Osmosensor ProP of *Escherichia coli* Responds to the Concentration, Chemistry, and Molecular Size of Osmolytes in the Proteoliposome Lumen[†,‡]

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ABSTRACT: Transporter ProP of *Escherichia coli* mediates the cellular accumulation of organic zwitterions in response to increased extracellular osmolality. We compared and characterized the osmoregulation of ProP activity in cells and proteoliposomes to define the osmotic shift-induced cellular change(s) to which ProP responds. ProP−$(His)_6$ activity in cells and proteoliposomes was correlated with medium osmolality, not osmotic shift, turgor pressure, or membrane strain. Both K_M and V_{max} for proline uptake via ProP−$(His)_6$ increased with increasing medium osmolality, as would be expected if osmolality controls the proportions of transporter with inactive and active conformations. The osmolality yielding half-maximal ProP−$(His)_6$ activity was higher in proteoliposomes than in cells. The osmolality response of ProP is also attenuated in bacteria lacking soluble protein ProQ. Indeed, the catalytic constant (k_{cat}) for ProP−$(His)_6$ in proteoliposomes approximated that of ProP in intact bacteria lacking ProQ. Thus, the proteoliposome system may replicate a primary osmosensory response that can be further amplified by ProQ. ProP−$(His)_6$ is designated as an osmosensor because its activity is dependent on the osmolality, but not the composition, of the assay medium to which the cell surface is exposed. In contrast, ProP−$(His)_6$ activity was dependent on both the osmolality and the composition of the lumen in osmolyte-loaded proteoliposomes. For proteoliposomes containing inorganic salts, glucose, or poly(ethylene glycol) 503, transporter activity correlated with total lumenal cation concentration. In contrast, for proteoliposomes loaded with larger poly(ethylene glycol)s, the osmolality, the lumenal cation concentration, and the lumenal ionic strength at half-maximal transporter activity decreased systematically with poly(ethylene glycol) radius of gyration (range 0.8−1.8 nm). These data suggest that ProP−$(His)_6$ responds to osmotically induced changes in both cytoplasmic K^+ levels and the concentration of cytoplasmic macromolecules.

Living cells respond to changes in extracellular osmolality by accumulating and releasing K^+ and organic osmolytes (e.g., polyols, betaines) (*1*−*7*). Transporters and mechanosensitive channels mediate the uptake and release of exogenous compounds (osmoprotectants) by bacteria that are subjected to osmotic shifts (*1*, *2*, *8*−*10*). Our goal is to understand how cells sense changes in extracellular osmolality and respond by modulating the activities of osmoregulatory transporters. Identification of the cellular alteration(s) sensed by osmoregulatory systems is difficult since changes in extracellular osmolality may alter many cellular properties (*1*). Recently three osmoregulatory transporters have been purified and reconstituted in artificial membrane systems: transporters ProP from *Escherichia coli* (*11*−*13*), BetP from *Corynebacterium glutamicum* (*14*−*16*), and OpuA from *Lactococcus lactis* (*17*, *18*). Each of these systems is an osmosensor and osmoregulatory transporter that mediates compatible solute accumulation in both cells and proteoliposomes. Recent studies suggest that systems BetP (*15*) and OpuA (*18*) become active when cations (particularly K^+) are concentrated in the cytoplasm of bacteria that have been introduced to high osmolality media.

ProP mediates the accumulation of several structurally related organic solutes (e.g., proline, glycine betaine, ectoine) in response to increased extracellular osmolality (*19*, *20*). It is a proton-solute symporter whose activity requires a membrane potential ($\Delta\Psi$) and is further stimulated by a proton gradient (ΔpH)[1] (*11*, *13*, *20*). Our previous data are consistent with a model in which ProP can exist in at least two states, inactive ($ProP^I$) and active ($ProP^A$) (*13*). Since no change in ProP protein structure has yet been correlated with osmosensing, we measure changes in the catalytic activity of ProP and infer the proportions of $ProP^I$ and $ProP^A$ from the transport rate.

The *proP* sequence predicts a 500 amino acid protein with 12 membrane-spanning domains that belongs to the major facilitator superfamily of transporter proteins (*21*). Both *proP* expression and ProP activity are osmoregulated (*22*). Defects in the unlinked locus *proQ* dramatically attenuate the osmoregulation of ProP activity in intact cells without influencing *proP* expression or ProP protein levels (*23*−*25*).

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[‡] This paper is dedicated to Dr. Karlheinz Altendorf on the occasion of his 60th birthday.

[1] Abbreviations: Π, osmotic pressure; $\Delta\Pi$, osmotic shift; $\Delta\Psi$, membrane potential, MOPS, 4-morpholinopropanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; PEG, poly(ethylene glycol).

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Since the activity of pure ProP−$(His)_6$ in proteoliposomes is osmoregulated, ProQ cannot be essential to that response. To focus on the osmoregulation of transporter activity, we constructed a system in which a ProP variant with a C-terminal histidine tag (ProP−$(His)_6$) is expressed in an osmolality-independent manner from the AraC-controlled P_{BAD} promoter. Bacteria cultivated in the absence of arabinose express ProP−$(His)_6$ at levels comparable to those in which *proP* is expressed from its own promoter in medium of moderate osmolality (*12*). Induction with arabinose yields high levels of ProP−$(His)_6$ for purification and reconstitution in proteoliposomes (*11*). This paper describes the use of such cells and proteoliposomes to compare the influence of various perturbations on ProP activity, in vivo and in vitro.

The experiments reported herein address the roles of ProQ, osmotic shifts versus osmolality, per se, turgor pressure mechanical deformation of the membrane, the osmolalities, and compositions of the media external and internal to cells and proteoliposomes in osmosensing by ProP−$(His)_6$. We conclude that the catalytic constant (k_{cat}) for ProP in proteolipsomes corresponds with that measured in *proQ*$^-$ bacteria and that osmolality determines the activity of sensor/transporter ProP by modulating the composition of the medium bathing the cytoplasmic (intact cells) or lumenal (proteoliposomes) surface of the membrane and/or the transporter. In contrast to the behavior of systems BetP and OpuA, we find ProP activity to be determined by the lumenal concentrations of both inorganic and organic solutes. The impact of lumenal organic solutes on activity is proportional to their molecular size and may simulate a role of cytoplasmic macromolecules in the osmoregulation of ProP activity in vivo. If that interpretation is correct, ProP activity would be determined by the concentration of cations (physiologically, K^+) and macromolecules in the bacterial cytoplasm.

EXPERIMENTAL PROCEDURES

Materials. E. coli phospholipids (polar lipid extract, acetone/ether washed) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Dodecyl β-D-maltoside and Triton X-100 were purchased from Anatrace (Maumee, OH). N−Octyl-1-β-D-glucoside was purchased from Calbiochem (San Diego, CA). Mixed high molecular weight poly(ethylene glycol)'s were purchased from Fluka (Oakville, ON). Monodisperse poly(ethylene glycol)'s, prepared by Polypure AS (Oslo, Norway), were purchased from LCC Engineering & Trading GmbH (Egerkingen, Switzerland). Calcein was purchased from Sigma (Oakville, ON). Other reagents were of the highest grade available. Buffers were prepared as described by Racher et al. (*13*), poly(ethylene glycol) solutions were prepared by dissolving poly(ethylene glycol)s in the specified buffers to attain the indicated osmolalities, and solution osmolalities were measured with a Wescor vapor pressure osmometer (Wescor, Logan, UT).

Bacteria and Culture Media. ProP−$(His)_6$ was expressed by *E. coli* strain WG710, a derivative of *E. coli* WG350 (F$^-$ *trp lacZ rpsL thi* Δ(*putPA*)*101* Δ(*proU*)*600* Δ(*proP-melAB*)*212*)(*21*) which contains plasmid pDC80 (a derivative of vector pBAD24 (*26*) in which expression of *proP−(His)*$_6$ is controlled by the arabinose-inducible P_{BAD} promoter (*12*)). *E. coli* strain RM2 is F$^-$ *trp rpsL thi lacZ* Δ*(putPA)101* (*27*) and strain WG174 is RM2 *proQ220*::Tn*5* (*23*, *24*). Bacteria were cultivated in LB (*28*) or in defined media based on NaCl-free MOPS medium, a variant of the MOPS medium described by Neidhardt et al. (*29*) from which all NaCl had been omitted. This base medium was supplemented with NH_4Cl (9.5 mM) as nitrogen source, glycerol (0.4% (v/v) as carbon source, L-tryptophan (245 μM) and thiamine hydrocholoride (1 μg/mL) to meet auxotrophic requirements, and NaCl as required to adjust the osmolality. NaCl-free MOPS medium and complete MOPS medium (*29*) had osmolalities of 0.12 and 0.21 mol/kg, respectively.

Transport Assays: Intact Bacteria. Bacteria were grown in LB medium at 37 °C for approximately 7 h, subcultured into fresh NaCl-free MOPS medium, and incubated for 16−18 h with rotary shaking (200 rpm) at 37 °C. They 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 0.9, harvested by centrifugation, resuspended in unsupplemented MOPS medium (NaCl-free MOPS medium devoid of organic supplements), and maintained at room temperature for no longer than 4 h prior to the transport assay. Replacement of standard MOPS medium (*29*) (used previously by this laboratory) with NaCl-free MOPS medium during this procedure did not alter the growth of *E. coli* WG710 (data not shown). However, this change reduced the (otherwise arbitrary) lower limit of the growth medium osmolality and allowed us to view the full titration curve for osmotic activation of ProP (see Results).

The standard transport assay medium was unsupplemented MOPS medium plus D-glucose (10 mM), chloramphenicol (0.09 μg/mL), and NaCl as desired to adjust the osmolality. Bacteria were introduced and the mixture was preincubated for 3 min at 25 °C with agitation. Transport was initiated by adding substrate L-[^{14}C] proline to a final concentration of 200 μM (unless otherwise indicated), creating a 500 μL assay mixture. The bacteria were isolated by filtration from 150 μL portions of the assay mixture 20, 40, and 60 s later. Each filter was washed once with 5 mL of unsupplemented MOPS medium to which NaCl had been added to render the assay and wash media isotonic. Filters were dried and counted as previously described (*30*). All assays were done in triplicate, and all experiments were performed at least twice. Each set of replicate assays was used to determine the rate of amino acid uptake over the 20−60 s interval. The rates are cited as the mean ± standard deviation. Protein concentrations were determined by the bicinchoninic acid assay (*31*) using the BCA Kit from Pierce (Rockford, IL) with bovine serum albumin (BSA) as standard. Levels of ProP or ProP−$(His)_6$ were determined by Western Blotting as previously described (*12*).

Transport Assays. Proteoliposomes. Proteoliposomes containing ProP−$(His)_6$ were prepared and proline uptake rates were measured as previously described (*11, 13*). Solvent additives (salts, D-glucose or poly(ethylene glycol)s) were introduced to the proteoliposome lumen by extrusion in the presence of additive during the final preparative step. The imposed membrane potential was clamped at −136 mV by adding K^+ to the transport assay medium. This manipulation was based on the assumption that the proteoliposomes responded as ideal osmometers to medium osmolality,

concentrating lumenal K^+ (see ref *13*). Assay medium osmolality was adjusted with D-glucose unless otherwise stated. The permeability of *E. coli* lipid liposomes (and hence proteoliposomes) was measured using calcein fluorescence as previously described (*13*).

Calculations. The following procedures were used to obtain data reported in Figures 2−6 and Tables 1−4. The initial PEG concentration of each proteoliposome preparation ($[PEG]_0$, g/L) was determined experimentally. The corresponding osmolality (Π_0/RT) and the osmolalities of transport assay media (Π/RT) were measured. The osmolality yielding half-maximal ProP−$(His)_6$ activity ($\Pi_{1/2}/RT$) was determined by fitting the initial rate of proline uptake via ProP−$(His)_6$ (a_0) at the corresponding assay medium osmolality (Π/RT) to eq 1 or 2 as described in the text.

The concentrations of K^+, HPO_4^{2-}, and $H_2PO_4^-$ in 0.1 M potassium phosphate, pH 7.4, were determined to be 0.178, 0.078, and 0.022 M, respectively, by titration of 0.1 M K_2HPO_4 with 0.1 M KH_2PO_4 (*13*). The initial cation concentrations (C_0) of the cosolvent loaded proteoliposomes were therefore 0.178 M (potassium phosphate only or potassium phosphate plus D-glucose or PEG) or 0.278 M (potassium phosphate plus NaCl, KCl, LiCl, or CsCl, each at 0.1 M). Since proteoliposomes behave as ideal osmometers, the concentrations of PEGs and cations in the lumen of proteoliposomes subjected to osmotic upshifts could be obtained by multiplying their initial values ($[PEG]_0$ and C_0) by the ratio of the assay medium osmolality and the initial osmolality ($(\Pi/RT)/(\Pi_0/RT)$). The cation concentration yielding half-maximal ProP−$(His)_6$ activity ($C_{1/2}$) was determined by fitting the initial rate of proline uptake via ProP−$(His)_6$ (a_0) at the corresponding lumenal cation concentration (C) to eq 3 as described in the text. Ionic strengths (I) were calculated with the assumption that the proportions of monobasic and dibasic phosphate did not vary significantly over the relevant ionic strength range (*13*).

The fraction of solution volume excluded to K^+ by PEG was calculated as $1/(1 - \Phi)$, where Φ is the fraction of the volume occupied by PEG (estimated as the product of the concentration and the partial specific volume for each PEG). The partial specific volumes (in mL g^{-1}) were 0.839 for PEG503, 0.838 for PEG591, 0.837 for PEG722, 0.836 for PEG1000, and 0.835 for PEG2000. These values were based on the empirical data of Bhat and Timasheff (*32*) with interpolation as necessary.

The Debye length (r_D) was calculated as described by Atkins (*33*), assuming that the dielectric constant of the relevant salt solutions at 20 °C (K_r) was 80. $K_{r,1/2}$ was defined as the dielectric constant of PEG-containing solutions required to render the Debye length in the proteoliposome lumen at half-maximal ProP−$(His)_6$ activity constant for unloaded and PEG-loaded proteoliposomes. It was calculated by multiplying the dielectric constant of water (80) by the ratio of the ionic strengths at half-maximal activity ($I_{1/2}$) for PEG-loaded and unloaded proteoliposomes.

RESULTS

ProP Activity is Determined by Medium Osmolality, not Osmotic Shift. Osmoregulatory transporters such as *E. coli* ProP have traditionally been described as respondents to osmotic upshifts (*1*, *8*, *9*). Yet the rate of ProP activation in intact cells (half-time approximately 1 min (*12*, *25*, *34*)) is slow relative to that of trans-membrane water flux in response to imposed osmotic gradients (e.g., ref *18*). This difference in time scale suggests that the osmotic gradient is dissipated early in the period during which the transporter responds. Furthermore, ProP activity is a function of growth medium osmolality (at constant transporter level) even after osmoadaptation during long-term cultivation of the bacteria (*12*). These observations contradict the view that ProP responds to imposed osmolality gradients.

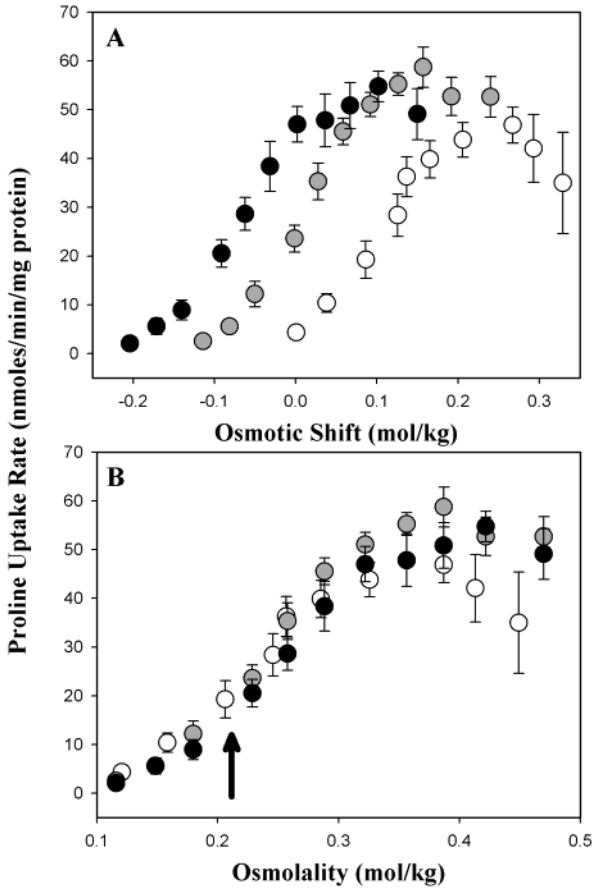

FIGURE 1: ProP senses and responds to osmolality, not osmotic shift. *E. coli* strain WG710 was cultivated, washed, and resuspended in NaCl-free MOPS minimal medium (0.12 mol/kg (open circles)) or in the same medium adjusted with NaCl to attain an osmolality of 0.23 (gray circles) or 0.32 mol/kg (black circles). The arrow in panel B indicates the osmolality of standard MOPS medium (*29*). The initial rate of proline uptake via ProP−$(His)_6$ was measured as described in Methods using assay media adjusted with NaCl to the indicated osmolalities and radiolabeled proline at a concentration of 200 μM. The same data are plotted vs the osmotic shift imposed upon delivery of the bacteria to the transport assay medium (A) and vs the absolute osmolality of that assay medium (B). Each data set was derived from experiments performed on two different days, and the parameters obtained by fitting the data to eq 1 are summarized in Table 1. The levels of ProP−$(His)_6$ in these bacteria were the same (data not shown).

To directly assess whether ProP responds to osmotic shifts or to osmolality, per se, *E. coli* strain WG710 was cultivated, harvested, and resuspended in media with three different osmolalities (0.12, 0.23, and 0.32 mol/kg), and the proline uptake rates of those bacteria were measured in media adjusted to a variety of osmolalities in the same range (Figure 1). ProP−$(His)_6$ activity correlated best with assay medium osmolality (Figure 1B), not with the imposed osmotic shift (Figure 1A). Using proteoliposomes, Racher et al. showed

Table 1: Characterization of the Osmolality Response of ProP−$(His)_6$

system			osmolality response parameter[a]		
bacteria or proteoliposomes	growth osmolality[b]	preparation	A_{max}	$\Pi_{1/2}/RT$	B
bacteria	0.12	I, II[c]	0.049 ± 0.002	0.222 ± 0.005	0.044 ± 0.004
bacteria	0.23	III, IV[c]	0.056 ± 0.001	0.235 ± 0.003	0.037 ± 0.002
bacteria	0.32	V, VI[c]	0.060 ± 0.001	0.243 ± 0.003	0.043 ± 0.002
proteoliposomes	N/A	I	1.77 ± 0.12	0.399 ± 0.009	0.034 ± 0.007
proteoliposomes	N/A	II	1.58 ± 0.10	0.380 ± 0.008	0.022 ± 0.006

[a] Proline uptake activity was measured in intact bacteria or proteoliposomes containing ProP−$(His)_6$ as described in Methods using 200 μM proline. Osmolalities of bacterial growth and transport assay media were adjusted with NaCl. Units of the osmolality response parameters are as follows: A_{max}, μmol min^{-1} (mg protein)$^{-1}$; $\Pi_{1/2}/RT$ and B, mol/kg. Values are cited plus or minus the standard deviation. [b] Bacteria were cultivated in MOPS medium supplemented with NaCl to attain the indicated osmolalities (mol/kg). [c] Each of these data sets was derived from two experiments performed with cell cultures prepared on two different days. The primary data are presented in Figure 1.

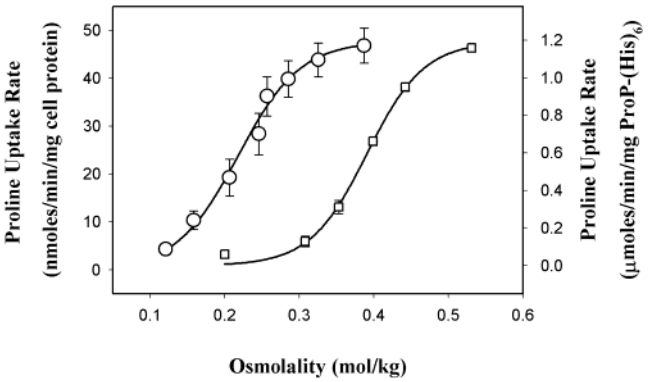

FIGURE 2: The osmotic activation profiles for ProP−$(His)_6$ in cells (open circles) and proteoliposomes (open squares) are similar. *E. coli* strain WG710 was cultivated, washed, and resuspended in NaCl-free MOPS minimal medium which has an osmolality of 0.12 mol/kg. ProP−$(His)_6$ was purified and reconstituted in proteoliposomes as described in Methods. The initial rate of proline uptake via ProP−$(His)_6$ (a_0) was measured as described in Methods using assay media adjusted with NaCl to the indicated osmolalities and radiolabeled proline at a concentration of 200 μM. The data for proteoliposomes are derived from Figure 4 of Racher et al. (*13*). Units for a_0 are nmoles/min/mg cell protein (left axis) or μmol min^{-1} (mg ProP−$(His)_6)^{-1}$ (proteoliposomes, right axis). Regression lines were obtained using eq 1 as the model.

that ProP−$(His)_6$ activity is proportional to assay medium osmolality, and not to the history of imposed osmotic shifts (*13*). These and other data indicate that ProP is not a turgor pressure sensor (see Discussion). As observed previously (*19*, *25*), the rate of proline uptake via ProP−$(His)_6$ declined if bacteria were subjected to very large osmotic shifts and/or very high osmolalities during the transport assay (Figure 1B, osmolalities greater than approximately 0.35 mol/kg^{-1}). Data representing this "inactivation phase" were omitted during further characterization of the transporter response (see below).

Quantitative Characterization of the Osmolality Response of ProP−(His)$_6$ in Cells and Proteoliposomes. The responses of ProP−$(His)_6$ to medium osmolality were qualitatively similar in intact bacteria and in proteoliposomes (Figure 2), fitting the relationship:

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

where Π is the osmotic pressure of the transport assay medium, a_0 is the initial rate of proline uptake measured with medium of 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 osmolality yielding half-maximal ProP−$(His)_6$ activity, and B is a constant inversely related to the slope of the response curve (see curves plotted in Figure 2). This empirical relationship supports extraction of parameters (see Table 1) which are useful descriptors for the osmotic activation of ProP−$(His)_6$ while we endeavor to identify the phenomena on which an appropriate physical model for this process would be based. Despite the close relationship between ProP activity and assay medium osmolality illustrated in Figure 1B, small but systematic increases in the calculated values for A_{max} and $\Pi_{1/2}/RT$ were observed as the osmolality of the bacterial growth medium increased. This variation may have arisen from a decreasing impact of the "inactivation phase" of the transporter response as the cells were cultivated in media of higher salinities (see Figure 1).

Significant variations in absolute solute uptake activity (reflected in A_{max}) are observed among proteoliposome preparations, whereas $\Pi_{1/2}/RT$ and B values are much more reproducible. Our current analyses of the osmosensory mechanism of ProP (like analyses of related systems by other laboratories (*8*, *15*, *18*)) therefore rely primarily on interpretation of the osmotic threshold for transporter activation, reflected here in $\Pi_{1/2}/RT$. Despite the qualitatively similar relationship between ProP−$(His)_6$ activity and medium osmolality in both cells and proteoliposomes, the osmolality yielding half-maximal activity ($\Pi_{1/2}/RT$) for proteoliposomes was almost 0.2 mol/kg higher than that for cells (Figure 2 and Table 1).

Impact of Transporter Kinetics on Characterization of the Osmolality Response. For ProP−$(His)_6$ in proteoliposomes, both K_M for proline and V_{max} increased as medium osmolality increased (*13*). These parameters should thus be designated as $K_{M,\Pi}$ and $V_{max,\Pi}$. To determine whether this phenomenon also occurred with intact bacteria, *E. coli* WG710 was cultivated, harvested, and resuspended in NaCl-free MOPS medium. The kinetic parameters were determined as the bacteria were exposed to media of varying osmolality during the transport assay. As in proteoliposomes, both K_M of ProP−$(His)_6$ for proline and the associated V_{max} increased with medium osmolality (Figure 3, panels A and B, open circles). $K_{M,\Pi}$ was systematically higher and more sensitive to osmolality in intact bacteria (Figure 3A, open circles) than in proteoliposomes (Figure 3A, open squares).

The interacting effects of substrate concentration and assay medium osmolality on the rate of proline uptake via ProP complicate the interpretation of a_0 values measured at a constant proline concentration and varying osmolality (see, for example, the data of Figures 1 and 2, which were obtained

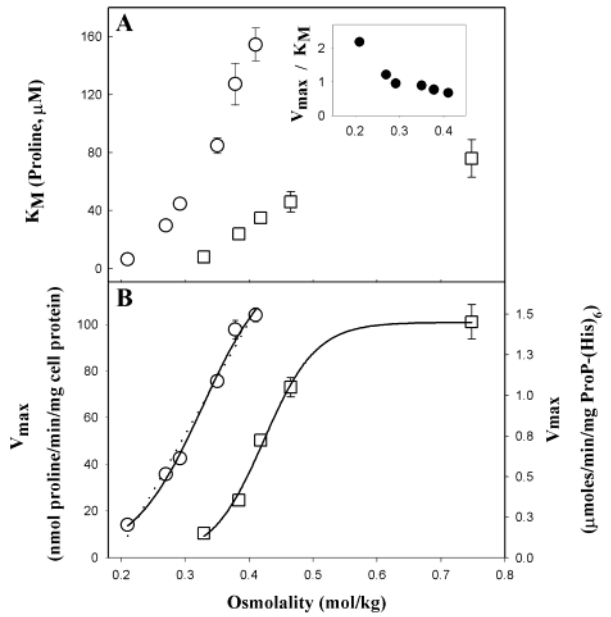

FIGURE 3: Kinetics of proline uptake via ProP−(His)$_6$ in bacteria (circles) and in proteoliposomes (squares). *E. coli* strain WG710 was cultivated, washed, and resuspended in NaCl-free MOPS medium (osmolality 0.12 mol/kg). ProP−(His)$_6$ was purified and reconstituted in proteoliposomes as described in Methods. The initial rate of proline uptake via ProP−(His)$_6$ was measured as described in Methods using assay media adjusted with NaCl to the indicated osmolalities and radiolabeled proline at an appropriate series of concentrations (11−900 μM for cells and 2.4−250 μM for proteoliposomes). K_M for proline (panel A) and V_{max} (panel B) were determined at each osmolality by nonlinear regression using the Michaelis−Menten equation as model. The data for proteoliposomes are derived from Figure 5 of Racher et al. (*13*). Lines were obtained by fitting data to eq 2 by nonlinear regression. Units of V_{max} are nmoles/min/mg cell protein (left axis) or μmoles/min/mg ProP−(His)$_6$ (proteoliposomes, right axis). In the inset to panel A, k_{cat}/K_M is represented by V_{max}/K_M for cells with constant ProP−(His)$_6$ levels. The dotted line in panel B was obtained by linear regression.

using a proline concentration of 200 μM). The parameters A_{max}, $\Pi_{1/2}/RT$ and B (defined above, eq 1) are clearly functions of the proline concentration used during the transport assay. Because the relationship between $K_{M,\Pi}$ and osmolality differs in cells and proteoliposomes, a_0 values measured at the same proline concentration and osmolality in these two systems also represent differing fractions of $V_{max,\Pi}$. In contrast to a_0, $V_{max,\Pi}$ is a proline-concentration-independent estimator of transporter activity. Like a_0 (represented in Figures 1 and 2), $V_{max,\Pi}$ for proline uptake via ProP−(His)$_6$ was a sigmoid function of osmolality (Figure 3B). The data in Figure 3B were fit to eq 2, which is analogous in form to eq 1:

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

except that $V_{max,\Pi}$ is the V_{max} for proline uptake measured with medium osmolality Π/RT. A_{max}, $\Pi_{1/2}/RT$, and B then become proline-concentration-independent parameters.

As would be expected, the resulting parameters differed from those presented in Table 1. For cells (open circles), A_{max} was 0.129 ± 0.015 μmol min^{-1} (mg cell protein)$^{-1}$, the osmolality at half that maximal activity was 0.327 ± 0.016 mol/kg, and the B value was 0.055 ± 0.009 mol/kg. For proteoliposomes (open squares), the corresponding values were 1.45 ± 0.05 μmol min^{-1} (mg ProP−(His)$_6$)$^{-1}$ and 0.423 ± 0.005 and 0.041±0.004 mol/kg, respectively. Since the $K_{M,\Pi}$ values for proteoliposomes were systematically lower than those for intact cells, this correction had less impact on the parameters estimated for proteoliposomes than for intact cells and the $\Pi_{1/2}/RT$ values for the two experimental systems became more similar. Nevertheless, a difference in $\Pi_{1/2}/RT$ of approximately 0.1 mol/kg remained.

Mechanical Deformation (Wrinkling) of the Lipid Bilayer and the Osmotic Activation of ProP−(His)$_6$. Many osmoregulatory systems respond to osmolality changes imposed with membrane-impermeant but not membrane-permeant solutes (*1*, *8*). For the reasons discussed above, this requirement for membrane-impermeant solutes does not indicate that ProP−(His)$_6$ responds to turgor pressure. Perhaps membrane-impermeant solutes are required because osmoregulatory systems respond to mechanical deformation (altered curvature or wrinkling) of the lipid bilayer in which they reside. The data illustrated in Figure 1 contradict this view of ProP−(His)$_6$ in intact bacteria. Although the shape of the cytoplasmic membrane surface in intact cells is not known, imposed osmotic upshifts and downshifts will certainly have very different effects on that shape. Despite such differences, the same ProP−(His)$_6$ activity can be attained in response to an upshift, a downshift, and no shift.

ProP is designated as an osmosensor because it is activated to the same degree when cells or proteoliposomes are exposed to external media adjusted to the same osmolality with chemically diverse, membrane-impermeant osmolytes (including NaCl and other inorganic salts, D-glucose, sucrose, and PEGs in the molecular weight range 600−1000 (*13*)). Recent data suggest that osmotic upshifts imposed with membrane-impermeant solutes activate transporters BetP (*15*) and OpuA (*18*) by concentrating cations in the proteoliposome lumen. Similarly, it is proposed that osmoregulatory sensor kinase KdpD of *E. coli* responds to changes ion concentration, ionic strength, and ATP levels (*35*). To determine whether a similar effect could account for its osmotic activation, the activity of ProP−(His)$_6$ was measured in proteoliposomes loaded with NaCl or with D-glucose (Figure 4). The initial rate of proline uptake via ProP−(His)$_6$ (a_0 of eq 1) was measured as extraliposomal osmolality was modulated with D-glucose, thereby avoiding adverse effects of NaCl on proteoliposome integrity (*36*). The proline concentration employed for these experiments (200 μM) minimized the impact of $K_{M,\Pi}$ modulation (Figure 3A) on the measured uptake rates and osmotic activation parameters in the proteoliposome system.

The simple correlation between ProP−(His)$_6$ activity and osmolality was not sustained when the composition of the proteoliposome lumen was varied (Figure 4A). Transporter activity was enhanced to a limited extent in NaCl- or D-glucose-loaded proteoliposomes even without osmotically induced proteoliposome shrinkage. Shrinkage was required to fully activate the transporter, however (Figure 4A, closed circles and triangles, respectively). In this system, neither osmolality nor fold shrinkage correlated with ProP−(His)$_6$ activity in an osmolyte-independent manner (Table 2). As first noted by Krämer and his colleagues for system BetP (*15*), the correlation between ProP−(His)$_6$ activity and the total cation concentration of the proteoliposome lumen was much more independent of lumenal solvent composition

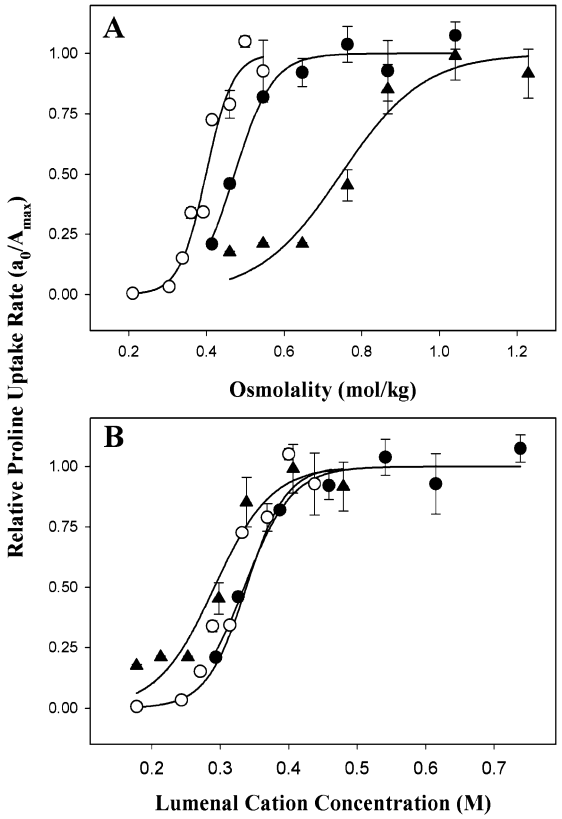

FIGURE 4: Effects of lumenal D-glucose or NaCl on the osmotic activation of ProP−$(His)_6$ in proteoliposomes. ProP−$(His)_6$ was purified and reconstituted in proteoliposomes which were used directly or loaded with NaCl or D-glucose as described in Methods. The unloaded proteoliposomes had an initial lumenal osmolality of 0.21 mol/kg (open circles). Those loaded with NaCl (closed circles) or D-glucose (closed triangles) had initial lumenal osmolalities of 0.414 and 0.461 mol/kg, respectively. Proline uptake activity was measured as described in Methods using L-proline (200 μM) as substrate. The transport assay medium was supplemented with D-glucose to titrate its osmolality, starting with the osmolality of the medium in which the proteoliposomes were prepared. The measured proline uptake rates were normalized on the maximum rate observed for each titration (A_{max} of eqs 1 and 3). Parameters obtained by fitting these data to eq 1 (panel A) or eq 3 (panel B) are listed in Table 3.

Table 2: Correlation of Liposome Deformation, Osmolality, and Cation Concentration with ProP−$(His)_6$ Activity[a]

lumenal cosolvent	$\Pi_{1/2}RT$ (mol/kg)	fold shrinkage at $0.5A_{max}$	$C_{1/2}$ (M)
Nil	0.380 ± 0.008	1.81	0.305 ± 0.006
NaCl	0.472 ± 0.011	1.14	0.335 ± 0.008
D-Glucose	0.747 ± 0.037	1.62	0.291 ± 0.014

[a] Proteoliposomes were prepared in a medium with an osmolality of 0.210 mol/kg, their lumenal solvent compositions were adjusted with the indicated cosolvent to an osmolality of 0.414 mol/kg (NaCl) or 0.461 mol/kg (D-glucose), and proline uptake activity was measured as described in Methods using L-proline (200 μM) as substrate and D-glucose to adjust the osmolality of the transport assay medium as indicated in Figure 4. The lumenal cation concentrations, $\Pi_{1/2}/RT$, and $C_{1/2}$ were calculated as described in Methods and are cited plus or minus the standard deviation. The fold shrinkage of the proteoliposomes at the osmolality yielding half-maximal activity (fold shrinkage at $0.5A_{max}$) was calculated by dividing $\Pi_{1/2}/RT$ by the osmolality of the medium in which the proteoliposomes were prepared (Π/RT).

(Figure 4B and Table 2) than the correlation between ProP−$(His)_6$ activity and either the imposed osmolality or the fold shrinkage of the proteoliposomes.

Table 3: Impacts of Lumenal Co−Solvents on the Activation of ProP−$(His)_6$[a]

proteoliposome preparation		osmolality response parameter			
#	cosolvent	Π_0/RT	A_{max}	$\Pi_{1/2}/RT$	B	$C_{1/2}$
I	Nil	0.22	1.77	0.40	0.034	0.34
I	Nil	0.22	1.58	0.38	0.022	0.31
I	D-glucose	0.46	1.01	0.75	0.107	0.29
I	NaCl	0.39	1.53	0.47	0.049	0.34
II	NaCl	0.40	1.51	0.53	0.079	0.37
I	KCl	0.39	1.44	0.54	0.080	0.38
I	LiCl	0.39	1.20	0.53	0.071	0.38
I	CsCl	0.39	1.36	0.54	0.096	0.39
II	PEG503	0.40	3.38	0.73	0.068	0.32
II	PEG591	0.40	3.45	0.60	0.083	0.27
II	PEG722	0.40	1.79	0.60	0.067	0.27
II	PEG1000	0.40	2.71	0.48	0.093	0.21
II	PEG1000	0.40	2.06	0.51	0.061	0.23
II	PEG2000	0.40	2.69	0.45	0.058	0.20

[a] For two proteoliposome preparations (numbers (#) I and II), the lumenal solvent composition was adjusted with the indicated cosolvents to the indicated initial osmolalities (Π_0/RT), and proline uptake activity was measured as described in Methods and the legends to Figures 4 and 5. The resulting data were fit to eq 1 to obtain parameters A_{max} (in μmol min^{-1} (mg ProP)$^{-1}$), $\Pi_{1/2}$/RT and B (each in mol/kg) or to eq 3 to obtain parameter $C_{1/2}$ (in M). On average, the standard deviation represented the following percent of the mean for each parameter: A_{max}, 7%; $\Pi_{1/2}/RT$ or $C_{1/2}$, 4%; B, 27%.

To obtain the $C_{1/2}$ values in Tables 2 and 3, the data illustrated in Figure 4 were fit to

$$a_0 = A_{max}\,[1 + \exp(-(C - C_{1/2})/B)]^{-1} \qquad (3)$$

where C is the total cation concentration of the proteoliposome lumen, calculated as described in Methods and including both the K^+ of the buffer system and the cation of any added salt (e.g., the Na^+ of NaCl). Parameter a_0 is the initial rate of proline uptake at lumenal cation concentration C, A_{max} is the uptake rate that would be observed at infinite lumenal cation concentration, $C_{1/2}$ is the lumenal cation concentration yielding half-maximal ProP−$(His)_6$ activity, and B is a constant related to the slope of the response curve (see curves plotted in Figure 4B). These data indicate that, in the proteoliposome system, osmotically induced shrinkage activates ProP−$(His)_6$ by modulating the composition of the proteoliposome lumen, not by mechanical deformation of the membrane itself.

Lumenal Solvent Composition and ProP−(His)$_6$ Activity in Proteoliposomes. To further explore the effects of lumenal solutes on ProP−$(His)_6$ activity, experiments were performed using proteoliposomes loaded with poly(ethylene glycol)s (PEGs) that had varying degrees of polymerization or with additional monovalent cation salts (KCl, LiCl, and CsCl). Particularly useful in this regard were monodisperse poly(ethylene glycol)s PEG503 ($n = 11$), PEG591 ($n = 13$), and PEG722 ($n = 16$), prepared by Polypure AS. PEG1000 and PEG2000 are mixtures of polymers with the indicated average molecular weights and degrees of polymerization (n) near 22 and 45, respectively. These PEGs were all membrane-impermeant as indicated by the calcein-based fluorescence quenching assay (*13*) (data not shown).

Like lumenal NaCl or D-glucose, lumenal PEGs enhanced the activity of ProP−$(His)_6$ even if the proteoliposome preparation and transport assay medium were isotonic (Figure 5). The relative activity of ProP−$(His)_6$ increased with PEG

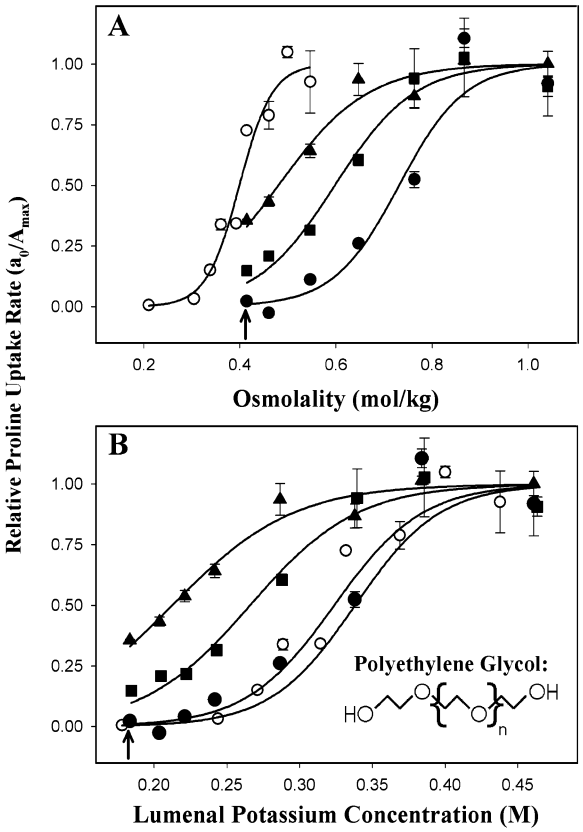

FIGURE 5: Effects of lumenal PEGs on the osmotic activation of ProP−$(His)_6$ in proteoliposomes. ProP−$(His)_6$ was purified and reconstituted in proteoliposomes, and the composition of the lumenal solvent was adjusted as described in Methods. The proteoliposomes were unloaded (open circles) or loaded with PEG503 (closed circles), PEG591 (closed squares), or PEG1000 (closed triangles). Each PEG-loaded preparation had an osmolality of 0.4 mol/kg and 0.1 M potassium phosphate, pH 7.4, as lumenal buffer (points indicated by the arrows in panels A and B). Proline uptake activity was measured using D-glucose to raise the osmolality of the assay buffer and proline at a concentration of 200 μM. The measured proline uptake rates were normalized on the maximum rate observed for each titration (A_{max} of eqs 1 and 3). The parameters obtained by fitting these data and similar results obtained with additional PEGs to eq 1 (panel A) or eq 3 (panel B) are listed in Table 3.

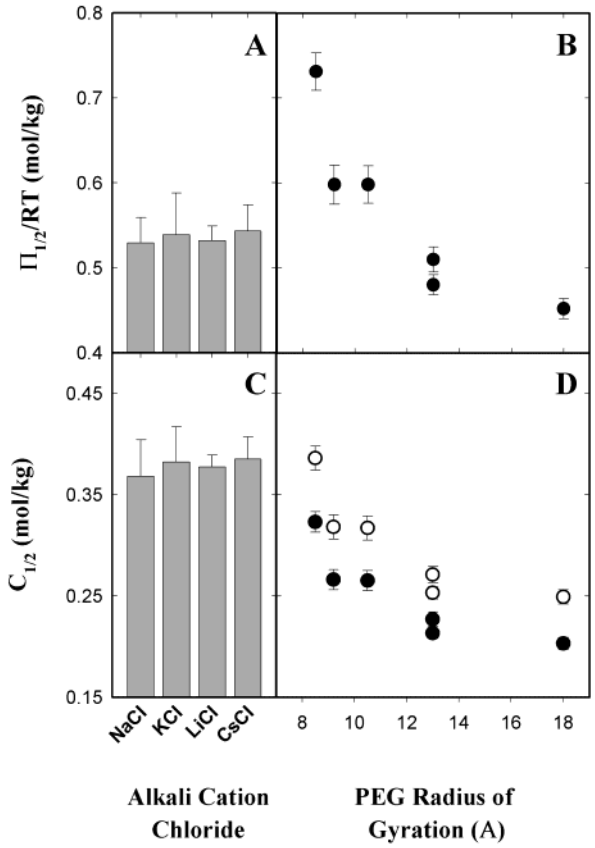

FIGURE 6: Impact of lumenal solvent composition on the osmotic activation of ProP−$(His)_6$. The assay medium osmolalities ($\Pi_{1/2}/RT$) (panels A and B) and the lumenal cation concentrations ($C_{1/2}$) (panels C and D) yielding half-maximal ProP−$(His)_6$ activity obtained by regression analysis of the data illustrated in Figures 4 and 5 are plotted vs the identity of the lumenal solute (panels A and C) or the radius of gyration of the lumenal PEG (panels B and D). The radii of gyration of the PEGs are based on values measured by Bhat and Timasheff (*32*) with interpolation as necessary. Open symbols in panel D represent values obtained after correction for volume exclusion by PEGs as described in Methods.

molecular weight in the absence of any osmotic shift, at constant osmolality and cation concentration (see arrows in Figure 5A,B). Osmotically induced proteoliposome shrinkage further enhanced activity, though (as for D-glucose-loaded proteoliposomes) higher osmolalities were required to attain full activation of PEG-loaded proteoliposomes (solid symbols) than of those containing only the potassium phosphate buffer (open circles). The osmolality yielding half-maximal activity ($\Pi_{1/2}/RT$) was now clearly osmolyte-dependent, and it decreased as PEG molecular weight increased (Figures 5A and 6A, Table 3). Furthermore, this osmolyte dependence could not be eliminated by relating activity to lumenal cation (K^+) concentration (Figures 5B and 6B, Table 3).

Each of the monvalent cation salts had a similar impact in terms of both osmolality and lumenal cation concentration at half-maximal ProP−$(His)_6$ activity (parameters $\Pi_{1/2}/RT$ and $C_{1/2}$, Figure 6 and Table 3). It should be noted that, regardless of cation composition (K^+ alone or K^+ plus Na^+, Li^+ or Cs^+), the lumenal cation concentration required to attain half-maximal activity was very high (0.35−0.40 M). Although this cation requirement was reduced in the presence of lumenal PEG, it remained high in biochemical terms (more than 0.2 M) (Figure 6B). Although the data are interpreted here in terms of cation concentration, these experiments do not rule out contributions of the anions phosphate and chloride to the observed transporter activation.

These data suggest that the semipermeable, cytoplasmic membrane acts as a transducer of osmotic signals (changes in extracellular osmolality imposed with membrane-impermeant solutes), translating them into parallel changes in concentration of all cytoplasmic solutes. In turn, ProP monitors the impact of extracellular osmolality on the composition of the cytoplasm or proteoliposome lumen.

Impact of Protein ProQ on ProP Activity in Intact Bacteria: The Turnover Number of ProP. The response of ProP to osmolality in intact bacteria is dramatically attenuated by genetic defects that eliminate soluble protein ProQ (*23*, *24*). ProQ deficiency does not alter *proP* transcription or ProP level (*25*). It is not yet known whether the effects of ProQ on ProP activity indicate direct interaction between these proteins in intact bacteria. It would be helpful to know whether the ProP−$(His)_6$ activity observed in proteoliposomes corresponds with the ProP activity of *proQ*$^+$ or of *proQ*$^-$ bacteria.

The turnover number (k_{cat}) for ProP−$(His)_6$ in proteoliposomes (osmolality, 0.33 mol/kg^{-1}) can be estimated as 0.14±0.02 s^{-1} on the basis of data published by Racher et al. (*13*). Given the range of ProP−$(His)_6$ activities observed in proteoliposomes to date, this value may represent a 2-fold underestimation. *E. coli* strains WG174 (*proQ*$^-$) and RM2 (*proQ*$^+$) were cultivated, washed, and resuspended in medium with an osmolality of 0.29 mol/kg. At this osmolality the K_M of ProP−$(His)_6$ for proline is 45 μM (Figure 3A). The ProP activities (a_0) of these bacteria were determined and the corresponding ProP levels were estimated by Western Blotting using purified ProP−$(His)_6$ as the standard (data not shown). These estimates yielded turnover numbers for ProP of 0.4 and 2.5 s^{-1} in ProQ-deficient and ProQ-containing bacteria, respectively. Thus the k_{cat} for ProP−$(His)_6$ in proteoliposomes (0.14 s^{-1}) corresponded better with that observed in ProQ-deficient bacteria (0.4 s^{-1}) than that observed in ProQ-containing bacteria (2.5 s^{-1}). The absence of ProQ (or ProQ-related molecules) from proteoliposomes may therefore account for some of the differences between cells and proteoliposomes noted above.

DISCUSSION

Osmoprotectant transporters are activated to mediate compatible solute accumulation when bacteria face environments of increasing osmolality (*1*, *22*). Since that response can be replicated with purified transporters, reconstituted in proteoliposomes, these proteins serve as both osmosensors and osmoregulators (*8*−*10*). We compared and characterized the osmoregulation of ProP activity in *E. coli* cells and proteoliposomes to further probe the osmoregulatory mechanism and define the osmotic shift-induced cellular change(s) to which ProP responds.

Microbiologists and plant physiologists have long maintained that walled cells osmoregulate to maintain turgor pressure (e.g., refs *8* and *22*). Since the same ProP−$(His)_6$ activity was attained when bacteria were subjected to an osmotic upshift, which would decrease turgor pressure, no osmotic shift, or an osmotic downshift, which would increase turgor pressure (Figure 1), ProP−$(His)_6$ did not respond to turgor pressure changes. Osmotically induced shrinkage of topologically closed systems such as cells, membrane vesicles, and proteoliposomes will change the shape of the membrane surface and concentrate cytoplasmic/lumenal contents. The data cited above as well as the lack of correlation between ProP−$(His)_6$ activity and the fold shrinkage of proteoliposomes (Table 2) show that ProP does not respond to mechanical deformation (wrinkling) of the phospholipid bilayer. ProP activity is osmoregulated in cytoplasmic membrane vesicles (*34*), and ProP−$(His)_6$ activity is osmoregulated in proteoliposomes (*11*), both of which can withstand hydrostatic pressure and the resulting in-plane membrane strain resulting from imposed osmotic downshifts (*36*−*38*). However these membrane systems are not strained after the osmotic lysis (membrane vesicles) or extrusion through microporous membranes (proteoliposomes) that is intrinsic to their preparation and osmotic upshifts, which would reduce any preexisting pressure and strain, are required for ProP activation. Thus neither changing turgor pressure nor changing membrane strain (either within or out of the membrane plane) is the correlate of osmolality sensed by ProP. Others have ruled out turgor pressure and membrane strain as the properties sensed by osmosensor/transporters BetP of *C. glutamicum* and OpuA of *L. lactis* (*8*, *9*). It therefore becomes critical to examine relationships among cytoplasmic/lumenal composition, structure and activity for these integral membrane proteins.

Since little structural information is currently available, the catalytic activities of osmoregulatory transporters are used to indicate (presumed) protein conformational changes associated with osmosensing. As expected for a model in which osmolality determines transporter conformation, both K_M and V_{max} for ProP−$(His)_6$ increased with medium osmolality in both cells and proteoliposomes (Figure 3). For cells in a medium with osmolality 0.29 mol/kg, the measured k_{cat} for ProP (2.5 s^{-1}) and corresponding K_M (45 μM) (Figure 3A) yielded a catalytic efficiency (k_{cat}/K_M) of approximately 10^5 s^{-1} M^{-1}. The catalytic efficiency of the transporter decreased as medium osmolality increased (Figure 3A, inset). This did not arise from solute effects on medium viscosity since the catalytic efficiency of ProP was orders of magnitude below those (10^8−10^9 s^{-1} M^{-1}) at which viscosity limits the rates of enzyme catalyzed reactions (*39*) and the viscosities of the media used for these measurements were low (*40*). The catalytic efficiency of ProP−$(His)_6$ appeared to be independent of medium osmolality in proteoliposomes (*13*), but low transporter activity precluded estimation of the kinetic parameters for osmolalities below 0.3 mol/kg.

Interesting differences in ProP−$(His)_6$ behavior between cells and proteoliposomes were detected. The k_{cat} for ProP−$(His)_6$ in proteoliposomes (0.14 s^{-1}) corresponded better with that observed in ProQ-deficient than in ProQ-containing bacteria (0.4 s^{-1} vs 2.5 s^{-1}), implying that ProQ acts in cells to enhance ProP activity (*10*) or to antagonize an inhibitor of ProP activity. The absolute values for K_M were lower and their osmolality dependence weaker in proteoliposomes than in cells (Figure 3A). The medium osmolality yielding half-maximal transporter activity for cells (V_{max} at $\Pi_{1/2}/RT$, 0.327 ± 0.016 mol/kg) was lower than that for proteoliposomes containing only 0.1 M potassium phosphate buffer (0.423 ± 0.005 mol/kg) (Figure 3B). However, the medium osmolality at half-maximal ProP−$(His)_6$ activity was a function of lumenal composition in proteoliposomes (Figure 6) (see further discussion below). Thus, differences in kinetic parameters between cells and proteoliposomes may arise from differences in composition between the cytoplasm and the proteoliposome lumen and/or from the absence of ProQ (or ProQ-dependent phenomena) from the proteoliposome system.

In proteoliposomes loaded with alkali metal halides or low molecular weight organic solutes (e.g., D-glucose), the activities of transporters ProP−$(His)_6$ (Figures 4 and 6), BetP (*15*), and OpuA (*18*) correlate better with the total monovalent cation concentration of the proteoliposome lumen than with osmolality. Increasing extracellular osmolality and long-term cultivation of bacteria in osmoprotectant-free, high osmolality media elicit transporter-mediated K^+ accumulation (*1*). It is has thus been argued that BetP of *C. glutamicum* is a sensor for cytoplasmic K^+ (*15*) and that OpuA of *L. lactis* senses the impact of changing cytoplasmic ionic strength on membrane lipids (*18*). ProP activity is specifically stimulated when K^+ is supplied to solute-depleted bacteria (*20*, *41*). Thus, it is possible that ProP activity responds to ion

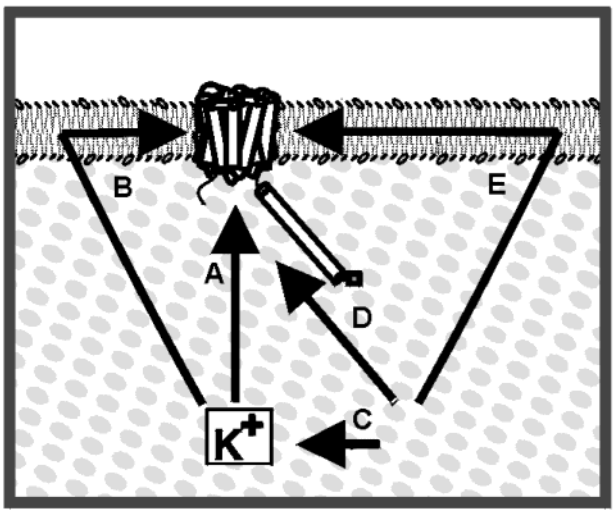

FIGURE 7: Pathways by which K^+ and PEGs may influence the activity of ProP. K^+ may act directly on ProP−$(His)_6$ (A), or it may affect the structure of the membrane in which ProP−$(His)_6$ is embedded (B). PEGs may act by influencing K^+ activity (C), by interacting directly with ProP−$(His)_6$ (D), or by affecting the structure of the membrane in which ProP−$(His)_6$ is embedded (E).

concentration or ionic strength and that the physiologically relevant ion is K^+ (see arrows A and B of Figure 7).

Multiple issues complicate efforts to further address the role of K^+ in ProP activation by comparing data obtained with intact cells and proteoliposomes. First, when osmotic shifts are imposed on intact cells (e.g., Figures 1 and 2), the cytoplasmic K^+ concentration is immediately altered, and enzymatic mechanisms respond quickly to adjust that concentration (*1*). Second, ProP−$(His)_6$ activity responds to the concentration and molecular size of both inorganic (e.g., K^+) and organic osmolytes in the proteoliposome lumen (Figure 6) and, presumably, also in the intact cell. Since osmotic shifts and osmoregulatory responses will influence the concentrations of all cytoplasmic solutes, analysis of cellular K^+ content alone is not sufficient to complete the comparison between cells and proteoliposomes. Finally, the distinction between K^+ concentration and K^+ activity must be addressed. Studies based on the release of free K^+ by hypoosmotic shock (*42*) and on NMR spectroscopy of intact bacteria (*43*) indicate that interactions with other cellular constituents profoundly influence the thermodynamic behavior of K^+. Record and his colleagues concluded that cellular K^+ activity increased from 0.14 to 0.76 M in cells grown in media with osmolarities from 0.1 Osm to 1.02 Osm (*43*−*45*). Given the complexity of these systems, it is interesting that the reported K^+ activity range overlaps the K^+ concentration range over which ProP−$(His)_6$ activity is modulated in proteoliposomes (0.15−0.45 M, Figures 4 and 6, Tables 2 and 3). The high cation concentration required to activate ProP (even in the presence of PEG) and the lack of specificity of the cation effect, in vitro (Figure 6), are inconsistent with the existence of a regulatory K^+ binding site on ProP. More definitive comparison of the cell and proteoliposome systems must await further assessment of the role of ProQ (noted above) and a more thorough description of the physical chemistry of the bacterial cytoplasm. However, progress can be made toward understanding the osmosensory mechanism of ProP−$(His)_6$ by imposing variations in lumenal composition using the proteoliposome system.

Table 4: Osmolality Response Parameters for Polyethylene Glycols[a]

proteoliposome preparation				osmolality response parameter				
cosolvent	$[PEG]_0$	Π_0/RT	I_0	$[PEG]_{1/2}$	$\Pi_{1/2}/RT$	$C_{1/2}$	$I_{1/2}$	$K_{r,1/2}$
Nil	N/A	0.22	0.256	N/A	0.40	0.34	0.49	N/A
NaCl	N/A	0.39	0.356	N/A	0.47	0.34	0.43	N/A
PEG503	55	0.40	0.256	100	0.73	0.32	0.47	80
PEG591	67	0.40	0.256	100	0.60	0.27	0.38	66
PEG722	68	0.40	0.256	101	0.60	0.27	0.38	66
PEG1000	84	0.40	0.256	100	0.48	0.21	0.31	53
PEG2000	100	0.40	0.256	114	0.45	0.20	0.29	51

[a] The lumenal solvent composition was adjusted with the indicated cosolvents (NaCl at 0.1 M and PEGs at the indicated concentration ($[PEG]_0$, g/L) to yield the indicated initial osmolalities (Π_0/RT) and ionic strengths (I_0). Proline uptake activity was measured and values for the PEG concentration ($[PEG]_{1/2}$, g/L), the osmolality ($\Pi_{1/2}/RT$, mol/kg), the total cation concentration ($C_{1/2}$, M), and the ionic strength ($I_{1/2}$, M) at half-maximal ProP−$(His)_6$ activity as well as the medium dielectric constant ($K_{r,1/2}$) required to maintain a constant Debye length at $I_{1/2}$ were determined as described in Methods.

In contrast to BetP and OpuA (*8*), ProP−$(His)_6$ did respond to changes in lumenal concentration of both ionic and nonionic solutes. Lumenal PEGs significantly stimulated ProP−$(His)_6$ activity in proteoliposomes on which no osmotic shift had been imposed, the proteoliposome lumen and external medium were 0.1 M in potassium phosphate and sodium phosphate, respectively, and all media were isotonic at an osmolality of 0.4 mol/kg (Figure 5). Thus, ProP−$(His)_6$ activity did not correlate with lumenal K^+ concentration in PEG-loaded proteoliposomes (Figures 5 and 6). Like intact bacteria, proteoliposomes are thermodynamically nonideal and the concentrations and activities of solutes in the proteoliposome lumen cannot be presumed equal. Can the effects of nonionic solutes on ProP−$(His)_6$ activity (Figures 5 and 6) be rationalized in terms of their impact on K^+ (or cation) activity (see arrow C of Figure 7)?

At high solute concentrations and/or when very high molecular weight solutes are used, solute occupancy of solution volume may significantly elevate the activities of all solutes (*46*). Correction for this excluded volume effect elevated estimates of the cation concentration required to attain half-maximal ProP−$(His)_6$ activity in the presence of PEGs (Figure 6, open symbols) but only slightly reduced the observed variation in $C_{1/2}$, suggesting that the PEGs did not act solely by modulating K^+ activity via volume exclusion.

Correlations between osmoregulatory transporter activity and lumenal ion concentration (e.g., Figure 4) could indicate that these enzymes respond to the ionic strength of the cytoplasm or proteoliposome lumen (*8*). ProP−$(His)_6$ does not respond simply to ionic strength since the ionic strength yielding half-maximal activity ($I_{1/2}$) varied with lumenal solvent composition (Table 4). Variations in ionic strength modulate macromolecular conformations and interactions by modulating the Debye length (r_D), the distance over which the electrostatic field of a charged functional group extends with appreciable strength. The r_D is a direct function of solution dielectric constant (K_r) and an inverse function of solution ionic strength (I) and density. Variations in solution density in our system were not significant (*47*) and variations in K_r are not likely to have complemented the variations in

I to the extent required for a constant net Debye length (Table 4) (*48*). Thus, ProP−$(His)_6$ activity does not appear to have been modulated solely via the impact of lumenal solvent composition on electrostatic interactions.

The impacts of PEGs on the osmolality and the cation concentration at half-maximal ProP−$(His)_6$ activity were inversely correlated with the PEG radius of gyration (R_G) (Figure 6). Macromolecular solutes may influence the structures and interactions of proteins through direct interactions, by increasing macromolecular crowding, through steric exclusion from macromolecule-associated water pools or through preferential exclusion based on nonsteric factors (*1*). PEGs similar in size range to those employed for this study are preferentially excluded from the surfaces of proteins (*32*, *49*−*51*) and phospholipid bilayers (*52*). The preferential exclusion of PEGs from protein surfaces is believed to be principally steric in origin since it can be correlated with the PEG radius of gyration (*32*, *51*). Nevertheless, PEGs do interact favorably with protein surfaces under some conditions (*32*, *51*), and such direct surface interactions will assume proportionally larger importance as PEG molecular weight, PEG size, and hence steric exclusion decrease. For this reason PEGs are not favored as tools for the analysis of macromolecular crowding (*53*). PEGs are well-known for their ability to induce fusion of natural and artificial membranes (*54*) but do not do so in the molecular size range and concentration used for this study of ProP−$(His)_6$ (*47*, *55*−*57*). PEGs are also known for their high water binding capacities (*47*). Notably, the PEG concentration (w/v) yielding half-maximal ProP−$(His)_6$ activity ($[PEG]_{1/2}$), and hence the water binding capacity of the lumenal PEG (*47*), was not correlated with PEG molecular size (Table 4).

It is tempting to conclude that the impact of PEG on ProP activity arises via its steric exclusion from the surface of ProP−$(His)_6$ or of the membrane in which it is embedded, and that osmotically induced concentration of PEGs in the proteoliposome lumen mimics the osmotically induced crowding of macromolecules in the cytoplasm of intact bacteria (Figure 7). However, given the multiple impacts of PEGs on macromolecular systems, this conclusion must remain contingent on studies of the impacts of other macromolecular solutes on ProP−$(His)_6$ activity. Modulation of ProP−$(His)_6$ structure due to concentration of macromolecular solutes could alter the protein's interaction with K^+ (arrow C of Figure 7) or the effects of the PEGs and K^+ may be independent (arrows D and E (PEG) vs arrows A and B (K^+) of Figure 7).

We have shown that transporter ProP−$(His)_6$ responds to the concentration, chemistry and molecular size of osmolytes in the proteoliposome lumen. These results pave the way for identification of the physical parameter(s) sensed by ProP and creation of a model for its osmoregulatory response based on that identification.

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