Roles of K^+, H^+, H_2O, and $\Delta\Psi$ in Solute Transport Mediated by Major Facilitator Superfamily Members ProP and LacY[†]

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ABSTRACT: H^+-solute symporters ProP and LacY are members of the major facilitator superfamily. ProP mediates osmoprotectant (e.g., proline) accumulation, whereas LacY transports the nutrient lactose. The roles of K^+, H^+, H_2O, and $\Delta\Psi$ in H^+-proline and H^+-lactose symport were compared using right-side-out cytoplasmic membrane vesicles (MVs) from bacteria expressing both transporters and proteoliposomes (PRLs) reconstituted with pure ProP-His_6. ProP activity increased as LacY activity decreased when osmotic stress (increasing osmolality) was imposed on MVs. The activities of both transporters decreased to similar extents when Na^+ replaced K^+ in MV preparations. Thus, K^+ did not specifically control ProP activity. As with LacY, an increasing extravesicular pH stimulated ProP-mediated proline efflux much more than ProP-mediated proline exchange from de-energized MVs. In contrast to that of LacY, ProP-mediated exchange was only 2-fold faster than ProP-mediated efflux and was inhibited by respiration. In the absence of the protonmotive force ($\Delta\mu_{H^+}$), efflux of lactose from MVs was much more sensitive to increasing osmolality than lactose exchange. Thus, H_2O may be directly involved in proton transport via LacY. In the absence of $\Delta\mu_{H^+}$, proline efflux and exchange from MVs were osmolality-independent. In PRLs with a ΔpH of 1 (lumen alkaline), ProP-His_6 was inactive when the membrane potential ($\Delta\Psi$) was zero, was active but insensitive to osmolality when $\Delta\Psi$ was -100 mV, and became osmolality-sensitive as $\Delta\Psi$ increased further to -137 mV. ProP-His_6 had the same membrane orientation in PRLs as in cells and MVs. ProP switches among "off", "on", and "osmolality-sensitive" states as the membrane potential increases. Kinetic parameters determined in the absence of $\Delta\mu_{H^+}$ represent a ProP population that is predominantly off.

Escherichia coli cells respond to increasing osmotic pressure by accumulating K^+ or an organic solute compatible with cellular functions (a "compatible solute") (*1*). K^+ accumulates rapidly, with glutamate as the counterion, if no suitable organic solutes are available exogenously. Bacterial growth slows and then resumes at a reduced rate as trehalose is synthesized and K^+ glutamate levels fall. K^+ glutamate inhibits transcription of most genes while selectively activating transcription of osmoregulatory genes, including the trehalose biosynthetic system (*2–7*).

If they are available prior to an osmotic upshift, organic osmoprotectants like proline are transported to the cytoplasm where they act as or are converted to compatible solutes (*1*). K^+ accumulation is attenuated, and growth may be restored to its prestress rate (*8, 9*). *E. coli* possesses at least four osmoprotectant transporters: H^+-solute symporter ProP, ABC transporter ProU, and Na^+-solute symporters BetT and BetU (*1, 10*). ProP catalyzes the accumulation of diverse osmoprotectants, including proline, glycine betaine, and ectoine (*11*). A member of the major facilitator superfamily, ProP is similar in structure and function to H^+-lactose symporter LacY (*12, 13*). However, ProP activity increases with medium osmolality in intact bacteria, in right-side-out cytoplasmic membrane vesicles (MVs),[1] and in proteoliposomes (PRLs) reconstituted with the purified protein (*14, 15*), while respiration, LacY, and other transporters are inhibited (*16*). Thus, ProP is an osmosensing transporter that acts to restore cellular hydration.

We would like to understand how osmotic pressure controls transporter activity. Three osmosensing transporters are being investigated in an effort to answer this question: ProP of *E. coli*, BetP of *Corynebacterium glutamicum*, and OpuA of *Lactococcus lactis*. BetP is a Na^+-glycine betaine symporter and a member of the betaine-carnitine-choline transporter family like *E. coli* BetT and BetU (*17*). OpuA is a glycine betaine-specific ABC transporter and an orthologue of *E. coli* ProU (*18*). Proteoliposome studies suggest that BetP and OpuA respond to osmotically induced changes in lumenal K^+ concentration and ionic strength, respectively.

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[1] Abbreviations: Π, osmotic pressure; $\Pi_{1/2}/RT$, osmolality at which ProP activity is half-maximal; A_{max}, rate of uptake of proline via ProP extrapolated to infinite osmolality; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MFS, major facilitator superfamily; MOPS, 4-morpholinopropanesulfonic acid; MV, membrane vesicle; PRL, proteoliposome; TM, transmembrane.

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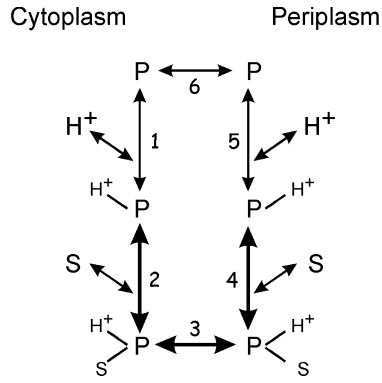

FIGURE 1: H^+-symport mechanism. The illustrated symport mechanism has been validated by extensive experimentation for H^+-lactose symporter LacY of *E. coli* (*13*). During active organic solute (S) uptake (steps 5 through 1, and then 6), binding of H^+ precedes binding of solute to the transporter (Y) in its periplasm-facing conformation and release of H^+ follows release of solute from the transporter in its cytoplasm-facing conformation. During efflux (steps 1 through 6), binding of H^+ precedes binding of solute to the transporter in its cytoplasm-facing conformation and release of H^+ follows release of solute from the transporter in its periplasm-facing conformation. If the solute is present in both compartments at a saturating concentration, the transporter may remain protonated throughout solute exchange (steps 2 through 4, and then 4 through 2, bold arrows).

ProP activity increases with the lumenal concentrations of diverse electrolytes or of high-molecular weight polyethylene glycols (the latter at a constant lumenal K^+ concentration) (*15*). Thus, ProP activity may be controlled by ProP hydration or macromolecular crowding. However, regulation of ProP by K^+ has not been ruled out since exogenous K^+ stimulates ProP activity in intact bacteria (*11, 19*). This paper provides the first report of in vitro ProP activity at a K^+ concentration lower than 0.18 M.

Here we report the impact of K^+ on ProP and LacY activities in MVs from *E. coli*. MVs are topologically closed, right-side-out sacs, prepared by the osmotic lysis of bacteria with weakened cell walls, each corresponding to the cytoplasmic membrane of an individual cell (*20*). MVs are filled with the solvent in which the bacteria were lysed. Respiration can be used to power secondary transporters in MVs since the cytoplasmic membrane also contains the respiratory chain. In contrast, primary transporters are active only in MVs provided with a phosphoenolpyruvate- or ATP-generating system (*21*). We used MVs containing both ProP and LacY to compare the impacts of K^+ and osmotic pressure on these two transporters. Our data show that K^+ stimulates active solute transport via both ProP and LacY, probably reflecting a K^+ requirement for respiration rather than direct involvement of K^+ in transport mediated by either protein. Thus, ProP activity is not specifically K^+-dependent.

Studies of LacY support the H^+-solute symport mechanism illustrated in Figure 1. Here we show that, in the absence of the protonmotive force ($\Delta\mu_{H^+}$), efflux of lactose from MVs was much more sensitive to increasing osmolality than lactose exchange. This suggests that H_2O may participate in a rate-limiting step of H^+-lactose symport. We provide evidence that Figure 1 also illustrates the transport mechanism of ProP and that the osmolality dependence of H^+-proline symport via ProP is contingent on respiration. Using purified ProP-His_6 in PRLs, we show that both osmosensing by ProP and H^+-solute symport require the membrane potential ($\Delta\Psi$). These data suggest that ProP senses both $\Delta\Psi$ and osmotic pressure, switching among "off", "on", and "osmolality-sensitive" states as the membrane potential increases in magnitude from 0 to −137 mV.

EXPERIMENTAL PROCEDURES

E. coli Strains, Culture Media, and Other Solutions. This study employed *E. coli* RM2 [F^- *trp lacZ rpsL thi* Δ*(putPA)101*] and derivatives of *E. coli* WG350 [F^- *trp lacZ rpsL thi* Δ*(putPA)101* Δ*(proU)600* Δ*(proP-melAB)212*], which is devoid of proline transporters (*22*). Each WG350 derivative contained a plasmid encoding ProP [pDC79 (*22, 23*)], ProP-His_6 [pDC80 (*24*)], or ProP*-T16C [pDC191 (*12*)] which encodes the T16C mutant derivative of cysteine-less ProP-His_6 [known as ProP* (*25*)]. These variants were expressed in an osmolality-independent manner from the AraC-controlled P_{BAD} promoter of vector pBAD24 (*26*).

The bacteria were cultivated at 37 °C in LB medium (*27*) or in NaCl-free MOPS medium, a variant of the MOPS medium described by Neidhardt et al. (*28*) from which all NaCl was omitted. MOPS medium was supplemented with NH_4Cl (9.5 mM) as a nitrogen source and glycerol (0.4%, v/v) as a carbon source. L-Tryptophan (245 μM) and thiamine hydrochloride (1 μg/mL) were added to meet auxotrophic requirements. Ampicillin (100 μg/mL) was included to maintain plasmids. D-Arabinose (0.4 mM) and IPTG (1 mM) were added to induce expression of *proP* and *lacY*, respectively.

The osmolalities of culture media and buffers were adjusted with NaCl or sucrose, as specified, and measured with a Wescor vapor pressure osmometer (Wescor, Logan, UT). The K^+ content of buffers was determined with a Perkin-Elmer model AA800 atomic absorption spectrophotometer.

Preparation of Right-Side-Out Cytoplasmic Membrane Vesicles. MVs were prepared from *E. coli* WG709 (WG350 pDC79). Strain WG709 is derived from strain RM2, which is *lacZ*$^-$. The *lacZ* mutation in RM2 and its derivatives was not polar on *lacY* since similar lactose uptake activities were observed after IPTG induction for WG709 [8 ± 1 nmol min^{-1} (mg of cell protein)$^{-1}$], RM2 [9 ± 1 nmol min^{-1} (mg of cell protein)$^{-1}$], and WG621, a spontaneous *lacZ*$^+$ revertant of RM2 [12 ± 2 nmol min^{-1} (mg of cell protein)$^{-1}$]. However, the level of expression of *proP* in strain WG709 decreased when IPTG was added to induce *lacY*, as indicated by reduced proline uptake activity [57.3 ± 0.4 and 8.8 ± 0.2 nmol min^{-1} (mg of cell protein)$^{-1}$, respectively]. Arabinose was therefore included in the culture media to restore *proP* expression to the level attained in the absence of both IPTG and arabinose as indicated by Western blots and transport assays.

K phosphate-loaded MVs (KMVs) were prepared using the lysozyme−EDTA method described by Kaback (*20*) with the following changes. Harvested cells were not washed. Lysozyme was added at a concentration of 50 μg/mL, and the resuspended cells were incubated at room temperature for 40 min. The spheroplast suspension was added dropwise to 375 volumes of 0.1 M K phosphate buffer (pH 6.6). K EDTA and $MgSO_4$ were added to final concentrations of 9.1 and 1.2 mM, respectively. MVs were collected by centrifugation at 28000*g* (not 45000*g*) and washed only twice. The

same procedure was used to prepare Na phosphate-loaded MVs (NaMVs) except that Na phosphate replaced K phosphate at every step.

Uptake Assays. Bacteria were cultivated and assays performed on intact cells as described by Culham et al. (*15*). Initial rates of solute uptake were measured using L-[U-^{14}C]proline and [D-glucose-1-^{14}C]lactose (Amersham Biosciences) (0.2 mM) as substrates. Protein concentrations were determined by the bicinchoninic acid assay (*29*) using the BCA kit from Pierce (Rockford, IL) with bovine serum albumin (BSA) as the standard. All assays were conducted in triplicate, and all experiments were performed at least twice. Representative rates are cited as means ± the standard error.

For MVs, proline and lactose uptake assays were carried out essentially as described by Kaback (*20*). KMVs were diluted into 0.1 M K phosphate (pH 6.6) with 10 mM $MgSO_4$ and sucrose to vary the osmolality. The samples were incubated at 24 °C for 5 min. Li D-lactate was added to a final concentration of 20 mM. The mixture was further incubated for 1.5 min, and L-[U-^{14}C]proline or [D-glucose-1-^{14}C]lactose (Amersham Biosciences) was added to a final concentration of 0.2 mM. The incubation was continued, and aliquots were removed, filtered, and washed with 10 mL of 0.1 M LiCl (with sucrose as needed to keep the wash buffer iso-osmolal with the assay buffer). The filters were transferred to scintillation vials, dried, covered in 5 mL of scintillation cocktail [diphenyloxazole (5 g/L) in xylenes], and counted. The same procedure was used for NaMVs except that Na phosphate replaced K phosphate at every step. Representative means and standard deviations are shown for initial rates of proline or lactose uptake measured in triplicate using one of at least two independent MV preparations.

Efflux and Exchange Assays. D-Lactate (20 mM)-energized uptake was used to load MVs with [^{14}C]proline or [^{14}C]lactose, each provided at a starting extravesicular concentration of 0.25 mM, following a procedure like those reported by Blancato et al. (*30*) and Dayem et al. (*31*). Unless otherwise indicated, MVs were incubated for 60 min in assay buffer supplemented with 0.6 M sucrose (1 mol/kg) for proline and for 20 min in assay buffer with no osmolyte (0.25 mol/kg) for lactose. Efflux and exchange assays were carried out essentially as described by Kaczorowski and Kaback (*32*) except that the loaded vesicles were diluted 20-fold into the indicated efflux or exchange buffer containing carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 10 μM). The loading and subsequent dilution yielded intravesicular solute concentrations of approximately 50 mM (proline) or 10 mM (lactose) with the extravesicular solute at approximately 10 μM. Aliquots were removed at the indicated times, filtered, and washed with 10 mL of 0.1 M LiCl supplemented with sucrose to keep the wash buffers iso-osmolal with the efflux and exchange buffers. Data were fit to the relationship $\ln s = s_{max} - kt$, where s is the measured quantity of intravesicular solute at time t, to yield parameters k (the efflux or exchange rate constant) and s_{max} (the amount of intravesicular solute at the time of MV dilution).

Purification of ProP-His$_6$, Preparation of PRLs, and Transport Assays. ProP-His$_6$ was purified; PRLs were prepared using the *E. coli* polar lipid extract from Avanti Polar Lipids, Inc. (Alabaster, AL), and proline uptake assays were performed as described previously (*15, 24, 33*).

Orientation of ProP in PRLs. The orientation of ProP in PRLs was determined by assessing the accessibility of a Cys residue in the N-terminal domain to covalent modification and that of the C-terminal domain to reaction with an antibody. The exposure of a Cys residue at position 16 of ProP was tested using fluorescent probe OGM (Oregon Green Maleimide) (Invitrogen Canada, Inc., Burlington, ON) and blocking agent MTSET (methanethiosulfonate ethyltrimethylammonium) (Toronto Research Chemicals Inc., Toronto, ON), as previously described (*12*). Following electrophoresis, protein fluorescence was determined by exposure to UV light using a Typhoon 9410 variable-mode imager (GE Healthcare Life Sciences, Picataway, NJ) with a CCD camera attachment. Each gel was also stained for protein using Coomassie Blue and imaged using a white light source.

Antibodies raised against ProP (anti-ProP) (*23*) or a synthetic peptide corresponding to the 15 C-terminal amino acids of ProP (anti-C) (*34*) were used to define the accessibility of the C-terminus as described by Schiller et al. for transporter BetP (*35*). To detect epitopes exposed on the PRL surface, preformed PRLs were diluted to 50 ng of ProP/μL, incubated separately for 90 min with each of the antibodies [anti-ProP:PRL ratio of 1000:1 and anti-C:PRL ratio of 100:1 (v:v)], harvested by centrifugation (70000*g* for 15 min at 4 °C), and washed four times with 10 mM Tris-buffered saline (TBS) [10 mM tris(hydroxymethyl)aminomethane and 0.15 M NaCl (pH 7.4)]. ProP−antibody complexes were separated by SDS−PAGE, and Western blotting was performed as described previously to detect the recovered antibodies (*33*). To detect intraliposomal epitopes, antibodies and PRLs were mixed as described above and antibodies were allowed to enter the lumen of the PRLs by two freeze−thaw cycles followed by extrusion through polycarbonate filters with 0.4 μm pores (Avanti Polar Lipids, Inc.). The PRLs were harvested by centrifugation, washed once with TBS, and incubated for 90 min at 30 °C to allow antibody binding. The same PRLs were subjected to more freeze−thaw cycles, extrusion, and washing, and then antibodies bound to ProP were detected by Western blot analysis as described above. The same procedure was also applied to liposomes as a control for nonspecific antibody binding or trapping.

RESULTS

ProP Activates as LacY Inactivates in MVs under Osmotic Stress. ProP activates in cells, MVs, and PRLs as the medium osmolality is increased with diverse inorganic salts or nonelectrolytes (*14, 24*). Osmotic stress imposed with NaCl or sucrose was shown to inhibit respiration, LacY, and a variety of other transporters in *E. coli* cells (*16*), but effects of osmolality on ProP and LacY were not directly compared. Inactivation of LacY and activation of ProP occurred over the same osmolality (and water activity) range in *E. coli* MVs expressing both transporters (Figure 2). The osmotic activation of ProP can be described by the empirical relationship

$$a_0 = A_{max}\{1 + \exp[-(\Pi - \Pi_{1/2})(RTB)]\}^{-1} \qquad (1)$$

where Π is the osmotic pressure of the transport assay medium, a_0 is the initial rate of proline uptake measured with medium osmolality Π/RT, A_{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

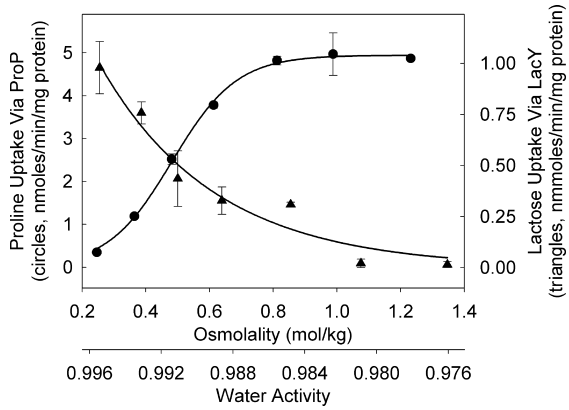

FIGURE 2: LacY activity decreased as ProP activity increased in membrane vesicles under osmotic stress. KMVs containing both LacY and ProP were prepared, and then proline (●) and lactose (▲) uptake activities were measured in 0.1 M K phosphate (pH 6.6) supplemented with sucrose to adjust the osmolality (and hence the water activity) as described in Experimental Procedures. The regression line obtained by fitting the initial rates of proline uptake to eq 1 had the following parameters: $A_{max} = 4.95 \pm 0.08$ nmol min^{-1} (mg of protein)$^{-1}$, $\Pi_{1/2}/RT = 0.483 \pm 0.009$ mol/kg, and $B = 0.101 \pm 0.008$ mol/kg. The regression line obtained by fitting the initial rates of lactose uptake to the exponential decay $v_0 = V_{max} \exp[-k(\Pi/RT)]$ had the following parameters: $V_{max} = 2.1 \pm 0.3$ nmol min^{-1} (mg of protein)$^{-1}$ and $k = 0.0030 \pm 0.0004$ kg/mol.

osmolality yielding half-maximal activity, and B is a constant inversely proportional to the slope of the response curve (*15*). In MVs, $\Pi_{1/2}/RT$ for ProP was 0.48 mol/kg. LacY activity decreased exponentially and was also reduced 2-fold at an osmolality of 0.48 mol/kg. In principle, this could result from effects of osmolality on respiration (and hence $\Delta\mu_{H^+}$) or on LacY itself. However, inhibition of LacY did not result solely from osmotic inhibition of respiration since ProP activity, which is also $\Delta\mu_{H^+}$-dependent (*24*), was maximal at the highest osmolality that was tested.

K^+ Dependence of ProP and LacY in MVs. MVs expressing ProP and LacY were prepared in 0.1 M K phosphate (pH 6.6) (the standard buffer) and in 0.1 M Na phosphate (pH 6.6) using cells from the same bacterial cultures. The K^+ concentrations of these preparations were 0.15 M and 35 μM, respectively. When each transporter was examined under optimal conditions (high osmolality for ProP and low osmolality for LacY), both were less active in vesicles prepared with Na^+ phosphate (NaMVs) than in those prepared with K^+ phosphate (KMVs) (Table 1).

We determined the osmolality dependence of each activity in each preparation (Figure 3). ProP had a lower maximal activity (A_{max}) and required a higher osmolality for activation (indicated by $\Pi_{1/2}/RT$) in NaMVs than in KMVs ($\Pi_{1/2}/RT$ of 0.574 ± 0.021 mol/kg for NaMVs vs 0.483 ± 0.009 mol/kg for KMVs) (Figure 3A). LacY activity was also lower in NaMVs than in KMVs, as noted above, and it fell 2-fold from the initial values when the osmolality reached 0.48 mol/kg for KMVs and 0.42 mol/kg for NaMVs (Figure 3B). We therefore compared the impacts of substituting Na for K on transport via ProP and LacY by plotting the ratio of the activity of each transporter in NaMVs to that of the same transporter in KMVs as a function of osmolality (Figure 3C). This approach accounts empirically for variations in $\Delta\mu_{H^+}$ with osmolality and accommodates the fact that LacY is most active at low osmolality whereas ProP is most active at high

Table 1: Activities of *ProP* and LacY in Membrane Vesicles[a]

	proline uptake activity		lactose uptake activity	
preparation[b]	Na vesicles	K vesicles	Na vesicles	K vesicles
I	0.25 ± 0.03	0.94 ± 0.06	0.26 ± 0.03	1.01 ± 0.04
II	1.49 ± 0.05	5.47 ± 0.51	0.47 ± 0.04	1.20 ± 0.02
III	0.92 ± 0.08	4.78 ± 0.37	0.31 ± 0.07	1.11 ± 0.05

[a] Lactose uptake activities were measured as described in Experimental Procedures using standard assay medium [0.1 M K or Na phosphate (pH 6.6), with an osmolality of 0.25 mol/kg]. Proline uptake activities were measured in the same way but with sucrose-supplemented medium (1 mol/kg). Uptake activities are reported as nanomoles per minute per milligram of vesicle protein. [b] Data are reported for three representative pairs of membrane vesicle preparations. The proline uptake activity of preparation I was low because *proP* expression was not induced with arabinose (see Experimental Procedures).

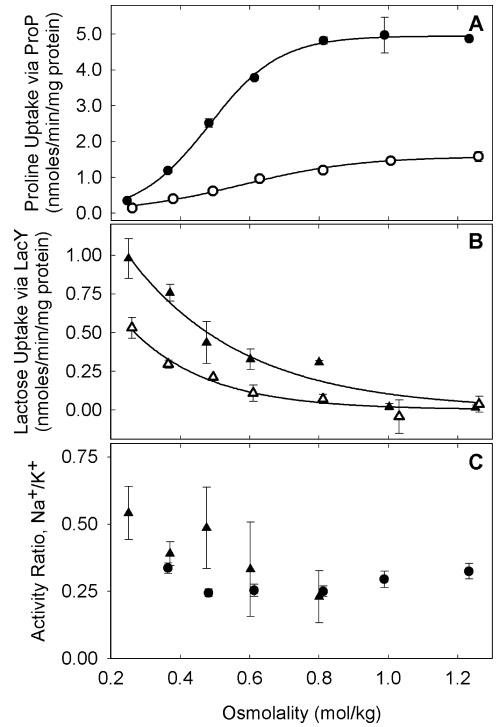

FIGURE 3: Impact of osmolality on ProP and LacY activities in MVs prepared with K or Na. KMVs and NaMVs containing both LacY and ProP were prepared using bacteria from the same culture, and the rate of solute uptake was measured as described in Experimental Procedures. Uptake activities were measured by diluting KMVs into 0.1 M K phosphate (black symbols) or NaMVs into 0.1 M Na phosphate (white symbols), at pH 6.6 and supplementing with sucrose to adjust the osmolality. Data analysis was as described in the legend of Figure 2. (A) D-lactate-dependent proline uptake activity, (B) D-lactate-dependent lactose uptake activity, and (C) ratio of the proline (●) or lactose (▲) uptake activity in Na phosphate to that in K phosphate plotted vs assay medium osmolality. Data points are not shown if the error exceeded the ratio.

osmolality. Presented in this way, the data show essentially the same effects of this cation substitution on both systems. We therefore conclude that K^+ stimulates active solute transport via ProP and LacY but ProP activity is not specifically K^+-dependent.

Efflux and Exchange Assays for ProP. Studies of proline efflux and exchange from de-energized MVs were employed to assess whether ProP and LacY follow similar transport mechanisms. In previous studies of LacY, MVs were loaded with lactose by passive equilibration at a variety of temper-

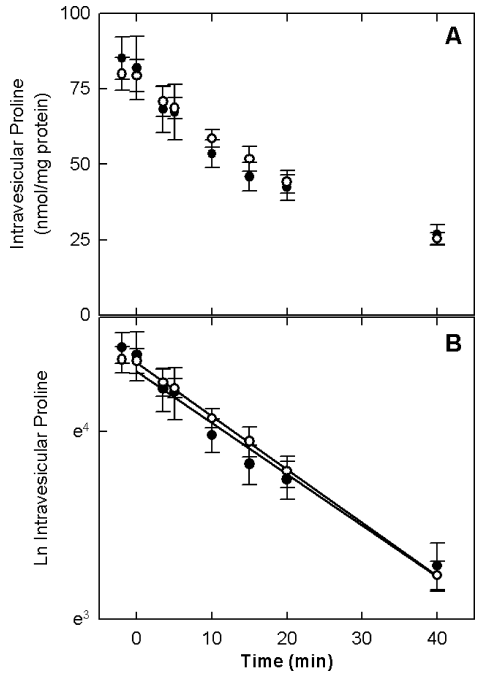

FIGURE 4: Proline efflux via ProP. KMVs were prepared in 0.1 M K phosphate (pH 6.6); they were loaded with [^{14}C]proline in the presence of D-lactate and sucrose, and proline efflux was measured as described in Experimental Procedures with the following modifications. The loaded MVs were diluted 20-fold into isotonic 0.1 M K phosphate (pH 6.6) containing D-lactate (20 mM). Two minutes later, CCCP (10 μM) (●) or valinomycin (25 nM) with nigericin (0.25 nM) (○) was added. The residual intravesicular proline (A) or the natural logarithm of the intravesicular proline (B) is plotted vs the time after uncoupler addition, and the lines were obtained by regression as described in Experimental Procedures.

atures for a variety of periods, in the presence of valinomycin (a K^+ ionophore) and nigericin (an ionophore mediating H^+-K^+ exchange) (*32, 36, 37*). Our MVs could not be loaded with proline or lactose using such treatments. MVs were therefore loaded with [^{14}C]proline or [^{14}C]lactose by D-lactate-energized active uptake (*30, 31*) and then diluted into D-lactate-, proline-, and lactose-free, uncoupler-supplemented medium to monitor efflux (Figure 4). Since efflux was similar when CCCP or valinomycin-nigericin was used to dissipate the protonmotive force (Figure 4), CCCP was used in all subsequent experiments.

ProP-Mediated Proline Fluxes across MV Membranes. For fluxes based on the mechanism illustrated by Figure 1, where H^+ binding precedes solute binding and H^+ release follows solute release, neither protonation nor deprotonation of the transporter is essential for solute exchange. The exchange rate is pH-independent if the solute saturates the transporter at both membrane surfaces, and exchange is $\Delta\mu_{H^+}$-independent if the intra- and extravesicular solute concentrations are also equal, as observed for LacY (*32, 38*). The following considerations validate the use of exchange assays in testing the applicability of this mechanism to ProP. The K_M for proline uptake via ProP varies with osmolality in the range of 0.01−0.10 mM (*24*). MVs were loaded with [^{14}C]proline at an osmolality of 1 mol/kg for a variety of times to produce a variety of intravesicular proline levels, and proline efflux rate constants were determined at the same osmolality. The efflux rate constant was independent of the initial intravesicular proline level in the range of 68−230 nmol/mg of protein (data not shown). On the basis of previous reports of passive lactose loading (*32*), 68 nmol/mg protein would correspond to an intravesicular proline concentration of >30 mM. Unless otherwise stated, initial intravesicular proline levels of >68 nmol/mg of protein were used for the reported efflux and exchange assays. The extravesicular concentration of nonradioactive proline was set at 10 mM for the exchange assays. The intravesicular and extravesicular proline concentrations could not be made equal as the intravesicular level could not be predetermined.

The reported pH dependence of unenergized lactose efflux via LacY (*32*) was replicated using the loading and efflux assay described above (data not shown). We monitored the impact of extravesicular pH on unenergized, ProP-mediated proline fluxes by diluting proline-loaded MVs into D-lactate- and proline-free, CCCP-supplemented medium to monitor efflux (Figure 5A) and into D-lactate-free medium supplemented with nonradioactive proline and CCCP to monitor exchange (Figure 5B). The rate constants for these reactions are summarized in Figure 5C. As for LacY, the rate constant for proline efflux via ProP increased with extravesicular pH much more than the rate constant for proline exchange. Proline exchange was approximately 2-fold faster than proline efflux. In contrast, lactose exchange via LacY is more than 20-fold faster than lactose efflux (*32*). As expected, proline efflux and exchange slowed when the vesicles remained energized (D-lactate substituting for CCCP) (white symbols in panels A and B of Figure 5, respectively). Thus, the effects of pH on substrate efflux and exchange via ProP and LacY were qualitatively similar, implying that both follow the mechanism illustrated in Figure 1. Key quantitative differences are explored below.

Osmolality Dependence of Unenergized Proline and Lactose Fluxes. MVs were used to determine the impact of osmolality on unenergized solute efflux and exchange via ProP and LacY. Lactose efflux slowed as osmolality increased, the efflux rate constant falling to half the initial value at an osmolality of 0.76 mol/kg (Figure 6A), but exchange was not affected. Thus, only H^+-linked lactose flux slowed as water activity fell. ProP behaved differently. Surprisingly, neither unenergized efflux nor exchange of proline via ProP was osmolality-sensitive. Thus, osmoregulation of ProP activity was respiration-dependent.

Membrane Potential Dependence of ProP Activation in PRLs. MVs were used for the experiments described above because respiration can generate $\Delta\mu_{H^+}$ in that system and MVs containing both ProP and LacY are readily prepared, facilitating their direct comparison. PRLs were used to further explore the role of $\Delta\mu_{H^+}$ in osmosensing by ProP. The response of ProP-His$_6$ in PRLs is analogous to the responses of ProP and ProP-His$_6$ to osmolality in cells (*15, 24, 33*) and MVs (ref *14* and Figure 2). In PRLs, a valinomycin-dependent K^+ diffusion gradient is used to generate $\Delta\Psi$, and pH gradients can be imposed by diluting PRLs prepared at one pH into a buffer with a different pH.

Racher et al. (*33*) showed that $\Delta\Psi$, alone, could support active uptake of proline into PRLs via ProP-His$_6$ (PRLs prepared in 0.1 M K phosphate diluted into 0.1 M Na phosphate with valinomycin) but ΔpH, alone, could not [PRLs prepared in 0.1 M K phosphate (pH 7.4) diluted into 0.1 M K phosphate (pH 6.4)]. However, ΔpH accelerated $\Delta\Psi$-dependent proline uptake and increased the level of

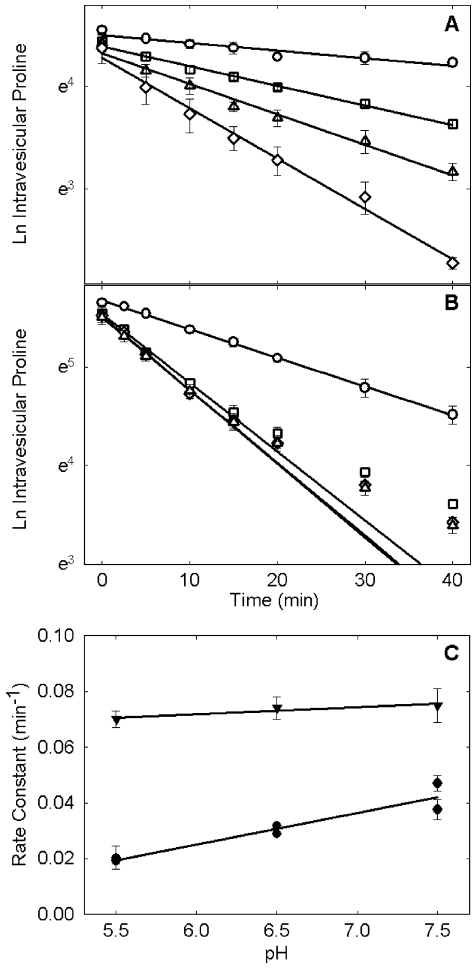

FIGURE 5: pH dependence of unenergized proline efflux and exchange via ProP. KMVs were prepared in 0.1 M K phosphate (pH 6.6); they were loaded with [^{14}C]proline in the presence of D-lactate and sucrose, and proline efflux and exchange were assessed as described in Experimental Procedures. The plots show the natural log of the residual intravesicular proline as a function of time after dilution into proline-free medium to measure efflux (A) or into proline-supplemented medium to measure exchange (B), and the resulting rate constants as a function of pH (C). Efflux rate constants were estimated by regression over the entire 40 min period shown, whereas exchange rate constants were estimated over 0−15 min (the linear range) as described in Experimental Procedures. (A) To measure proline efflux, the [^{14}C]proline-loaded MVs were diluted into isotonic 0.1 M K phosphate at pH 5.5 (□), 6.5 (△), or 7.5 (◇) with CCCP (10 μM) or at pH 6.5 with D-lactate (20 mM) and no CCCP (○). (B) To measure proline exchange, the MVs were treated as described for panel A except that the dilution buffers also contained nonradioactive proline (10 mM). (C) Efflux (●) and exchange (▼) rate constants determined as described for panels A and B, respectively, are plotted vs pH.

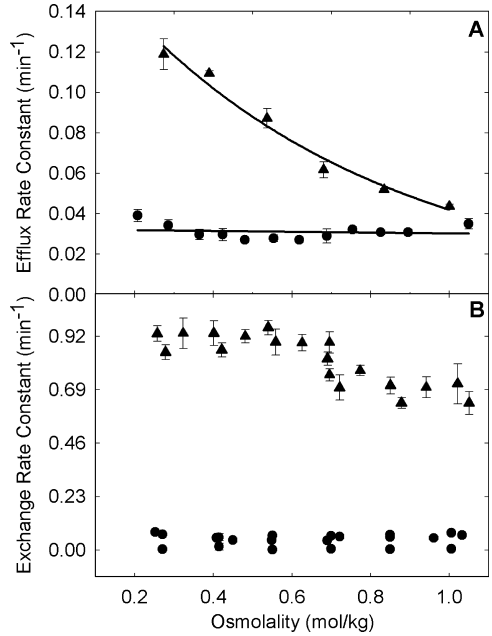

FIGURE 6: Osmolality dependence of unenergized proline and lactose fluxes. KMVs were prepared in 0.1 M K phosphate (pH 6.6), and they were loaded with [^{14}C]proline or [^{14}C]lactose in the presence of D-lactate with or without sucrose as described in Experimental Procedures. Solute efflux and exchange were assessed as described in Experimental Procedures, via dilution of the loaded MVs into 0.1 M K phosphate (pH 6.6) supplemented with sucrose to produce the indicated osmolality and CCCP (10 μM) (solute efflux) or CCCP (10 μM) with nonradioactive proline or lactose (10 mM) (solute exchange). (A) Proline efflux rate constants (●) and lactose efflux rate constants (▲) are plotted vs efflux medium osmolality. (B) Proline exchange rate constants (●) and lactose exchange rate constants (▲) are plotted vs exchange medium osmolality. To obtain the proline efflux data shown in Figure 5A, MVs were loaded with [^{14}C]proline in medium adjusted to 0.97, 0.72, or 0.48 mol/kg and diluted into medium of the same or slightly lower osmolality. Analogous data were obtained when the loading and efflux buffers were isosmolal. Proline exchange was assessed using isosmolal loading and exchange buffers. Proline loading at osmolalities of <0.5 mol/kg yielded initial intravesicular proline levels of <68 nmol/mg of protein (see the text). MVs were loaded with [^{14}C]lactose using a 0.25 mol/kg buffer and then diluted into buffers of the same or higher osmolality to measure efflux or exchange. Rate constants were determined by regression analysis as described in Experimental Procedures and illustrated in Figures 4 and 5 and then plotted vs efflux or exchange medium osmolality.

accumulation of proline in the steady state (*33*). In addition, the initial rate of proline uptake into PRLs via ProP-His_6 increased with ΔpH at constant $\Delta\Psi$ and with $\Delta\Psi$ at constant ΔpH (*24*). Since energized proline uptake was osmolality-dependent in MVs (Figures 2 and 3) but unenergized proline fluxes were not (Figure 6), we used PRLs to further elucidate the roles of $\Delta\Psi$ and ΔpH in osmosensing and H^+-solute symport. As the magnitude of $\Delta\Psi$ was systematically reduced from −137 to 0 mV by supplementing the transport assay medium with K^+, the maximum activity attained by ProP-His_6 fell to zero (despite a constant ΔpH of 59 mV) (Figure 7). This is consistent with our earlier observations (*24*). As $\Delta\Psi$ fell from −137 to 0 mV, ProP-His_6 attained its maximum activity at a lower osmolality ($\Delta\Psi$ = −120 mV), then became osmolality-independent ($\Delta\Psi$ = −100 mV), and finally became inactive ($\Delta\Psi$ = 0 mV). Thus, ProP appears to progress from an off to an on and then an osmolality-sensitive state as the membrane potential increases in magnitude from zero to approximately −100 mV and above. The pH gradient did not contribute to H^+-proline symport unless $\Delta\Psi$ exceeded −100 mV because ProP was in its off state.

Membrane Orientation of ProP-His_6 in PRLs. The membrane orientation of ProP-His_6 in PRLs was determined to further validate their use in this study and the interpretation of the resulting data. The N- and C-termini of ProP are cytoplasmic in cells (*12*). This was shown in part by covalently labeling ProP* variants containing single Cys residues with fluorescent, thiol-reactive probe OGM. ProP variants with Cys only at a position in the N- or C-terminal hydrophilic domain (including N-terminal position 16) or in a predicted cytoplasmic loop could be labeled only if

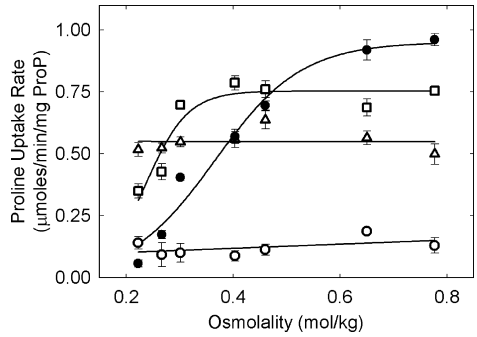

FIGURE 7: Impacts of $\Delta\Psi$ on the osmotic activation of ProP-His_6 in PRLs. The impact of osmolality on ProP-His_6 activity in PRLs was determined as described by Racher et al. (*24*) with ΔpH fixed at 1 pH unit (−59 mV) as $\Delta\Psi$ varied from 0 to −137 mV. $\Delta\Psi$ was adjusted by adding K^+ to the uptake assay buffers, taking the progressive concentration of K^+ in the PRL lumen due to osmotic shrinkage into account (*24*). The initial rate of proline uptake via ProP-His_6 is plotted vs osmolality, with $\Delta\Psi$ at −137 (●), −120 (□), −100 (△) and 0 mV (○).

cytoplasmic membranes were isolated and permeabilized when OGM was added (*12, 25*). This labeling occurred despite pretreatment of the intact bacteria with nonfluorescent thiol-blocking reagent MTSET. In contrast, Cys residues in predicted periplasmic loops were readily labeled in intact cells. The same approach was used to determine the orientation of ProP*-T16C in PRLs. Negligible fluorescence was associated with ProP*-T16C when intact PRLs were treated with OGM (Figure 8A, lane 1). However, fluorescence was detected when OGM was added and allowed to penetrate the PRLs by freeze−thaw−extrusion treatments, with or without pretreatment of the intact PRLs with MTSET (Figure 8A, lanes 2 and 3, respectively). Thus, the hydrophilic N-terminal domain is sequestered in the lumen of PRLs.

Antibodies raised against full-length ProP (anti-ProP) (*23*) or a peptide replicating its C-terminus (anti-C) (*34*) were used to further test the orientation of ProP-His_6 in PRLs. Anti-C antibodies sedimented with PRLs after they were enclosed in the PRL lumen by freezing, thawing, and extrusion (Figure 8B, top panel, lanes 3−5) but not after incubation with intact liposomes or PRLs (Figure 8B, top panel, lanes 1 and 2). To ensure that these studies detected binding to ProP and not just enclosure in the PRL lumen, PRLs and liposomes were subjected to two to six freeze−thaw−extrusion cycles after the initial freeze−thaw−extrusion process and incubation, thereby allowing antibody that was enclosed but not bound to equilibrate with the external medium. After the fourth cycle, no antibodies could be detected in protein-free liposomes [in Figure 8B, top panel, compare lane 6 (two cycles) with lanes 7 and 8 (four and six cycles)], whereas in ProP-containing PRLs, the amount of antibodies decreased but antibodies remained even after six washes (Figure 8B, top panel, lanes 3−5). In analogous experiments, anti-ProP sedimented with PRLs that had or had not been subjected to the freeze−thaw−extrusion process (Figure 8B, bottom panel, lanes 3−5 and lane 2, respectively). This indicated that the C-terminal domain of ProP-His_6 was accessible from only the lumenal side. Both site-directed fluorescence labeling and immunolabeling showed that ProP is integrated in a right-side-out orientation with the N- and C-termini facing the lumen of proteoliposomes.

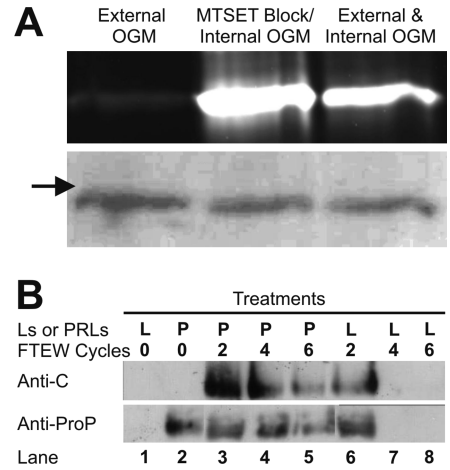

FIGURE 8: Orientation of ProP in PRLs. Site-directed OGM labeling and an immunoassay were used to show that the N- and C-termini of ProP-His_6 are sequestered in the PRL lumen. (A) Cys16 was labeled with OGM after purification of variant ProP*-T16C and its reconstitution in PRLs (see Experimental Procedures). This transporter retained activity after purification and reconstitution [$A_{max} = 1.34 \pm 0.14$ μmol min^{-1} (mg of ProP-His_6)$^{-1}$, and $\Pi_{1/2}/RT = 0.377 \pm 0.003$ mol/kg] (see eq 1). The top panels show fluorescence and the bottom panels Coomassie Blue staining of this protein in the same SDS−PAGE gels. In lane 1, PRLs were labeled externally with OGM. In lane 2, PRLs were blocked externally with MTSET, and then OGM was added and introduced into the PRL lumen by freezing, thawing, and extrusion. In lane 3, OGM was added and introduced into the PRL lumen by freezing, thawing, and extrusion, with no prior MTSET blocking. The position of the 50 kDa protein standard is indicated by the arrow. (B) Liposomes (L) and PRLs (P) were incubated with the indicated antibodies either directly to detect the external accessibility of the C-terminal domain (lanes 1 and 2) or after the antibodies were enclosed within the proteoliposomes (lanes 3−5) or liposomes (lanes 6−8) to determine accessibility of ProP domains from the lumenal side (see Experimental Procedures). In the latter case, the freeze−thaw−extrusion−wash cycle (FTEW) was repeated two, four, or six times to ensure release of entrapped but not bound antibodies from the PRLs or liposomes.

DISCUSSION

This study showed that ProP and LacY activities are similarly K^+-dependent in MVs (Figure 3). These effects may reflect the K^+ dependence of respiration in this system. Houssin et al. reported that respiration and lactose transport via LacY in *E. coli* cells were inhibited as salinity increased, yet $\Delta\mu_{H^+}$ remained remarkably unaffected (*16*). Meury found that respiration recovered as K^+ accumulated after an osmotic upshock (a sudden increase in osmotic pressure) (*2*). He also explored the same phenomena with bacteria lacking K^+ transporters Kdp and Trk. For those cells, K^+ could accumulate and respiration could be restored after an osmotic upshock, but only if the exogenous K^+ concentration was very high (0.6 M) (*2*). In the absence of Kdp and Trk, the required K^+ may have been supplied by residual K^+ transporter Kup (*39*).

Transporters Kdp and Trk were not active in the MVs used for this study. ATP, an essential substrate for P-type ATPase Kdp, is not present in MVs unless an ATP-generating system is provided. Respiration would be expected to support $\Delta\mu_{H^+}$-dependent Trk activity in MVs. However Trk activity was not detected when Kdp activity was restored to *E. coli* MVs by providing an ATP-generating system (ref *40* and personal communication with K. Altendorf). Peripheral membrane

component TrkA may be lost during MV preparation, or Trk may be off in MVs because regulatory nucleotides are not available (*39*). Respiration-dependent transport (e.g., via LacY and ProP) is observed in KMVs (e.g., Figure 2) so the K^+ present in these preparations must meet the K^+ requirement for respiration, even in the absence of Kdp and Trk activities. However, NaMVs may behave like K^+ uptake-deficient bacteria. The K^+ requirements for ProP and LacY may arise indirectly from a K^+ requirement for respiration and $\Delta\mu_{H^+}$ generation.

This study shows that H^+-linked lactose fluxes via LacY slow as osmolality increases [Figures 2 and 6A (▲)], whereas lactose exchange, occurring under conditions that do not require coupled H^+ translocation, is essentially osmolality-independent [Figure 6B (▲)]. Thus, water may be directly involved in a rate-determining step of H^+ translocation via LacY. ProP and LacY likely share an alternating access mechanism in which two helix bundles rock around an axis in the membrane plane, opening the substrate binding site sequentially to the periplasm and cytoplasm (*13, 41*). H^+-linked lactose transport is known to involve a cluster of acidic and basic residues located within the C-terminal helix bundle close to the N-terminal bundle of LacY. H^+ translocation might involve associated water molecules, though none have yet been visualized (*42*). Water molecules are known or believed to play key roles in other H^+ transport processes (*43*). The arrival and departure of water molecules are evident in structures representing the photocycle (and H^+ transport cycle) intermediates of light-driven H^+ pump bacteriorhodopsin (*44, 45*), which is also osmotic pressure-sensitive (*46*). Thus, data suggest that increasing osmotic pressure (decreasing water activity) may inhibit LacY and other proteins by decreasing the availability of water molecules.

This study revealed that $\Delta\Psi$ contributes in two ways to ProP activity: as a component of $\Delta\mu_{H^+}$, the thermodynamic driving force for solute uptake, and by converting ProP from an inactive (off) to an active (on) and then to an osmolality-sensitive (OS) state (Figure 7). The role of $\Delta\Psi$ as a driving force for transport, reported previously (*24*), is illustrated by the increasing amplitude of the curves in Figure 7 with increasing $\Delta\Psi$. The $\Delta\Psi$ dependence of osmosensing by ProP is evident in the impact of $\Delta\Psi$ on the curve shapes. We postulate that the following equilibria link the off, on, and OS states of ProP:

$$ProP_{off} \leftrightarrow ProP_{on} \leftrightarrow ProP_{OS} \quad (2)$$

Most ProP molecules would assume conformations associated with the off and OS states in the absence of $\Delta\Psi$ and at -137 mV, respectively. The on, osmolality-insensitive state (evident in PRLs at a $\Delta\Psi$ of -100 mV, white triangles in Figure 7) could represent a distinct conformer that mediates osmolality-insensitive transport (as suggested by eq 2), or it could constitute a mixture of transporters that are on and osmotically inhibited (like LacY) or OS (like ProP at high $\Delta\Psi$). Since $\Delta\Psi$ is required for ProP activation, proline efflux and exchange from unenergized MVs must represent residual activity of the transporter in its off conformation, or the activity of a small fraction of the transporter population that is on in the absence of $\Delta\Psi$. The fact that those reactions are osmolality-insensitive (Figure 6) implies that a small proportion of an on, osmolality-insensitive conformer exists when $\Delta\Psi$ is zero and predominates as $\Delta\Psi$ approaches -100 mV. Notably, ProP activity at low osmolality is suppressed as ProP activity at high osmolality continues to increase when the magnitude of $\Delta\Psi$ exceeds 120 mV. The osmolality at which ProP activity is half-maximal ($\Pi_{1/2}/RT$) also increases with $\Delta\Psi$ in that range (Figure 7), perhaps reflecting the increasing proportion of $ProP_{OS}$ at the expense of $ProP_{on}$. In contrast to its effects on LacY and other H^+ translocators, dehydration may be required to remove excess water molecules from ProP and create a pathway for tightly coupled, unidirectional H^+ translocation in response to $\Delta\mu_{H^+}$.

The structural features responsible for the $\Delta\Psi$- and osmolality-dependent behavior of ProP are not yet known, but candidates can be identified on the basis of our ProP model and experimental data (*12*). As noted above, ionizable residues implicated in H^+ translocation and coupling to lactose transport fall within transmembrane helices VII, VIII, IX, and X in the C-terminal helix bundle of LacY (*42*). Residues that are both ionizable and oriented toward the protein interior occur in the N-terminal but not the C-terminal helix bundle of ProP (*12*). Experiments that are in progress are testing the specific roles of these residues in H^+ translocation, proline transport, and osmosensing.

ProP orthologues fall into two groups on the basis of the sequences of their cytoplasmic C-termini (*18, 47*). All orthologues examined to date possess extended C-termini relative to those of the most similar paralogues that are not osmosensors or osmoregulators (*47*). Members of the group represented by *E. coli* ProP terminate in series of heptad repeats characteristic of α-helical coiled-coil-forming proteins, whereas the others have shorter C-terminal extensions without heptad repeats (*47*). Peptides replicating the putative coiled-coil sequences form antiparallel, homodimeric coiled-coil structures in vitro (*23, 48–50*). ProP dimerizes, and antiparallel coiled-coil structures also form in vivo (*50–52*). Orthologues with the antiparallel coiled coil activate at lower osmolalities than orthologues and variants without it (*47, 50*). These and other data suggest that the coiled coil adjusts the transporter's osmolality response to a lower range and that it is not the osmosensor, per se. Increasing $\Delta\Psi$ could elevate the osmolality at which ProP activates by favoring ProP molecules that are not linked by coiled coils over coiled-coil-containing ProP dimers.

Tran and Unden reported that $\Delta\Psi$ fell only from -140 to -105 mV as *E. coli* K-12 cells were cultivated in MOPS medium with various electron donor/acceptor pairs or fermentatively on glucose (*53*). Thus, ProP would serve as an osmosensory transporter under these diverse conditions of active growth, but it would contribute only weak, osmolality-insensitive uptake of proline or other osmolytes if $\Delta\Psi$ fell to lower magnitudes. The task of osmoprotectant uptake could then fall to ABC transporter ProU or Na^+-solute symporter BetT or BetU, if the ATP supply or Na^+ motive force were sustained.

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REFERENCES

1. Wood, J. M. (1999) Osmosensing by Bacteria: Signals and Membrane-Based Sensors. *Microbiol. Mol. Biol. Rev. 63*, 230–262.
2. Meury, J. (1994) Immediate and transient inhibition of the respiration of *Escherichia coli* under hyperosmotic shock. *FEMS Microbiol. Lett. 121*, 281–286.
3. Meury, J., Robin, A., and Monnier-Champeix, P. (1985) Turgor-controlled K^+ fluxes and their pathways in *Escherichia coli*. *Eur. J. Biochem. 151*, 613–619.
4. Ding, Q., Kusano, S., Villarejo, M., and Ishihama, A. (1995) Promoter selectivity control of *Escherichia coli* RNA polymerase by ionic strength: Differential recognition of osmoregulated promoters by E sigma D and E sigma S holoenzymes. *Mol. Microbiol. 16*, 649–656.
5. Gralla, J. D., and Vargas, J. R. (2006) Potassium glutamate as a transcriptional inhibitor during bacterial osmoregulation. *EMBO J. 25*, 1515–1521.
6. Lee, S. J., and Gralla, J. D. (2004) Osmo-regulation of bacterial transcription via poised RNA polymerase. *Mol. Cell 14*, 153–162.
7. Rosenthal, A. Z., Hu, M., and Gralla, J. D. (2006) Osmolyte-induced transcription:−35 region elements and recognition by sigma38 (rpoS). *Mol. Microbiol. 59*, 1052–1061.
8. Dinnbier, U., Limpinsel, E., Schmid, R., and Bakker, E. P. (1988) Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. *Arch. Microbiol. 150*, 348–357.
9. Meury, J. (1988) Glycine betaine reverses the effects of osmotic stress on DNA replication and cellular division in *Escherichia coli*. *Arch. Microbiol. 149*, 232–239.
10. Ly, A., Henderson, J., Lu, A., Culham, D. E., and Wood, J. M. (2004) The osmoregulatory systems of *Escherichia coli*: Identification of BCCT family member BetU and distributions of betU and trkG among pathogenic and non-pathogenic isolates. *J. Bacteriol. 186*, 296–306.
11. MacMillan, S. V., Alexander, D. A., Culham, D. E., Kunte, H. J., Marshall, E. V., Rochon, D., and Wood, J. M. (1999) The ion coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*. *Biochim. Biophys. Acta 1420*, 30–44.
12. Wood, J. M., Culham, D. E., Hillar, A., Vernikovska, Ya. I., Liu, F., Boggs, J. M., and Keates, R. A. B. (2005) Structural model for the osmosensor, transporter, and osmoregulator ProP of *Escherichia coli*. *Biochemistry 44*, 5634–5646.
13. Guan, L., and Kaback, H. R. (2006) Lessons from Lactose Permease. *Annu. Rev. Biophys. Biomol. Struct. 35*, 67–91.
14. Milner, J. L., Grothe, S., and Wood, J. M. (1988) Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. *J. Biol. Chem. 263*, 14900–14905.
15. Culham, D. E., Henderson, J., Crane, R. A., and Wood, J. M. (2003) Osmosensor ProP of *Escherichia coli* responds to the concentration, chemistry and molecular size of osmolytes in the proteoliposome lumen. *Biochemistry 42*, 410–420.
16. Houssin, C., Eynard, N., Shechter, E., and Ghazi, A. (1991) Effect of osmotic pressure on membrane energy-linked functions in *Escherichia coli*. *Biochim. Biophys. Acta 1056*, 76–84.
17. Krämer, R., and Morbach, S. (2004) BetP of *Corynebacterium glutamicum*, a transporter with three different functions: Betaine transport, osmosensing, and osmoregulation. *Biochim. Biophys. Acta 1658*, 31–36.
18. Poolman, B., Spitzer, J. J., and Wood, J. M. (2004) Bacterial Osmosensing: Roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions. *Biochim. Biophys. Acta 1666*, 88–104.
19. Koo, S.-P., Higgins, C. F., and Booth, I. R. (1991) Regulation of compatible solute accumulation in *Salmonella typhimurium*: Evidence for a glycine betaine efflux system. *J. Gen. Microbiol. 137*, 2617–2625.
20. Kaback, H. R. (1971) Bacterial membranes. *Methods Enzymol. 22*, 99–120.
21. Wood, J. M. (2007) Bacterial Osmosensing Transporters. *Methods Enzymol. 428*, 77–107.
22. Culham, D. E., Lasby, B., Marangoni, A. G., Milner, J. L., Steer, B. A., van Nues, R. W., and Wood, J. M. (1993) Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. *J. Mol. Biol. 229*, 268–276.
23. Culham, D. E., Tripet, B., Racher, K. I., Voegele, R. T., Hodges, R. S., and Wood, J. M. (2000) The role of the carboxyl terminal α-helical coiled-coil domain in osmosensing by transporter ProP of *Escherichia coli*. *J. Mol. Recognit. 13*, 1–14.
24. Racher, K. I., Culham, D. E., and Wood, J. M. (2001) Requirements for osmosensing and osmotic activation of transporter ProP from *Escherichia coli*. *Biochemistry 40*, 7324–7333.
25. Culham, D. E., Hillar, A., Henderson, J., Ly, A., Vernikovska, Ya. I., Racher, K. I., Boggs, J. M., and Wood, J. M. (2003) Creation of a fully functional, cysteine-less variant of osmosensor and proton-osmoprotectant symporter ProP from *Escherichia coli* and its application to assess the transporter's membrane orientation. *Biochemistry 42*, 11815–11823.
26. Guzman, L.-M., Belin, D., Carson, M. J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol. 177*, 4121–4130.
27. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.
28. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) Culture medium for enterobacteria. *J. Bacteriol. 119*, 736–747.
29. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150*, 76–85.
30. Blancato, V. S., Magno, C., and Lolkema, J. S. (2006) Functional characterization and Me ion specificity of a Ca-citrate transporter from *Enterococcus faecalis*. *FEBS J. 273*, 5121–5130.
31. Dayem, M. A., Basquin, C., Pourcher, T., Cordat, E., and LeBlanc, G. (2003) Cytoplasmic loop connecting helices IV and V of the melibiose permease from *Escherichia coli* is involved in the process of Na^+-coupled sugar translocation. *J. Biol. Chem. 278*, 1518–1524.
32. Kaczorowski, G. J., and Kaback, H. R. (1979) Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. I. Effect of pH on efflux, exchange and counterflow. *Biochemistry 18*, 3691–3697.
33. 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., Henderson, P. J. F., White, G., and Hallett, F. R. (1999) Purification and reconstitution of an osmosensor: Transporter ProP of *Escherichia coli* senses and responds to osmotic shifts. *Biochemistry 38*, 1676–1684.
34. Culham, D. E., Emmerson, K. S., Lasby, B., Mamelak, D., Steer, B. A., Gyles, C. L., Villarejo, M., and Wood, J. M. (1994) Genes encoding osmoregulatory proline/glycine betaine transporters and the proline catabolic system are present and expressed in diverse clinical *Escherichia coli* isolates. *Can. J. Microbiol. 40*, 397–402.
35. Schiller, D., Rübenhagen, R., Krämer, R., and Morbach, S. (2004) The C-terminal domain of the betaine carrier BetP of *Corynebacterium glutamicum* is directly involved in sensing K^+ as an osmotic stimulus. *Biochemistry 43*, 5583–5591.
36. Schuldiner, S., Kerwar, G. K., Kaback, H. R., and Weil, R. (1975) Energy-dependent binding of dansylgalactosides to the β-galactoside carrier protein. *J. Biol. Chem. 250*, 1370.
37. Persson, B., Roepe, P. D., Patel, L., Lee, J., and Kaback, H. R. (1992) Site-directed mutagenesis of lysine 319 in the lactose permease of *Escherichia coli*. *Biochemistry 31*, 8892–8897.
38. Kaczorowski, G. J., Robertson, D. E., and Kaback, H. R. (1979) Mechanism of lactose translocation in membrane vesicles from *Escherichia coli*. 2. Effect of imposed $\Delta\Psi$, ΔpH and $\Delta\mu_{H+}$. *Biochemistry 18*, 3697–3704.
39. Stumpe, S., Schlösser, A., Schleyer, M., and Bakker, E. P. (1996) In *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) pp 473−499, Elsevier Science BV, Amsterdam.
40. Kollman, R., and Altendorf, K. (1993) ATP-driven potassium transport in right-side-out membrane vesicles via the Kdp system of *Escherichia coli*. *Biochim. Biophys. Acta 1143*, 62–66.
41. Kaback, H. R., Dunten, R., Frillingos, S., Venkatesan, P., Kwaw, I., Zhang, W., and Ermolova, N. (2007) Site-directed alkylation and the alternating access model for LacY. *Proc. Natl. Acad. Sci. U.S.A. 104*, 491–494.

42. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) Structure and mechanism of the lactose permease of *Escherichia coli*. *Science 301*, 610–615.
43. Wraight, C. A. (2006) Chance and design-proton transfer in water, channels and bioenergetic proteins. *Biochim. Biophys. Acta 1757*, 886–912.
44. Lanyi, J. K. (2006) Proton transfers in the bacteriorhodopsin photocycle. *Biochim. Biophys. Acta 1757*, 1012–1018.
45. Lanyi, J. K. (2004) Bacteriorhodopsin. *Annu. Rev. Physiol. 66*, 665–688.
46. Cao, Y., Varo, G., Chang, M., Ni, B., Needleman, R., and Lanyi, J. K. (1991) Water is required for proton transfer from aspartate-96 to the bacteriorhodopsin Schiff base. *Biochemistry 30*, 10972–10979.
47. Tsatskis, Y., Khambati, J., Dobson, M., Bogdanov, M., Dowhan, W., and Wood, J. M. (2005) The osmotic activation of transporter ProP is tuned by both its C-terminal coiled-coil and osmotically induced changes in phospholipid composition. *J. Biol. Chem. 280*, 41387–41394.
48. Hillar, A., Tripet, B., Zoetewey, D., Wood, J. M., Hodges, R. S., and Boggs, J. M. (2003) Detection of α-helical coiled-coil dimer formation by spin-labeled synthetic peptides: A model parallel coiled-coil peptide and the antiparallel coiled-coil formed by a replica of the ProP C-terminus. *Biochemistry 42*, 15170–15178.
49. Zoetewey, D. L., Tripet, B. P., Kutateladze, T. G., Overduin, M. J., Wood, J. M., and Hodges, R. S. (2003) Solution structure of the C-terminal antiparallel coiled-coil domain from *Escherichia coli* osmosensor ProP. *J. Mol. Biol. 334*, 1063–1076.
50. Tsatskis, Y., Kwok, S. C., Becker, E., Gill, C., Smith, M. N., Stalker, L., Hodges, R. S., and Wood, J. M. (2008) Coiled-coil orientation switching places osmosensory transporter ProP in an osmolality-refractory state. *Biochemistry 47*, 60–72.
51. Liu, F., Culham, D. E., Vernikovska, Ya. I., Keates, R. A. B., Boggs, J. M., and Wood, J. M. (2007) Structure and function of the XIIth transmembrane segment in osmosensor and osmoprotectant transporter ProP of *Escherichia coli*. *Biochemistry 46*, 5647–5655.
52. Hillar, A., Culham, D. E., Vernikovska, Ya. I., Wood, J. M., and Boggs, J. M. (2005) Formation of an antiparallel, intermolecular coiled-coil is associated with in vivo dimerization of osmosensor and osmoprotectant transporter ProP in *Escherichia coli*. *Biochemistry 44*, 10170–10180.
53. Tran, Q. H., and Unden, G. (1998) Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. *Eur. J. Biochem. 251*, 538–543.

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