiled-coil and, in bacteria cultivated in low osmolality medium, these transporters have Π1/2/RT values of ∼0.5 mol/kg (as compared with 0.2 mol/kg for the wild type transporter) (25). As reported previously (8) and illustrated here in Figs. 2 and 5 (left panels), concentration of wild type ProP at the cell poles correlated with CL content for cls− bacteria and it was evident at all CL levels in cls+ bacteria. Strikingly, mutations R488I and I474P essentially eliminated the polar localization of ProP even for cls+ bacteria cultivated at high osmolality (Fig. 5). Thus mutations that disrupt the C-terminal coiled-coil of ProP also disrupt its polar localization.

observed for minC+ bacteria under the same conditions (Table 2 and Ref. 8). We compared the phospholipid compositions of rod-shaped cells and minicells that had been cultivated to exponential or stationary phase in low and high osmolality media. The proportion of CL in minicells was consistently higher than that in rod-shaped cells from the same culture. The proportion of CL varied widely, from 4 mol % in cells cultured to exponential phase in low osmolality medium to 13 mol % in minicells cultivated to stationary phase in high osmolality medium, the proportion of PG ranging from ∼21 to 4 mol %, and the proportion of PE remaining essentially constant at ∼80 mol % (Table 2).
Dimerization, the Coiled-coil, and ProP Function—Covalent cross-linking experiments indicate that ProP is a dimer in vivo (28). The dimer interface includes transmembrane (TM) helix XII and the C-terminal coiled-coil (28, 48). Residues 419–437 of ProP constitute the membrane-embedded portion of TM XII (28). The dimer interface includes transmembrane (TM) helix XII and the C-terminal coiled-coil (28, 48). The dimer interface includes transmembrane (TM) helix XII and the C-terminal coiled-coil (28, 48).

DTME is a bifunctional thiol reagent with a cross-link length of ~1.3 nm and a cleavable disulfide. To determine whether membrane composition and subcellular localization influence ProP dimerization or coiled-coil formation, we used DTME to cross-link ProP variants with cysteine (Cys) only at positions 420 (at the periplasmic end of TM XII), 439 (in the cytoplasm, just beyond TM XII), or 480 (in the coiled-coil). Replacement of these residues with Cys had little effect on ProP activity (28, 48). DTME cross-linking of these Cys (but not of Cys at other positions) was previously shown to occur when ProP was expressed in E. coli cells cultivated to exponential phase at low osmolality (28, 48). The degree of cross-linking remained essentially constant when each variant was expressed in cls+ or cls– bacteria that were grown in low or high osmolality medium to exponential or stationary phase (Fig. 6A and data not shown). Thus a significant fraction of ProP could be cross-linked despite large changes in membrane phospholipid composition and the subcellular location of the transporter. These data suggest that ProP is dimeric regardless of its subcellular location. However, covalent cross-linking shifts monomer-dimer equilibria, favoring dimerization. It is therefore possible that DTME cross-linking overestimated the proportion of dimeric ProP or of dimers containing coiled-coils in these preparations.

We also determined the impact of DTME cross-linking on the osmotic

**TABLE 2**

Phospholipid head group compositions and ratios of E. coli cells and minicells

| Growth phase | Osmolarity | PE Cells | PE Minicells | PG Cells | PG Minicells | CL Cells | CL Minicells | PG:PE ratio | CL:PG ratio |
|--------------|------------|----------|--------------|----------|--------------|----------|--------------|-------------|-------------|
| Exponential  | Low        | 75.1 ± 2.1 | 809 ± 0.6    | 213 ± 2.4 | 11.7 ± 0.5    | 3.6 ± 0.6 | 7.4 ± 0.5    | 0.28 ± 0.03 | 0.15 ± 0.01 |
|              | Stationary | 89.9 ± 2.4 | 834 ± 1.3    | 4.4 ± 1.5 | 6.8 ± 0.9    | 5.7 ± 0.9 | 9.8 ± 0.4    | 0.05 ± 0.02 | 0.08 ± 0.01 |
| Exponential  | High       | 76.3 ± 3.8 | 818 ± 1.1    | 17.0 ± 3.1 | 8.3 ± 0.4    | 6.8 ± 1.0 | 9.9 ± 0.8    | 0.22 ± 0.04 | 0.10 ± 0.01 |
| Stationary   | High       | 84.1 ± 0.8 | 805 ± 2.4    | 6.5 ± 0.7 | 6.8 ± 1.5    | 9.4 ± 0.1 | 12.8 ± 1.3   | 0.08 ± 0.01 | 0.08 ± 0.02 |

E. coli strain ECK1164 (minC::kan) was cultivated to exponential or stationary phase in MOPS medium adjusted to low osmolality (0.15 mol/kg) or high osmolality (0.77 mol/kg) with NaCl. Minicells and rod-shaped cells were separated and their phospholipid compositions were determined as described under “Experimental Procedures.”

**FIGURE 4.** The anionic lipid content of the membrane determines the osmotic required to activate ProP. A, II/1/2RT is plotted versus the CL content for cls+ bacteria (closed circles, PG constant at ~18 mol %, data previously reported by Tsatskis et al. (7)), and versus the PG content for cls– bacteria (open circles, CL constant at ~0.4 mol %, phospholipid compositions previously reported by Romantsov et al. (8)). B, II/1/2RT is plotted versus the fraction of phosphate in anionic lipid head groups (CL plus PG, data from A), C, II/1/2RT is plotted versus the CL content (closed circles) and versus the PG content (open circles) of PRLs. D, II/1/2RT is plotted versus the fraction of phosphate in anionic lipid head groups (CL plus PG, data from C). The proline uptake activity of ProP was determined in cells cultivated to exponential phase in media of increasing osmolality (A and B) or in PRLs with varying phospholipid compositions (C and D). Cells and PRLs were prepared as described under “Experimental Procedures.” Proline uptake was measured as described in the legend to Fig. 3. II/1/2RT was determined by fitting the data to Equation 1 as described under “Experimental Procedures” and the resulting values are plotted versus phospholipid compositions determined as described under “Experimental Procedures.” The plots include single II/1/2RT determinations for cells and averages of replicate determinations of II/1/2RT for PRLs, including 3 replicates for PRLs with 1.5% CL, 1 for 3.4% CL, 10 for 4.6% CL (the unmodified polar lipid extract), 4 for 27.3% CL, and 2 for the other CL proportions. The corresponding values for A max are given in supplemental Table S2.
activation of ProP. Bacteria expressing ProP* (a cysteine-less ProP variant) and its derivative with Cys only at coiled-coil position 480 were cultivated in high osmolality medium to elevate their CL content and the osmotic activation of ProP was evaluated with and without DTME treatment (Fig. 4B). Without DTME, these ProP variants showed the high osmotic activation threshold characteristic of ProP in a high CL environment (\(\Pi_{1/2}/RT\)) values of 0.367 ± 0.009 for ProP* and 0.375 ± 0.010 mol/kg for ProP*-E480C. Each activates at low osmolality in bacteria cultivated at low osmolality (\(\Pi_{1/2}/RT\) values of 0.230 ± 0.006 and 0.227 ± 0.007 mol/kg, respectively (28)). For both variants DTME treatment reduced the activation amplitude ~2-fold, presumably by attenuating respiration (Fig. 6B). However, DTME treatment dramatically decreased \(\Pi_{1/2}/RT\) for ProP*-E480C without affecting \(\Pi_{1/2}/RT\) for ProP* (the values were 0.280 ± 0.003 and 0.359 ± 0.021 mol/kg, respectively) (Fig. 6B). Thus covalent stabilization of the coiled-coil decreased \(\Pi_{1/2}/RT\) even though the transporter was expressed in high-CL bacteria.

**DISCUSSION**

The phospholipid head group composition of *E. coli* is influenced by multiple growth parameters and phospholipids are not uniformly distributed over the cell surface. The proportion of phosphatidate in anionic lipids (% PG + 2% CL) increases with growth medium osmolality and decreases as cultures enter stationary phase, but the CL:PG ratio increases in both cases (7, 8). CL is concentrated near the poles and septa of *E. coli* cells, whereas PG is not (8). *E. coli* minicells are believed to represent the poles of rod-shaped *E. coli* cells. Here we compared the phospholipid compositions of *E. coli* minicells and cells from the same cultures (Table 2). The PG:PE ratio of minicells was markedly lower than that of cells from the same exponential phase cultures and the CL:PG ratio of minicells was higher than that of cells cultivated under all conditions. An unknown mechanism maintains a CL-rich environment at the cell poles and a more PG-rich environment elsewhere. Thus the cytoplasmic membrane at the cell poles is a specialized, CL-enriched structure, its CL content varying with growth conditions from at least 7 to 13 mol % as indicated by minicells from CL+ bacteria. By comparison, the mitochondrial and cytoplasmic membranes of rat liver contain 18 and 1 mol % CL, respectively (49).

Bacteria take up and release osmoprotectants to control cellular hydration despite fluctuating extracellular osmotic pressure (1). We showed that *E. coli* protein ProP is both an osmosensor and an osmoprotectant transporter by demonstrating that the initial rate of osmoprotectant uptake via ProP is a sigmoid function of medium osmolality in both cells and PRLs (22). In addition, as *E. coli* is cultivated in media of increasing osmolality, the osmolality at which ProP attains half-maximal activity (\(\Pi_{1/2}/RT\)) increases with the CL content of the membrane (7). This osmotic adaptation may ensure that ProP senses and responds to fluctuations around the ambient osmolality.
Cardiolipin Controls ProP Localization and Function in E. coli

### TABLE 3

**Impacts of phospholipid headgroup composition on ProP function in vivo and in vitro**

Based on data reported by Tsatsakis et al. (7) and Fig. 4.

| Experimental system | Phospholipid headgroup composition at which $\Pi_{1/2}/RT$ for ProP was 0.35 mol/kg | PG | CL | % of Phospholipid in CL and PG | mol % |
|---------------------|---------------------------------------------------------------------------------|----|----|-------------------------------|-------|
| $cls^{-}$ bacteria  | 26                                                                              | 0.4 | 27 |
| PRLs, CL:PE of 0.05$^a$ | 35                                                                              | 3   | 40 |
| $cls^{-}$ bacteria$^b$ | 17                                                                              | 5.5 | 27 |
| PRLs, PG:PE of 0.2$^a$ | 14                                                                              | 14  | 37 |

$^a$ PRLs were prepared as described under "Experimental Procedures." The CL:PE mole ratio was fixed at 0.05 and the PG content was varied or the PG:PE mole ratio was fixed at 0.2 and the CL content was varied.

$^b$ Under these conditions the mole fraction of CL at the cell poles were estimated to be in the range 7–10%, with PG at 12-8% (Table 2 and Fig. 4).

Here we show that $\Pi_{1/2}/RT$ for ProP is a direct function of mole percent CL in $cls^{-}$ bacteria (at constant mol % PG), of mol % PG in $cls^{-}$ bacteria (at constant mol % CL), and of anionic lipid content in both intact bacteria and PRLs (Figs. 3 and 4). Thus E. coli may modulate the osmosensory function of ProP by modulating phospholipid head group composition.

In wild type ($cls^{-}$) bacteria under osmotic stress, elevation of $\Pi_{1/2}/RT$ correlated with increasing CL content and with co-localization of ProP and CL at the cell poles (8). Here we show that $\Pi_{1/2}/RT$ also increases with the proportion of PG as $cls^{-}$ bacteria are cultivated in media of increasing osmolality (Fig. 4), yet neither PG nor ProP is concentrated at the cell poles (8) (Fig. 2). Thus elevation of $\Pi_{1/2}/RT$ occurs as ProP clusters with CL but elevation of $\Pi_{1/2}/RT$ is not contingent on placement of ProP at the cell poles.

Although both anionic lipids modulated the osmolality at which ProP became active, $\Pi_{1/2}/RT$ was much more sensitive to CL content than to PG content in both cells and PRLs (Fig. 4). ProP was intrinsically more sensitive to CL than to PG. Furthermore, a much lower (average) CL content was sufficient to raise $\Pi_{1/2}/RT$ for ProP to 0.35 mol/kg in cells than in PRLs (Table 3). This occurred at least in part because, in vivo, ProP is embedded in the CL-enriched membrane localized at the cell poles. These observations indicate that, physiologically, osmotic induction of CL synthesis and concentration of ProP with CL at the cell poles adjust the osmotic activation threshold of ProP to match ambient conditions.

We showed elsewhere that ProP orthologues with and without C-terminal coiled-coils are osmosensory transporters but orthologues and variants with the coiled-coil activate at lower osmolalities than those without (7). We hypothesize that association of the C termini with the CL-rich membrane and homomeric coiled-coil formation are mutually exclusive. ProP-CL association is favored over coiled-coil formation at the CL-rich cell poles, raising the osmotic activation threshold. Mutations that prevent coiled-coil formation raise the activation threshold regardless of the membrane environment of the transporter or location on the cell surface.

The data presented here support that model. ProP variants with coiled-coil disrupting mutations R488I and I474P do not concentrate with CL at the poles of exponential phase bacteria (Fig. 5). If these mutations were to favor ProP-CL interaction only by destabilizing the coiled-coil, they would be expected to enhance, not diminish polar localization of ProP. We therefore suggest that these mutations also lower the affinity of the ProP C-terminal domain for the CL-rich membrane and their redistribution over the cell surface results from the latter effect.

If association of the ProP C termini with the CL-rich membrane and homomeric coiled-coil formation are mutually exclusive, the proportion of ProP proteins engaged in coiled-coils should decrease as membrane CL content increases and ProP co-localizes with CL at the cell poles. Homodimerization of ProP can be demonstrated by covalent cross-linking of single Cys variants (28, 29, 48). Residue Cys-480 is at a heptad “g” position, flanking the hydrophobic core of the ProP coiled-coil (26). Cys replacement at position 480 does not impair coiled-coil formation (28) and cross-linking of ProP dimers with Cys only at position 480 correlates with antiparallel coiled-coil formation (27). In contrast to the above prediction, the proportion of covalently cross-linked, dimeric ProP did not vary as phospholipid head group composition and ProP localization varied with growth conditions (Figs. 2 and 6A). However, intermolecular cross-linking traps proteins in their dimeric form so cross-linking may have overridden the impact of the membrane environment on coiled-coil formation.

We returned to functional analysis to determine whether cross-linking had indeed perturbed this system. When cross-linked within bacteria cultivated at high osmolality, variant ProP*-E480C required a much lower osmolality to activate than Cys-less variant ProP* (Fig. 6B). We believe that cross-linking of residue Glu-480 did override the impact of the high CL membrane, restoring coiled-coil formation and lowering the osmotic activation threshold of ProP.

The mechanisms concentrating CL and some proteins at bacterial cell poles are not well understood. When protein synthesis was inhibited and the nucleoid condensed with chloramphenicol, CL remained concentrated at cell poles, whereas ProP and its parologue LacY did not (Fig. 2). Thus LacY may be passively excluded from the “transit zone” or its polar localization may require continuing synthesis of labile proteins. Additional factors must contribute to the polar co-localization of CL and ProP. The C terminus of ProP may interact directly with CL or it may interact with an unidentified ProP partner protein that colocalizes with CL at cell poles. Mutations R488I and I474P would then impair association of the ProP C termini with that CL-associated protein. For example, ProP could form heteromeric coiled-coils with an unknown partner (cf. the competition for homomeric or heteromeric leucine zipper formation by bZIP proteins in eukaryotes) and such interactions could also be disrupted by mutations R488I and I474P.

Localization of ProP at cell poles may have objectives or consequences that extend beyond provision of a CL-rich environment. For example, polar localization of proteins that sense and/or respond to osmotic stress may influence cell growth or morphogenesis. In non-spherical cells, wall and membrane strain vary with cell surface location and cell size. Yeast and plant cells grow anisotropically as their walls yield locally to the global force of turgor (50) and some osmosensory protein complexes are directed to sites of polarized growth, perhaps to sense and maintain membrane strain at those locations (51). Bacterial
mechanosensitive channels (e.g. MscL (52)) are believed to detect membrane strain in vivo because their function is known to depend on membrane curvature in vitro. Thus their polar localization in rod-shaped bacteria would be expected to affect their responses to osmotic downshifts. In PRLs, ProP and related osmorsensory transporters BetP and OpuA can be activated by increasing luminal solute concentration without imposing osmotic shifts (41, 42, 53). This indicates that changing the membrane strain is not necessary for their activation but it does not rule out effects of membrane strain on their activities. Further work is required to fully elucidate the relationships among subcellular location, phospholipid environment, protein structure, osmorsensory function, and osmoregulatory transport activity for ProP.

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REFERENCES

1. Wood, J. M. (1999) Microbiol. Mol. Biol. Rev. 63, 230–262
2. Bolen, D. W. (2001) Methods Mol. Biol. 168, 17–36
3. Poolman, B., Spitzer, J. J., and Wood, J. M. (2004) Biochim. Biophys. Acta 1666, 88–104
4. Sleator, R. D., Wood, J. M., and Hill, C. (2003) J. Bacteriol. 185, 7140–7144
5. Cronan, J. E. (2003) Annu. Rev. Microbiol. 67, 1–9
6. Cronan, J. E. (2003) Annu. Rev. Microbiol. 57, 203–224
7. Tsatskis, Y., Khambati, J., Dobson, M., Bogdanov, M., Dowhan, W., and Wood, J. M. (2005) J. Biol. Chem. 280, 41387–41394
8. Romantsov, T., Helbig, S., Culham, D. E., Gill, C., Stalker, L., and Wood, J. M. (2005) Mol. Microbiol. 64, 1455–1465
9. Mileykovskaya, E., and Dowhan, W. (2000) J. Bacteriol. 182, 1172–1175
10. Koppelman, C.-M., den Blauwen, T., Duursma, M. C., Heeren, R. M. A., and Nanninga, N. (2001) J. Bacteriol. 183, 6144–6147
11. Huang, K. C., Mukhopadhyay, R., and Wingren, N. S. (2006) PLoS Comput. Biol. 2, e151
12. Nagamori, S., Smirnova, I. N., and Kaback, H. R. (2004) J. Cell Biol. 165, 53–62
13. Lai, E.-M., Nair, U., Phadke, N. D., and Maddock, J. R. (2004) Mol. Microbiol. 54, 1029–1044
14. Matsutomo, K., Kusaka, J., Nishibori, A., and Harai, H. (2006) Mol. Microbiol. 61, 1110–1117
15. Norris, V. (1995) Mol. Microbiol. 16, 1051–1057
16. Binenbaum, Z., Parola, A. H., Zaitsevsky, A., and Fishov, I. (1999) Mol. Microbiol. 32, 1173–1182
17. Kentner, D., and Sourjik, V. (2006) Curr. Opin. Microbiol. 9, 619–624
18. Thiem, S., Kentner, D., and Sourjik, V. (2007) EMBO J. 26, 1615–1623
19. Guan, L., and Kaback, H. R. (2006) Annu. Rev. Biophys. Biomol. Structure 35, 67–91
20. Culham, D. E., Lasby, B., Marangoni, A. G., Milner, J. L., Steer, B. A., van Nues, R. W., and Wood, J. M. (1993) J. Biol. Chem. 268, 276–287
21. Wood, J. M., Culham, D. E., Hillar, A., Vernikovska, Ya. I., Liu, F., Boggs, J. M., and Keates, R. A. B. (2005) Biochemistry 44, 5534–5546.
22. Racher, K. I., Voegele, R. T., Marshall, E. V., Culham, D. E., Wood, J. M., Jung, H., Bacon, M., Cairns, M. T., Ferguson, S. M., Liang, W.-J., Hender-