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Contributions of Coulombic and Hofmeister Effects to the Osmotic Activation of *Escherichia coli* Transporter ProP

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Abstract

Osmosensing transporters mediate osmolyte accumulation to forestall cellular dehydration as the extracellular osmolality increases. ProP is a bacterial osmolyte-H⁺ symporter, a major facilitator superfamily member, and a paradigm for osmosensing. ProP activity is a sigmoid function of the osmolality. It is determined by the osmolality, not the magnitude or direction of the osmotic shift, in cells and salt-loaded proteoliposomes. The activation threshold varies directly with the proportion of anionic phospholipid in cells and proteoliposomes. The osmosensory mechanism was probed by varying the salt composition and concentration outside and inside proteoliposomes. Data analysis was based on the hypothesis that the fraction of maximal transporter activity at a particular luminal salt concentration reflects the proportion of ProP molecules in an active conformation. ProP attained the same activity at the same osmolality when diverse, membrane-impermeant salts were added to the external medium. Contributions of Coulombic and/or Hofmeister salt effects to ProP activation were examined by varying the luminal salt cation (K⁺ and Na⁺) and anion (chloride, phosphate, and sulfate) composition and then systematically increasing the luminal salt concentration by increasing the external osmolality. ProP activity increased with the sixth power of the univalent cation concentration, independent of the type of anion. This indicates that salt activation of ProP is a Coulombic, cation effect resulting from salt cation accumulation and not site-specific cation binding. Possible origins of this Coulombic effect include folding or assembly of anionic cytoplasmic ProP domains, an increase in local membrane surface charge density, and/or the juxtaposition of anionic protein and membrane surfaces during activation.

Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.5b01169. Dependence of the rate of proline uptake on the luminal ion composition of proteoliposomes ($A_{\max}$ and R^2 values) (Table S1) and Impact of data linearization (Figure S1) (PDF)

The authors declare no competing financial interest.

To thrive, cells must detect and respond to changing physical properties of their environments. Osmolality changes cause transmembrane water fluxes that impair cellular functions. Osmotic equilibration occurs within seconds because most phospholipid membranes are highly water permeable and cell membranes include aquaporins.¹ As a result, increasing osmolality causes immediate cellular dehydration and concentrates cellular constituents. Cytoplasmic osmolality, the concentrations of all cytoplasmic solutes, and excluded volume or confinement effects on biopolymers all increase. Cytoplasmic membrane strain changes and, for walled cells, turgor pressure may decrease.

Osmoregulatory responses enable cells to tolerate osmolality changes. Representatives of diverse phylogenetic groups share the following strategy.²⁻⁵ Increasing osmolality triggers the uptake of inorganic ions as well as the uptake or synthesis of organic solutes that can accumulate to high cytoplasmic levels without impairing cellular functions. This osmolyte accumulation forestalls cellular dehydration. Decreasing osmolality causes water influx and strains cytoplasmic membranes. Mechanosensitive channels open, releasing osmolytes and preventing cell lysis.

Studies of bacterium *Escherichia coli* have contributed to our understanding of osmoregulation,⁶ and osmotically-induced perturbation of the cytoplasmic composition of *E. coli* has facilitated the analysis of physicochemical requirements for cellular processes.⁷ Transporters promote the growth of *E. coli* K-12 at high osmolality by mediating the uptake of glycine betaine, proline, and other organic osmolytes. Transporter activity is regulated at the transcriptional and post-translational levels. This report concerns the post-translational osmoregulation of transporter ProP, which serves as a paradigm for the study of osmosensing transporters.^{6,8}

ProP is an osmolyte-proton symporter and a member of the major facilitator superfamily (MFS).⁹ ProP transports various zwitterionic osmolytes, including proline, glycine betaine, dimethylsulfoniopropionate, γ -butyrobetaine, carnitine, and ectoine.¹⁰ The ProP protein includes a membrane-integral domain with small periplasmic loops, larger cytoplasmic loops, and a cytoplasmic C-terminal domain that is larger than those of orthologues that are not osmosensors (Figure 1A). The membrane-integral domain can be modeled on the crystal

structures of MFS members that are comprised of N- and C-terminal six-helix bundles flanking a substrate-binding cleft^{11,12} (Figure 1B). Experimental data support such structural models^{11,13,14} and an alternating access mechanism for proline-proton symport via ProP.¹⁵ The C-terminal domains of some ProP orthologues form intermolecular, antiparallel α -helical coiled coils stabilized by interchain salt bridges¹⁶⁻¹⁸ (Figure 1D). A peptide replica of the coiled coil from *E. coli* ProP has limited stability *in vitro*.¹⁶ Data indicate that the antiparallel coiled coil and TMXII participate in the dimer interface of ProP *in vivo*,¹⁷ but the role of dimerization in ProP function is unknown. Some ProP orthologues lack the coiled coil and retain post-translational osmoregulation.^{19,20}

ProP activity increases as a sigmoid function of the osmolality ($\Pi/\rho RT$). Both K_M and V_{max} for proline uptake increase with assay medium osmolality.²¹ The initial rate of proline uptake via ProP increases from near zero to near maximal as the osmolality increases from approximately 0.10 to 0.35 mol/kg [bacteria cultivated at low osmolality (0.12 mol/kg) and offered proline at 0.2 mM].²¹ Analogous responses are seen in intact cells, cytoplasmic membrane vesicles, and proteoliposomes prepared with purified ProP-His₆ and a polar lipid extract from *E. coli*.^{15,21,22}

Systematic efforts to correlate ProP activity with osmotically induced changes narrowed the extensive list of cellular properties to which ProP might respond.^{6,23} ProP does not respond to a specific exogenous solute. The same activity was attained when the same osmolality was imposed with diverse, membrane-impermeant organic and inorganic solutes.²⁴ In addition, the same ProP activity was attained at the same osmolality when an increase, a decrease, or no change in osmolality was imposed on *E. coli* cells²¹ or proteoliposomes.²⁴ Thus, ProP activity did not correlate with turgor pressure or membrane tension, and its regulation does not require cellular constituents other than the phospholipid membrane.

ProP function is phospholipid-dependent.⁶ The major phospholipid species in *E. coli* are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL, or diphosphatidylglycerol) in proportions of approximately 75:20:5 (mole percent).²⁵ The proportion of CL doubles at the expense of PE as the growth medium osmolality increases from approximately 0.1 to 0.7 mol/kg.¹⁹ The osmolality at which ProP attains half-maximal activity varies directly with the proportion of anionic phospholipid (PG plus CL) in both intact cells and proteoliposomes.^{19,26} Because ProP concentrates with CL at the poles of *E. coli* cells, CL is believed to modulate ProP function *in vivo*.^{26,27}

ProP has the same, periplasmic-surface-out orientation in cells, cytoplasmic membrane vesicles, and proteoliposomes.¹⁵ The luminal compositions of membrane vesicles and proteoliposomes can be manipulated; osmotic upshifts imposed with membrane-impermeant solutes then concentrate the selected luminal solutes. Such systems allowed ProP activity to be correlated with the luminal concentrations of diverse solutes. ProP became active as inorganic salts, but not D-glucose, were concentrated in the proteoliposome lumen.²¹ ProP activation was not monovalent cation-specific.^{15,21} The K⁺ salts of kosmotropic anions activated ProP at concentrations lower than those of the K⁺ salts of chaotropic anions, suggesting that ProP activation followed the Hofmeister series.²⁸ Furthermore, ProP activity was stimulated when bovine serum albumin (BSA) was incorporated in the proteoliposome

lumen at a concentration that simulated volume exclusion by cytoplasmic biopolymers (0.18 g/mL).²⁸ Similar results were obtained when large poly(ethylene glycol)s (PEGs) were incorporated.²¹ These observations led to the hypothesis that ProP senses osmotically induced increases in the concentration of protein-stabilizing cytoplasmic salts and biopolymers that result from osmotically induced water efflux.²⁸

Extensive research delineating the structural basis for solute effects on protein, nucleic acid, and model processes provides context for this analysis.²⁹ Effects of salts on protein conformational equilibria can result from ion binding at specific sites, from conformation-specific, Coulombic interactions of salt ions with fixed charges, and from preferential interactions of salt ions with the protein surfaces that are buried (or exposed) in the process (Hofmeister effects). There is no evidence that ProP activity responds to ion binding at specific sites. For many processes involving soluble proteins, Coulombic effects predominate in the concentration range relevant for ProP activation in proteoliposomes (0.1–0.3 M).^{29,30} Furthermore, ProP activity depends on the anionic lipid content of the membrane (as discussed above). The solvent-exposed surfaces of ProP abut and may interact with the polyanionic membrane surface. Thus, Coulombic effects may play a role in transporter activation.

This study evaluated the roles of Coulombic and Hofmeister effects in ProP activation. Proteoliposomes comprised of ProP and a polar lipid extract from *E. coli* were loaded at low osmolality with relatively low (subthreshold) concentrations of K⁺ and/or Na⁺ salts, including anions with varying Hofmeister effects (sulfate, phosphate, and chloride). The rate of proline uptake via ProP was measured as a function of the external osmolality. The results indicate that nonspecific, Coulombic cation effects dominate the osmotic activation of ProP in this system. They provide new insights into the structural basis for osmosensing.

EXPERIMENTAL PROCEDURES

Proteoliposome Preparations and Transport Assays

ProP-His₆ was expressed by *E. coli* strain WG710, a derivative of *E. coli* WG350 [*F*[−] *trp lacZ rpsL thi (putPA)*101 (*proU*)600 (*proP-melAB*)212]⁹ that contains plasmid pDC80¹⁶ that is a derivative of vector pBAD24.³¹ Liposomes were prepared with a polar lipid extract from *E. coli* (Avanti Polar Lipids, Inc.). Strain WG710 was cultivated, and ProP-His₆ was purified; proteoliposomes containing ProP-His₆ were prepared in 0.1 M K phosphate, 0.5 mM K ethylenediaminetetraacetic acid (K EDTA), and 2 mM 2-mercaptoethanol (pH 7.4), and initial rates of proline uptake at room temperature were measured in duplicate as previously described (L-proline at 200 μM).^{21,24,28,32} For the experiments reported in Table 2, the assay medium osmolality was adjusted with the sodium salt of chloride, bromide, sulfate, or citrate rather than sorbitol. Samples were taken at 16, 32, 48, and 64 s or 20, 40, 60, and 80 s to detect linear initial uptake rates. Osmolalities were measured with a Wescor Vapro 5520 vapor-pressure osmometer (Wescor, Logan, UT).

The proportions of K phosphate and Na phosphate in the proteoliposome lumen were adjusted, and the proline uptake activity of the resulting preparations was measured as described by Culham et al.²⁸

To vary the anion composition of the proteoliposome lumen, the K^+ phosphate concentration was lowered by diluting the proteoliposomes 10-fold with 0.5 mM K EDTA (pH 7.4), harvesting them by centrifugation at 300000g for 22 min at 20°C, resuspending them in a buffer of the desired composition, and extruding them through Nucleopore Track-Etch Membranes with a 0.4 μ m (diameter) pore size. The resuspension buffers were prepared by titrating a mixture of KOH, 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis Tris Propane or BTP), and EDTA with H_2SO_4 , H_3PO_4 , or HCl to attain a pH of 7.4. This resulted in buffers with the following concentrations: 0.11 M K^+ , 0.04 M BTP, 0.5 mM EDTA, and 68 mM sulfate, 88 mM phosphate, or 140 mM chloride.

To measure proline uptake activity as a function of luminal anion concentration, the resulting proteoliposomes were diluted into pH 6.4 assay buffers prepared with NaOH, BTP, EDTA, and H_2SO_4 , H_3PO_4 , or HCl as described above. Sorbitol was included to adjust the osmolality. This resulted in assay buffers with the following concentrations: 110 mM Na^+ , 40 mM BTP, 0.5 mM EDTA, and 86 mM sulfate, 140 mM phosphate, or 183 mM chloride. Valinomycin (0.19 μ M) and KCl were added to the assay mixtures to clamp the membrane potential (Ψ) at -137 mV as previously described.²⁴ Initial rates were determined by diluting the proteoliposomes into the corresponding valinomycin-supplemented, pH 6.4 medium in which Na^+ replaced K^+ , thereby imposing a protonmotive force of -196 mV (see Experimental Strategy). Analogous, valinomycin- and KCl-free buffers were used to wash the proteoliposomes during the filtration assay.

Experimental Strategy

The following experimental system was devised to evaluate anion effects on ProP activity. Proteoliposomes are usually prepared in 0.1 M K phosphate (pH 7.4) and diluted into 0.1 M Na phosphate (pH 6.4) for measurements of proton-solute symport (e.g., ref 32). High buffer capacity is required to stabilize the luminal pH despite proline-linked proton uptake mediated by ProP,²⁸ but the presence of phosphate at a high concentration obscures Hofmeister effects of other chemical species because Hofmeister effects are additive and phosphate is strongly kosmotropic.²⁹ First, the anionic luminal buffer, phosphate, was replaced with the cationic luminal buffer, 1,3-bis[tris-(hydroxymethyl)methylamino]propane (BTP), which has pK_a values of 6.8 and 9. BTP provided good buffer capacity and the freedom to explore the Hofmeister effect with selected anions. Second, luminal K^+ (110 mM) was retained so that a membrane potential (Ψ) could be imposed by adding K^+ ionophore valinomycin. The imposed Ψ (-137 mV) combined with the imposed pH gradient (1 unit, pH 7.4 to 6.4) to power proline-proton symport by providing a protonmotive force of -196 mV.

Data Analysis

The fraction of maximal transporter activity is defined as

$$f = A/A_{max} \quad (1)$$

where A is the initial rate of substrate (radiolabeled proline) uptake at a given osmolality and A_{max} is the maximal initial rate of proline uptake obtained at high osmolality. If the

dependence of ProP activity on the osmolality (or an osmolality-dependent parameter) represents the transition from an inactive (ProP_I) to an active (ProP_A) conformation of the monomeric transporter

then the observed equilibrium constant $K_{obs}^{monomer}$ for the activation process of eq 2A (active monomer), at the osmolality where the fraction of maximal activity is f , is given by

$$K_{obs}^{monomer} = f / (1 - f) \quad (3A)$$

If activation involves dimerization of the transporter, so that

then the equilibrium constant K_{dimer} for the activation process of eq 2B (active dimer) would be

$$K_{obs}^{dimer} \propto f / (1 - f)^2 \quad (3B)$$

where the proportionality constant is related to the effective concentration of membrane-bound ProP.

In the salt concentration range relevant for this analysis, Coulombic and Hofmeister effects of salts on the equilibrium constants, K_{obs} , for protein conformational changes or related processes are well approximated by³³

$$\ln K_{obs} = \ln K_0 + SK_{obs} \ln C - (m_G / RT) C \quad (4)$$

where K_{obs} is $K_{obs}^{monomer}$ or K_{obs}^{dimer} at the osmolality where the luminal salt concentration is C (which is equal to the salt anion concentration for the 1:1 and 2:1 salts investigated here), R is the gas constant, and T is the temperature in kelvin. SK_{obs} quantifies the Coulombic effect of luminal salt on $\ln K_{obs}$; m_G / RT quantifies the Hofmeister effect of luminal salt on $\ln K_{obs}$ (m_G is the derivative of the observed standard free energy change for the process with respect to salt concentration at high salt concentrations where Coulombic effects are negligible), and K_0 is equal to the extrapolated value of K_{obs} at $C = 1$ M salt for a salt exhibiting no net Hofmeister effect ($m_G = 0$).²⁹

Dominant Coulombic Effects—If Coulombic effects of luminal salts were dominant, and ProP activation were to involve interactions among functional groups on the protein or phospholipid with the same charge, activation would be favored by an increase in salt concentration C . In that case, the salt effects would be Coulombic, m_G would be zero, and the logarithm of the equilibrium constant K_{obs} would vary with the logarithm of the salt concentration C :

$$\ln K_{obs} = \ln K_0 + SK_{obs} \ln C \quad (5)$$

where K_0 in eq 5 is the value of K_{obs} at 1 M salt. For this case, SK_{obs} and K_0 for the active monomer model (eqs 2A and 3A) were obtained by fitting values of activity A at each salt concentration C to the following combined relationship (from eqs 3A and 5):

$$A = A_{max} K_{obs} / (1 + K_{obs}) \quad (6)$$

$$A = \frac{A_{max} \exp(\ln K_0 + SK_{obs} \ln C)}{1 + \exp(\ln K_0 + SK_{obs} \ln C)} \quad (7)$$

by nonlinear regression performed with SigmaPlot 12.5.

If ProP activation involves the interaction of highly negatively charged surfaces on the protein or phospholipid, then the Coulombic effects of salts on activation will be primarily cation effects.²⁹ For the salts investigated here, with univalent cations M^+ and uni- and/or divalent anions, eq 5 becomes

$$\ln K_{obs} = \ln K_0 + SK_{obs} \ln [M^+] \quad (8)$$

where K_0 in eq 8 is the extrapolated value of K_{obs} at a salt cation concentration $[M^+]$ of 1 M. In a mixed salt solution, where all salt cations are univalent, the symbol $[M^+]$ refers to the total salt cation concentration. The analog of fitting eq 7 for this case is obtained by replacing salt concentration C with total salt cation concentration $[M^+]$:

$$A = \frac{A_{max} \exp(\ln K_0 + SK_{obs} \ln [M^+])}{1 + \exp(\ln K_0 + SK_{obs} \ln [M^+])} \quad (9)$$

Dominant Hofmeister Effects—If Hofmeister effects of luminal salts were dominant, and osmotic activation was triggered predominantly by Hofmeister effects of the salt ions on uncharged surfaces of ProP ($SK_{obs} = 0$), the logarithm of the equilibrium constant K_{obs} (or equivalently the standard free energy change, G) for ProP activation would vary linearly with salt or salt ion concentration C , with slope $-m_G/RT$ (defined above):

$$\ln K_{obs} = \ln K_0 - (m_G/RT) C \quad (10)$$

The intercept, K_0 , would be the equilibrium constant at $C = 0$.²⁹ For this case, to obtain m_G/RT and K_0 for the active monomer model (eqs 2A and 3A), values of activity A at each salt concentration C were fit to the following combined relationship (from eqs 3A and 10):

$$A = \frac{A_{max} \exp[\ln K_0 - (m_G/RT) C]}{1 + \exp[\ln K_0 - (m_G/RT) C]} \quad (11)$$

by nonlinear regression performed with SigmaPlot 12.5.

Data Acquisition and Analysis—The contributions of Coulombic and Hofmeister effects to the osmotic activation of ProP were assessed as follows. For each data set

(obtained as described above), A was fit to the logarithm of the luminal ion concentration according to eq 9, or to the luminal ion concentration according to eq 11, for the monomer model (eq 3A), by nonlinear regression. The resulting $A_{\max}$ values are reported with the correlation coefficients (R^2) in Table S1 of the Supporting Information. The $A_{\max}$ values were used to compute the fraction of active ProP (f) according to eq 1. Next, the pooled values of f for up to nine replicate experiments (as indicated in Results) were fit to eqs 9 and 11, with f as the dependent variable, as described above. Resulting, global estimates for K_0 , SK_{obs} , and m_G/RT for these two limiting cases of the more general eq 4 are reported in Table 1. Figures 2, 5, and 6 illustrate these analyses with direct and linear plots of the relevant data and regression lines, as indicated. Some data points shown in direct plots of f versus the logarithm of the ion concentration or of the ion concentration (Figures 2A, 5A, and 6A) are absent from the corresponding linearized plots (Figures 2B, 5B, and 6B,C) because $\ln K_{\text{obs}}$ cannot be calculated for data points where experimental scatter results in $f < 0$ or $f > 1$ (monomer model) or in $f < 0$ only (dimer model) (illustrated by Figure S1 of the Supporting Information).

Dimer Model—The expression corresponding to Coulombic eq 9 for the quadratic, dimer model is

$$A = A_{\max} + 0.5e^E - \sqrt{A_{\max}e^E + 0.25e^{2E}} \quad (12)$$

where $E = \ln K_0 + SK_{\text{obs}} \ln[M^+] \equiv -G/(RT)$. Fits of the pooled data to eqs 9 and 12 were employed to assess whether the data better fit the monomer or the dimer version of the Coulombic model.

Previous Analyses—In previous work, data illustrating the dependence of ProP activity on the osmolality, the luminal ionic strength, or the concentration of a luminal solute were analyzed by nonlinear regression with eq 13, which was selected because it has the appropriate functional form:

$$a_0 = A_{\max} / \left[1 + e^{-(X - X_{1/2})/B} \right] \quad (13)$$

where a_0 is the initial rate of substrate (radiolabeled proline) uptake, X is the measured osmolality ($\Pi/\rho RT$), the calculated luminal ionic strength, or the calculated concentration of a luminal solute, $A_{\max}$ is the rate that would be attained at infinite osmolality, B is a constant, and $X_{1/2}$ is the value of X at which $a_0 = 1/2 A_{\max}$. Equation 13 is equivalent to eq 9; eqs 7, 9, and 11 are used here because they have the same functional form but provide thermodynamic interpretations of the parameters.

RESULTS

Dependence of ProP Activity on Luminal Cations

Salt ions modulate the equilibrium constants for protein processes by exerting Coulombic and Hofmeister effects (see eq 4 in Data Analysis).^{29,33} Coulombic effects of salt ions on processes involving charged biopolymers, typically dominant at low salt concentrations

(<0.5 M), are valence-specific but not otherwise ion-specific. For processes in which the number and/or density of biopolymer charges changes appreciably, Coulombic effects are described by eq 5 with a constant value of SK_{obs} , the slope of a log-log plot of K_{obs} versus salt concentration. Typically, SK_{obs} is determined by the change in the number and/or density of biopolymer charges in the process. Hofmeister effects of salt ions on biopolymer processes, typically most significant at moderately high salt concentrations (>0.5 M), are ion-specific. For processes in which the water accessible surface area (ASA) of the biopolymer changes significantly, Hofmeister salt effects are described by eq 10 with a constant value of the quantity $-m_G/RT$, the slope of a semilog plot of K_{obs} versus salt concentration. The value of m_G (the m -value) is determined by the non-Coulombic interactions of the salt ions with the biopolymer surface exposed or buried during the process. The m -value is proportional to the change in ASA with the proportionality constant describing the strength of the interaction.

Previous work suggested that luminal cations Na^+ , K^+ , Li^+ , and Cs^+ were equivalent contributors to ProP activation.^{21,28} To further explore the cation specificity of ProP and define the thermodynamics of ProP activation by salts, initial rates of proline uptake were determined with phosphate-buffered proteoliposomes that included Na^+ and K^+ in varying proportions. The assay medium osmolality was adjusted with sorbitol, and the membrane potential (Ψ) was clamped at -137 mV (see Experimental Strategy). Initial rates of proline uptake determined at each osmolality were fit to the monomer model (eqs 9 and 11) by nonlinear regression as described in Data Analysis. $[\text{M}^+]$ (eq 9) and C (eq 11) were the total cation (K^+ plus Na^+) concentration, calculated from the water loss at each osmolality. The regression parameters are listed in Table 1.

In Figure 2A, the relative activity attained at each osmolality ($f = A/A_{\text{max}}$) is plotted versus the logarithm of the total luminal cation concentration and the regression line was obtained with eq 9. This f versus $\ln[\text{M}^+]$ plot is sigmoidal, indicating the applicability of the simple two-state active monomer or active dimer model [eqs 3A and 3B, respectively (see Data Analysis)]. The K_0 value obtained by fitting the pooled data to eq 11 is small ($K_0 = 0.0017$), indicating that only a small fraction (0.17%) of transporters are in the active state under this initial condition. The convergence of the data representing different K^+/Na^+ ratios indicates that the contributions of Na^+ and K^+ to ProP activation are additive (Figure 2A), consistent with previous evidence that cation effects on ProP are not cation-specific. The data representing all K^+/Na^+ ratios were therefore pooled and are represented as a linear plot in Figure 2B. The similar or identical statistical parameters R^2 and F in Table 1 show that this regression analysis did not further distinguish between a Coulombic (eqs 5 and 9) and a Hofmeister (eqs 10 and 11) model for ProP activation.

Development of an Experimental System for Detecting Hofmeister Anion Effects on ProP

To test the hypothesis that luminal anions exert Hofmeister effects on ProP activity, anion phosphate was replaced with cation Bis Tris Propane (BTP) as the luminal buffer in proteoliposomes (see Experimental Strategy). This allowed luminal phosphate to be replaced by other anions (chloride and sulfate) in ProP activation experiments. Anions were used for these experiments as cations sodium and potassium fall together in the Hofmeister series. In

addition, the variety of cations that can be used for the analysis of membrane systems is limited by cation effects on lipid phase behavior (e.g., see ref 34).

The replacement of phosphate with BTP as the luminal buffer was validated as follows. ProP-His₆-containing proteoliposomes, prepared in K phosphate, were treated as described in Experimental Procedures to load them with K phosphate (0.178 M K⁺, 0.100 M phosphate, pH 7.4) or K BTP phosphate (0.110 M K⁺, 0.040 M BTP, 0.088 M phosphate, pH 7.4). Initial rates of proline uptake were determined as described above. The data obtained with these two preparations were very similar. They yielded similar plots of the relative activity attained at each osmolality ($f = A/A_{\max}$) versus the internal (luminal) phosphate concentration when fit to the monomer model (eq 11) (Figure 3).

Next, luminal phosphate was replaced with other anions. Initial rates of proline uptake were determined, as described above, for ProP-His₆-containing proteoliposomes that were buffered to pH 7.4 with BTP (0.04 M) and loaded with sulfate (0.07 M), phosphate (0.09 M), or chloride (0.14 M). The initial, internal K⁺ concentration was fixed at 0.11 M. The activity of the transporter was negligible for this initial condition. The assay medium osmolality was then adjusted with sorbitol, while the membrane potential (Ψ) was clamped at -137 mV (see Experimental Procedures). As for fully phosphate-buffered proteoliposomes, proline uptake was a linear function of time that increased as a function of external osmolality (and hence increasing internal salt concentration) (Figure 4). These observations suggest that BTP effectively replaced phosphate as the luminal buffer and that K⁺ at an initial concentration of 0.11 M was sufficient to generate the required membrane potential (Ψ).

Dependence of ProP Activity on Luminal Anions

The uptake rates attained with sulfate, phosphate, or chloride as the anion (e.g., Figure 4) were fit to the monomer model for Hofmeister effects (eq 11) by nonlinear regression as described in Data Analysis. The relative activity attained at each osmolality ($f = A/A_{\max}$) was plotted versus internal salt concentration C for each internal potassium salt. Figure 5A shows such normalized data, pooled from three independent osmolality titrations conducted with a single proteoliposome preparation and each salt anion. The K_0 values for each initial low-osmolality, low-salt condition were again small ($K_0 = 0.0015$), indicating that only a small fraction (0.15%) of transporters were in the active state under the initial condition. The data in Figure 5A are more scattered than those in Figure 2A, but the dependence of f on C is again sigmoidal, fitting eq 9 or 11 and indicating the applicability of the simple two-state active monomer or active dimer model (eq 3A or 3B, respectively).

Figure 5B shows the alternative plot of $-\ln K$ versus internal salt concentration C . The slopes of the plots in Figure 5B are negative and increase in magnitude from chloride to phosphate (a mixture of H₂PO₄⁻ and HPO₄²⁻ at pH 7.4) to sulfate (Figure 5B). This series could indicate that the osmotic activation of ProP is a Hofmeister effect, reflecting a conformational change that decreases the cytoplasm-exposed, solvent accessible, nonpolar surface area (ASA) of ProP.

However, analysis of the dependence of ProP activity on potassium ion concentration revealed an alternative interpretation. The data converged when the uptake rates attained with chloride, phosphate, or sulfate as the anion were plotted together as activity A versus luminal $[K^+]$ (Figure 6A; regression parameters for the pooled data listed in Table 1). This convergence indicates that the apparently different slopes (m_G/RT) of the regression lines obtained with sulfate, phosphate, and chloride (Figure 5B) arise primarily from their different valences, not from their different positions in the Hofmeister series. The regression parameters for the fit to eq 9 (relating A to $\ln C$) were similar to (F) or the same as (R^2) those for the fit to eq 11 (relating A to C) (Table 1 and the corresponding linear plot in Figure 6B). Thus, the regression analysis did not further distinguish between a Coulombic (eqs 5 and 9) and a Hofmeister (eqs 10 and 11) model for ProP activation.

Within error, the same SK_{obs} value of -6 was obtained with potassium in the presence of BTP (Figure 6 and Table 1), with potassium and sodium as luminal cations (Figure 2 and Table 1), and with the effects of external ions [discussed below (Table 2)]. Data for each salt anion, plotted separately as $\log K_{\text{obs}}$ versus $\log$ salt concentration, gave separate, parallel lines with slopes near -6 (Figure 6C). Collectively, these data indicate that the salt effect on ProP structure and activity is specifically a Coulombic, cation effect, a reasonable concept if the charged regions involved in activation are highly negatively charged.

Role of Dimerization in ProP Activation

Evidence summarized above indicates that *E. coli* ProP forms homodimers in which the dimer interface includes both transmembrane helix XII and the carboxyl-terminal, antiparallel homodimeric coiled coil (Figure 1). However, it is not clear whether dimerization is required for the transport activity or for the osmotic activation of ProP. To address that issue, the data presented in Figures 2 and 6 were fit to both the active monomer model (eq 3A) (as discussed above) and the active dimer model (eq 3B) for ProP activation. The statistical parameters obtained for the monomer model (fit to eq 9, Table 1) and for the dimer model (fit to eq 12) were similar. [For the dimer model, the data in Figure 2 yielded values of 0.93 (R^2) and 850 (F), whereas the data in Figure 6 yielded values of 0.77 (R^2) and 341 (F), all essentially the same as the corresponding values for the monomer model in Table 1.] Thus, these data do not discriminate between the monomer and the dimer model for the osmotic activation of ProP.

Dependence of ProP Activity on External Ions

In previous work, the same ProP activity was attained when diverse, membrane-impermeant salts or nonelectrolytes were used to impose the same osmotic upshift on proteoliposomes loaded with 0.1 M K phosphate.²⁴ This conclusion was verified and extended as follows. ProP activity was measured by using the sodium salt of chloride, bromide, sulfate, or citrate, rather than sorbitol, to vary the osmolality of the medium external to proteoliposomes prepared in 0.1 M K phosphate. Again, similar values of the equilibrium constant for ProP activation at 1 M K^+ (K_0) and similar SK_{obs} values were obtained when these data were fit to the monomer model with luminal K^+ as ion M^+ (eq 9, Table 2). These data reinforced the conclusion that ProP responds to the osmolality and the luminal cation concentration, not the chemical composition of the external medium.

DISCUSSION

In proteoliposomes, transporter ProP mediates proline-proton symport powered by an imposed protonmotive force. ProP activity is a sigmoid function of the assay medium osmolality and hence of the concentrations of luminal ions in that system. Here this phenomenon was interpreted in terms of a two-state model in which increasing osmolality favors the transition from an inactive to an active ProP monomer (eq 2A) or from an inactive ProP monomer to an active ProP dimer (eq 2B). The free energy (or the logarithm of the equilibrium constant, K_{obs}) for this transition was a logarithmic function of luminal cation concentration in proteoliposomes with diverse luminal ion compositions (Figures 2B and 6B) (see further discussion below). The data did not discriminate between the active monomer and active dimer models for ProP activation. These observations suggest that Coulombic effects of luminal cations activate ProP by increasing negative charge density via folding and/or association of negatively charged functional groups.

Each salt ion can exert both Coulombic and Hofmeister effects on biopolymer processes. Coulombic effects dominate many protein processes (e.g., protein denaturation) at low salt concentrations, whereas Hofmeister effects dominate such process at high salt concentrations.²⁹ The data reported in Figures 2 and 4–6 were analyzed according to the alternative hypotheses that either Coulombic or Hofmeister effects would account for the reported response. The statistical parameters for the alternative analyses were similar (Table 1), so those analyses did not rule out the possibility that the salt effects are Hofmeister anion effects. However, the conclusion that ProP activation results from a Coulombic salt cation effect is favored for the following reasons. First, osmotic activation was not luminal cation- or anion-specific, and hence, the Coulombic model describes the data in terms of one parameter (SK_{obs}) instead of three m -values for the three different salts (KCl, K/Na phosphate, and K sulfate). Second, very large changes in protein or membrane ASA would be predicted by the Hofmeister interpretation of the m -values obtained from these analyses (Table 2). Estimation as described by Record et al.²⁹ would indicate a change in solvent-exposed surface area (ΔASA) corresponding to folding of more than 200 amino acid residues. Third, the large ΔASA values arise because the salt concentration range for ProP activation is lower than that in which large Hofmeister effects are typically observed.

We find that cations activate ProP with an SK_{obs} of -6 . This means thermodynamically that six cations (K^+ and/or Na^+) are taken up in the conversion of the inactive transporter to the active transporter. In principle, this could mean that six cation-binding sites are present on the active form of the transporter (and not on the inactive form), that these bind Na^+ and K^+ very strongly, and that all six must be occupied for activation. This is possible but would be unprecedented for any protein process. On the other hand, nucleic acid conformational changes and protein–DNA interactions with SK_{obs} values equal to or exceeding ± 6 are common. They are interpreted as Coulombic effects on processes involving changes in charge density or number, not site binding.

Indeed, Coulombic effects are typically more pronounced for processes involving polyelectrolytes (nucleic acids and/or lipid bilayers) than for polyampholytes (proteins). Solvent cations accumulate at a high local concentration at polyanionic surfaces, powerfully

stabilizing high-charge density nucleic acid duplexes and lipid phases characterized by close packing of anionic headgroups (particularly those enriched with CL).³⁵ Such effects are clearly relevant here because the osmolality (and hence the luminal ion concentration) required to activate ProP varies directly with the proportion of anionic lipid (PG or CL) in proteoliposomes.²⁶ Such behavior is consistent with a mechanism in which activation requires an anionic region of ProP to approach the anionic membrane surface (see further discussion below).

Additional observations suggest that Hofmeister effects do not contribute to the osmotic activation of ProP *in vivo*. The cytoplasmic membranes of *E. coli* cells cultivated in high-osmolality, MOPS medium (0.77 mol/kg) and used for the analysis of ProP function *in vivo* included PE, PG, and CL with mole fractions 0.76, 0.17, and 0.07, respectively. In addition, ProP concentrates with CL at the cell poles where the mole fractions of PG and CL are at least 0.08 and 0.10, respectively (estimated via the analysis of minicells representing the cell poles).²⁶ In this study, ProP function was analyzed using proteoliposomes prepared with purified ProP-His₆ and a polar lipid extract from *E. coli* that includes PE, PG, and CL with mole fractions 0.71, 0.24, and 0.05, respectively (weight fractions reported by Avanti Polar Lipids, Inc., corrected with dominant molecular masses determined by Romantsov et al.³⁶). Thus, the anionic lipid content of the proteoliposomes used for this study exceeded that attained during analyses of ProP function, *in vivo*. This would have elevated the osmolality (and luminal ion concentration) at which ProP became active. In addition, cytoplasmic biopolymers are not present to impose volume exclusion effects on ProP in proteoliposomes, as they do in cells. If present, such volume exclusion would decrease the osmolality (and the luminal ion concentration) at which the transporter would become active.²⁸

These considerations reinforce the conclusion that the Coulombic effects of cytoplasmic cations modulate the conformation and function of ProP *in vivo* and *in vitro*, and that the contributions of the Hofmeister effects of these ions would be minimal. This conclusion differs from that based on previous work with the proteoliposome system.²⁸ The earlier work was based primarily on parameter $X_{1/2}$ of eq 13, which was selected because it has the appropriate functional form, rather than on the more appropriate parameter SK_{obs} of eq 9, which is derived above. In that work, as in this study, the effectiveness of luminal anions in activating ProP correlated with both their valences and their positions in the Hofmeister series. The full analysis presented here reveals that the anion valence determines the effectiveness of luminal salts by determining the cation concentration at a given anion concentration.

These observations have important implications for both the physiology of the osmotic stress response and the structural mechanism of osmosensing. Glutamate and other metabolites are the predominant cytoplasmic anions in osmotically stressed *E. coli*,²³ ranking in the Hofmeister series between sulfate and chloride. K^+ is the dominant cytoplasmic cation, and increasing external osmolality triggers K^+ uptake.²³ These considerations led to the early proposal that K^+ uptake is the primary response to increasing osmolality and K^+ glutamate serves as a second messenger. Osmolyte accumulation was seen as a secondary response, triggered by effects of K^+ glutamate on osmosensing transporters and the transcriptional machinery.³⁷ Our data support the proposal that an osmotically induced increase in

cytoplasmic cation concentration activates transporter ProP and that the physiologically relevant cation is K^+ . However, they also show that this response need not be secondary to active K^+ uptake. Rather, the increase in K^+ concentration that occurs upon osmotically induced, passive water efflux from proteoliposomes (and presumably cells) is sufficient. That sensitivity is achieved through the action of cytoplasmic K^+ on ionic functional groups present on the cytoplasmic surfaces of the transporter and/or the phospholipid membrane. Thus, osmolyte uptake can attenuate both K^+ uptake³⁸ and cytoplasmic dehydration. Other transporters (e.g., LacY¹⁵) are inactivated as ProP is activated in response to osmotic stress. Perhaps K^+ -induced conformational changes are also harnessed to inactivate such systems, transiently reserving available energy supplies for the osmoregulatory response.

Specification of a structural mechanism for osmosensing by ProP will require identification of the relevant, anionic functional groups as well as the inactive and active protein conformations. The cation dependence of ProP activation is characterized by an SK_{obs} of -6 (Table 1). An analysis of cation effects on a DNA oligonucleotide coil to helix transition suggested that, for that system, an SK_{obs} of -6 would reflect clustering of approximately 12–15 negative charges. This would be consistent with association of two coils, each with a net charge of -7 .³⁹

Figures 1B–D and 7 illustrate the occurrence of acidic residues residing on ProP surfaces that may be exposed to the cytoplasm. Those in the N- and C-terminal domains and loop C3 are most obviously available to fold and/or interact with each other or the membrane surface in response to an increasing cytoplasmic cation concentration (Figures 1A,B and 7). Loop C3 (Figure 1B) and the C-terminal domain (Figure 1C,D) include clusters of anionic residues. Figure 8 highlights the charged functional groups that are conserved among the ProP orthologues from *E. coli* (ProPEc), *Dickeya dadantii* (previously *Erwinia chrysanthemi*) (OusA), *Agrobacterium tumefaciens* (ProPA_t), and *Xanthomonas campestris* (ProPX_c), all of which function as osmoregulatory transporters.^{18,20,40,41}

The C-terminal sequences that form antiparallel, intermolecular coiled coils in proteins ProPEc and ProPA_t (and likely OusA) include regions with high anion charge density (Figure 8),⁴² suggesting that an increasing cation concentration would influence the balance among coiled-coil formation, dissociation of the coiled coil, and association of the C-termini with other anionic molecular surfaces.²⁶ However, the activities of ProPEc, ProPA_t, and ProPX_c respond very similarly to the osmolality upon expression in *E. coli*,^{20,43} even though most of the coiled-coil sequence is replaced with a weakly charged, nonpolar peptide in ProPX_c. Furthermore, ProPEc activity remained osmolality-sensitive after deletion of 11 or 18 C-terminal residues.¹⁹ Evidence implicates the coiled coil in targeting ProP to the bacterial cell poles and modulating the osmolality response.²⁶ Taken together, these observations suggest that the anionic residues essential for osmosensing reside elsewhere.

The membrane-integral domain of ProPEc is linked to the coiled-coil-forming sequence by residues M438–Y471. This peptide is predicted to include α -helical, coil, and intrinsically disordered segments (Figure 7). The proximity of this peptide to the membrane and experimental data⁴⁴ suggest that the structure of this peptide will be influenced by interaction with phospholipid headgroups. Acidic residues are conserved among all known

ProP orthologues at four positions within this sequence (Figure 8); conserved residue E440 is essential for ProP activity,¹³ and some of the conserved residues would cluster if this peptide became α -helical (Figure 1D). Increasing the cytoplasmic cation concentration may favor clustering of such carboxylates with other cytoplasmic surfaces of ProP and/or the phosphates of adjacent lipid headgroups, thereby favoring an active transporter conformation. Phospholipid headgroup clustering may also modulate transporter conformation by altering the physical properties of the membrane.²³

Past efforts to understand osmosensing have focused on osmosensing transporters ProP, OpuA, and BetP.⁶ Extensive studies have been conducted with OpuA from *Lactococcus lactis* (an ABC transporter related to osmoregulatory transporter ProU of *E. coli*) and BetP from *Corynebacterium glutamicum* (a Na⁺-betaine symporter and a member of the betaine-carnitine-choline transporter family like osmoregulatory transporters BetT and BetU of *E. coli*). Despite fundamental differences in structure and energy coupling mechanism, the activities of ProP, OpuA, and BetP are sigmoid functions of the osmotic pressure in intact cells and, after purification, in proteoliposomes. In each case, the proteoliposome system can mimic the intact cell, the cytoplasmic transporter surface being exposed to the proteoliposome lumen. (For OpuA, transporters with “cytoplasmic surface in” topology are selected by supplying ATP in the proteoliposome lumen.) For each transporter, the osmotic activation threshold varies directly with the anionic lipid content of the proteoliposome membrane^{26,45,46} and a cytoplasmic domain has been implicated in osmosensing.⁶

Transporter OpuA activates as inorganic ions (and not sucrose) concentrate at the transporter's cytoplasmic surface, and half-maximal activity was attained at a (total) cation concentration of approximately 0.17 M for OpuA reconstituted in proteoliposomes comprised of *E. coli* lipid.^{34,47} BetP activity correlates with the luminal K⁺ concentration in proteoliposomes, reaching a half-maximal level at 0.22 M K⁺ in proteoliposomes comprised of *E. coli* lipid.⁴⁸ Each of these systems differs from ProP in showing cation specificity: Na⁺ did not activate Na⁺ symporter BetP, while Rb⁺ and Cs⁺ inhibited OpuA. Neither system has been analyzed in the manner reported here, however, and no K⁺-specific, cytoplasm-facing binding site has been identified for either system. An additional signal transmitted via the membrane was invoked because the K⁺ response was insufficient to account for BetP activation *in vivo*.⁴⁹

Despite the published evidence of cation-specific activation of OpuA and BetP, it is appealing to suggest that ProP, BetP, and OpuA are activated as cations (particularly K⁺) concentrate in the cytoplasm of bacterial cells under osmotic stress, and that this effect is Coulombic in origin. The avoidance of secondary effects that become apparent when solutes are provided at high concentrations, as cosolvents, is a major challenge for analyses of osmosensory mechanisms. For this study of proton symporter ProP, such effects precluded the use of divalent cations, or of anions that are membrane-permeant in the ionized and/or neutral state (e.g., SCN⁻ and acetate), that interfere with scintillation counting (I⁻), or that are contaminated with oxidizing agents (e.g., HBr, which is contaminated with Br₂). Despite rigorous efforts to avoid such issues, perhaps the apparent cation specificity of OpuA and BetP arises from secondary ion effects on their ion coupling mechanisms (ATP hydrolysis

and Na⁺ symport, respectively). If so, this report may present a unified mechanism for osmosensing by ProP, OpuA, and BetP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ψ	membrane potential
ASA	water accessible surface area of a biopolymer
A	initial rate of substrate (radiolabeled proline) uptake at a given osmolality
$A_{\max}$	maximal rate of proline uptake via ProP extrapolated to infinite osmolality
BSA	bovine serum albumin
BTP	1,3-bis[tris(hydroxymethyl)-methylamino]propane
CL	cardiolipin
EDTA	ethylenediami-netetraacetic acid
f	fraction of the maximal transport rate ($A_{\max}$) attained under particular conditions
MFS	major facilitator superfamily
MOPS	4-morpholinopropanesulfonic acid
PDB	Protein Data Bank
PE	phosphatidylethanolamine
Osm	osmolality in moles per kilogram (osmolality is $-55.5 \ln a_w = \Pi/\rho RT$, where a_w is the water activity, Π is the osmotic pressure, and ρ is the water density)
PEG	poly(ethylene glycol)
PG	phosphatidylglycerol
R	gas constant

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Figure 1.

Structure of ProP. (A) Schematic representing the membrane topology of ProP that was validated by LacZ and PhoA fusion analysis and chemical labeling of introduced Cys residues.^{11,13,14} P1–P6 designate periplasmic loops 1–6, respectively, and C1–C5 designate cytoplasmic loops 1–5, respectively. The red rectangle designates the extended C-terminal domain common among ProP orthologues, but not among paralogues that are not osmosensory transporters.⁴⁰ The gray rectangle designates the further-extended C-terminal domain, shared among a subset of ProP orthologues, that forms the intermolecular antiparallel α -helical coiled coil illustrated in panel D. See Figures 7 and 8 for further illustrations of these C-terminal sequences. (B) Homology model of ProP (residues D19–S454) based on a crystal structure of *E. coli* XylE (PDB entry 4GBZ). XylE was the best match to ProP according to searches performed with Modeler via the ModWeb Server and with Protein Homology/analogY Recognition Engine version 2.0 (Phyre 2) in July 2015. The illustrated Phyre 2 model retains the intramembrane cluster of functionally important, ionizable residues identified earlier (circled in panel B).^{12,14} Unlike earlier models based on

GlpT,^{11,12} this image shows cytoplasmic loop 3 (Loop C3). However, the cytoplasmic N- and C-termini remain truncated. Acidic and basic residues are colored red and blue, respectively. The solvent-inaccessible portions of transmembrane helices I and XII^{13,14} are otherwise colored black to illustrate the position of the interface between the acyl chains and the phospholipid headgroups at the cytoplasmic membrane surface (marked with a dotted line). (C) Helical wheel representation of the amphipathic α -helical peptide corresponding to residues E440–E471 of *E. coli* ProP (drawn with heliQuest⁵⁰). Positions 440, 455, 458, 465, and 471 are labeled because they are occupied by Asp or Glu in each of the sequences illustrated in Figure 8. Residues are colored as follows: red, Asp and Glu; pink, Asn and Gln; purple, Thr and Ser; blue, Lys, Arg, and His; green, Pro; yellow, Ile, Leu, Val, and Tyr; gray, Gly and Ala. The subscript N designates N-terminal E440, the subscript C C-terminal E471, and the arrow the hydrophobic moment. This peptide is predicted to be moderately surface-seeking on the basis of its hydrophobic moment ($\langle \mu_H \rangle = 0.339$) and hydrophobicity ($\langle H \rangle = 0.170$).⁵¹ (D) NMR structure of the homodimeric antiparallel coiled coil formed by residues 468–497 of ProP (PDB entry 1R48). Residues are colored red (acidic), blue (basic), green (polar), or yellow (nonpolar). Residue R488 (ball and stick) resides in a coiled-coil heptad “a” position and forms salt bridges that stabilize the antiparallel coiled-coil orientation *in vitro* and *in vivo*.^{17,18,42,52}

Figure 2.

Dependence of ProP activity on luminal K^+ and Na^+ in proteoliposomes. Proteoliposomes were prepared and proline uptake rates measured as described in Experimental Procedures. (A) Proteoliposomes prepared in 0.1 M K phosphate were loaded with 0.10 M K phosphate ($\diamond$), 0.07 M K phosphate with 0.03 M Na phosphate ($\square$), or 0.06 M K phosphate with 0.04 M Na phosphate ($\circ$), as described. Data derived from two replicate experiments for each luminal composition were fit to eq 9 by nonlinear regression with $[M^+]$ as the total luminal cation concentration (K^+ plus Na^+) as described in Data Analysis. The plot shows f vs the

logarithm of the total luminal cation concentration and the corresponding regression line. The regression parameters are listed in Table 1. (B) $\ln K_{\text{obs}}$ for the active monomer model plotted vs the $\ln$ of the total cation concentration ($\ln[M^+]$) according to eq 8. The line is drawn according to regression parameters K_0 and SK_{obs} obtained as described in the legend for panel A and reported in Table 1.

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Figure 3.

Replacement of phosphate with Bis Tris Propane (BTP) as the luminal buffer.

Proteoliposomes prepared in 0.1 M K phosphate were kept in that buffer or loaded with K BTP phosphate, and initial rates of proline uptake were measured as described in Experimental Procedures. Filled symbols and lines denote data obtained with K BTP phosphate, whereas empty symbols and dashed lines represent data obtained with K phosphate. The lines were obtained by fitting each data set to eq 11 by nonlinear regression with salt concentration C .

Figure 4.

Measurement of the osmolality dependence of proline uptake via ProP. Proteoliposomes were prepared and proline uptake rates measured as described in Experimental Procedures. Proteoliposomes prepared in 0.1 M K phosphate were loaded with K BTP X where X was sulfate (top), phosphate (middle), or chloride (bottom). Initial rates of proline uptake were measured as described in Experimental Procedures by diluting proteoliposomes into the corresponding Na BTP X buffers supplemented with sorbitol to attain the indicated osmolalities. Uptake rates were obtained as the slopes of the lines generated by linear

regression of the resulting data. In this filtration assay, proline in the assay solution external to the trapped cells and binding of radiolabeled proline to the cells and filters contribute to a background value that can be estimated by extrapolation to time zero (the intercept of each regression line).

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Figure 5.

Dependence of ProP activity on luminal anions in proteoliposomes. Proteoliposomes were prepared and proline uptake rates measured as described in Experimental Procedures. Proteoliposomes prepared in 0.1 M K phosphate were loaded with K BTP X, where anion X was sulfate (○), phosphate (□), or chloride (◇). Initial rates of proline uptake were measured by diluting proteoliposomes into the corresponding Na BTP X buffers as described in Experimental Procedures. Three independent experiments were performed with duplicate assays in each experiment. Parameters K_0 and m_G/RT for the active monomer

model (eqs 2A and 3A) were obtained by fitting the pooled data for each anion to eq 11 by nonlinear regression as described in Data Analysis, yielding the regression lines shown in panel A (see also Table S1 of the Supporting Information). In panel B, $\ln K_{\text{obs}}$ for the active monomer model is plotted vs salt concentration according to eq 10.

Figure 6.

Origin of the effect of luminal K^+ on ProP activity in proteoliposomes. Data introduced in Figure 5 were further analyzed and are presented as follows. (A) Parameters K_0 and m_G/RT for the active monomer model (eqs 2A and 3A) were obtained by fitting the pooled data to eq 11 by nonlinear regression with K^+ as ion concentration C as described in Data Analysis. The regression parameters are listed in Table 1, and the data are plotted with the corresponding regression line in panel A. Again, the anion was sulfate ($\circ$), phosphate ($\square$), or chloride ($\diamond$). (B) Parameters K_0 and SK_{obs} for the active monomer model (eqs 2A and

3A) were obtained by fitting the pooled data to eq 9 with K^+ as ion M^+ as described in Data Analysis. The $\ln K_{\text{obs}}$ for the active monomer model is plotted vs the logarithm of the potassium concentration, according to eq 8. The symbols are defined as for panel A, and the line drawn was according to regression parameters K_0 and SK_{obs} (Table 1). (C) Parameters K_0 and SK_{obs} for the active monomer model (eqs 2A and 3A) were obtained separately for the data set representing each salt anion by fitting the data to eq 9 with K^+ as ion M^+ by nonlinear regression. The $\ln K_{\text{obs}}$ for the active monomer model is plotted vs the logarithm of the salt (anion) concentration, according to eq 5. The symbols are defined as for panel A, and the lines were drawn according to regression parameters K_0 and SK_{obs} obtained separately for each salt.

N-Terminus	+6	<-TMI
2° Structure:	CCCCCCCCCCCCCHCCCCCHHHHHHHHHHHHHHHHH	
Sequence: 1-	<u>MLKRKKVKPITLRDVTIIDDGKLRKAITAASLGNA</u>	-35
Loop C1	TMII-> +2 <-TMIII	
2° Structure:	HHHHHHHHHHCCHHHHH	
Sequence: 84-	<u>LFFGMLGDKYGRQKILA</u>	-100
Loop C2	TMIV-> -1 <-TMV	
2° Structure:	HHHHHHHHHHHHHHHHHHCCCHHHHHHHHHHHHHHH	
Sequence: 139-	<u>FSVGGEYTGASIFVAEYSPDKRGFMGSWLD</u> FG	-171
Loop C3	TMVI-> +1 <-TMVII	
2° Structure:	HHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHHHHHHCCCHHHHHHHHHCCCHHHHHHHHH	
Sequence: 212-	<u>IIIGLYLRHAL EETPAFQQHVDKLEQGDREGLQDGP</u> KVSFKEIATKYWRSLTLCIGL	-267
Loop C4	TMVIII-> +2 <-TMIX	
2° Structure:	HHHHHHHHHHHHHHHHHHCCCHHHHHHHHH	
Sequence: 305-	<u>MIGMLFVQPVMGLLSDRFGRRPFVLLGS</u>	-332
Loop C5	TMX-> +2 <-TMXI	
2° Structure:	HHHCHHHHHHHHHCCCHHHHHHHHHHHHHHH	
Sequence: 367-	<u>CFTGVMASLTLPAMFP</u> THIRYSALAAAFNI	-395
C-terminus:	TMXII-> +2	
2° Structure:	HHHHHHHCCCCCCCCCCCCCHHHHC	
Sequence: 431-	<u>GLITGVTMKETANRPLKGATPAAS</u>	-454
C-terminus:	-7 +3	
CC Heptads:	-defgabcdefgabcdefgabcdefgabcdefgabc---	
2° Structure:	CHHHHHHHHHHHCCCHHCCCCCCCCCCCCCHHHHHHHHHHHCCCCC	
Sequence: 455-	<u>DIQEAK EILVEHYDNIEQKIDDI</u> DEIADLQAKRTRLVQQHPRIDE	-500

Figure 7.

Properties of cytoplasm-exposed ProP loops and terminal domains. Sequences and net charges of the cytoplasm-exposed loops [C1–C5 (see Figure 1A)] and terminal domains of *E. coli* ProP (AAC77072.1) are shown with letters designating acidic residues (D and E) colored red and letters designating basic residues (H, K, and R) colored blue. The C-terminal domain includes a cluster of negatively charged residues (455–483, net charge of –7) flanked by sequences with net positive charges. The coiled-coil (CC) heptad repeats are shown above the C-terminal sequence. Bold font designates the part of this sequence (residues 468–497) that forms an antiparallel coiled coil as illustrated in Figure 1D.⁴² The limits of the loops are assigned on the basis of the homology model described by Wood et al.¹¹ or determined experimentally.¹³ Secondary structure is also designated according to the homology model¹¹ (H, helix; C, coil). Underlined sequences are predicted to be disordered according to DisEMBL 1.5, with the Hot-loops definition.⁵³

Figure 8.

Conservation of large, cytoplasm-exposed ProP loops and terminal domains. Sequences of the cytoplasmic N- and C-termini and the large, central cytoplasmic loops [loops C3 (Figure 1A)] of ProP orthologues from *E. coli* (ProPEc), *D. dadantii* (previously *Er. chrysanthemi*) (OusA), *A. tumefaciens* (ProPA_t), and *X. campestris* (ProPX_c) are aligned according to a full length sequence alignment performed with CLUSTALW.⁵⁴ All of these proteins function as osmoregulatory transporters.^{18,20,40} Letters designating acidic residues (D and E) are colored red, and letters designating basic residues (H, K, and R) are colored blue. Conserved residues are marked with asterisks. The alignment of the N-termini includes the first 10 residues of TMI to illustrate conservation of functionally essential residues E37 and D40.¹⁴ The heptad repeats yielding the known coiled-coil structures of ProPEc and ProPA_t^{16-18,42,52} and the predicted coiled-coil structure of OusA are shown above the alignment of the C-termini. Bold font designates the part of this sequence that forms the antiparallel coiled coil illustrated in Figure 1D.⁴²

Table 1
Dependence of Global Regression Parameters on Luminal Cation Concentration

luminal ion(s) ^a	Coulombic analysis ^b					Hofmeister analysis ^c			
	$-\ln K_0$	SK_{obs}	R^2 ^d	F ^d	ν_1/ν_2 ^d	$-\ln K_0$	$-m_G/RT$ (M ⁻¹)	R^2 ^d	F ^d
K/Na	-7.3 ± 0.6	-6.0 ± 0.5	0.93	851	2/129	6.4 ± 0.4	21.5 ± 1.5	0.93	821
BTP K	-10.1 ± 1.1	-6.0 ± 0.6	0.77	343	2/205	6.4 ± 0.6	34.5 ± 3.4	0.77	341

^aProteoliposomes were prepared; their luminal composition was adjusted, and transport assays were performed as described in Experimental Procedures. The proteoliposomes contained K and/or Na phosphate (K/Na) or BTP K sulfate, phosphate, or chloride (BTP K).

^bFor each experiment, parameter A_{max} was obtained by fitting A to $\ln[M^+]$ (where $[M^+]$ was the luminal K^+ plus Na^+ concentration) by nonlinear regression according to eq 9. The resulting values for A_{max} , listed in Table S1, were used to compute $f(A/A_{\text{max}})$ according to eq 1. For the pooled data, $\ln K_0$, SK_{obs} , R^2 , F , and ν_1/ν_2 were then obtained by fitting f to $\ln[M^+]$ by nonlinear regression according to eq 9. This analysis is illustrated in Figures 2, 5, and 6 as discussed in the text.

^cFor each experiment, parameter A_{max} was obtained by fitting A to C (where C was the luminal K^+ plus Na^+ concentration) by nonlinear regression according to eq 11. The resulting values for A_{max} , listed in Table S1, were used to compute $f(A/A_{\text{max}})$ according to eq 1. For the pooled data, $\ln K_0$, m_G/RT , R^2 , F , and ν_1/ν_2 were then obtained by fitting f to C by nonlinear regression according to eq 11. This analysis is illustrated in Figures 2, 5, and 6 as discussed in the text.

^dThe statistical parameters are R^2 , the square of the correlation coefficient; F , the variance ratio (treatments mean square/error mean square); and ν_1 and ν_2 the degrees of freedom of the numerator and denominator of the variance ratio, respectively. The ν_1 and ν_2 values are the same for analyses based on eqs 9 and 11.

Table 2
Impacts of External Anions on the Osmotic Activation of ProP^a

external anion	$A_{\max}$ [$\mu\text{mol min}^{-1}$ (mg of ProP) ⁻¹]	$-\ln K_0$	SK_{obs}
chloride	2.35 ± 0.26	-6.0 ± 1.7	-5.5 ± 1.4
bromide	2.03 ± 0.11	-6.4 ± 1.1	-5.8 ± 0.8
sulfate	1.76 ± 0.10	-7.2 ± 1.4	-6.1 ± 1.1
citrate	2.36 ± 0.14	-8.0 ± 1.9	-6.8 ± 1.5

^aProteoliposomes were prepared in K phosphate buffer (0.1 M), and transport assays were performed in Na phosphate buffer (0.1 M) supplemented with the Na salt of the indicated anion to adjust osmolality and hence the luminal K phosphate concentration. Parameters $A_{\max}$, $-\ln K_0$, and SK_{obs} were obtained by fitting A to $\ln[M^+]$ (the luminal K^+ concentration) according to eq 9 as described in Experimental Procedures.