Purification and Reconstitution of an Osmosensor: Transporter ProP of *Escherichia coli* Senses and Responds to Osmotic Shifts†

K. I. Racher, R. T. Voegele,‡ E. V. Marshall, D. E. Culham, and J. M. Wood*

Department of Microbiology and Guelph Waterloo Centre for Graduate Work in Chemistry, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

H. Jung

Abteilung Mikrobiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany

M. Bacon, M. T. Cairns, S. M. Ferguson, W.-J. Liang, and P. J. F. Henderson

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

G. White and F. R. Hallett

Department of Physics, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

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ABSTRACT: The ProP protein of *Escherichia coli* is an osmoregulatory H^+-compatible solute cotransporter. ProP is activated by an osmotic upshift in both whole cells and membrane vesicles. We are using biochemical and biophysical techniques to explore the osmosensory and catalytic mechanisms of ProP. We now report the purification and reconstitution of the active transporter. Protein purification was facilitated by the addition of six histidine (His) codons to the 3′ end of *proP*. The recombinant gene was overexpressed from the *E. coli galP* promoter, and ProP−$(His)_6$ was shown to be functionally equivalent to wild-type ProP by enzymatic assay of whole cells. ProP−$(His)_6$, purified by Ni^{2+} (NTA) affinity chromatography, cross-reacted with antibodies raised against the ProP protein. ProP−$(His)_6$ was reconstituted into Triton X-100 destabilized liposomes prepared with *E. coli* phospholipid. The reconstituted transporter mediated proline accumulation *only* if (1) a membrane potential was generated by valinomycin-mediated K^+ efflux *and* (2) the proteoliposomes were subjected to an osmotic upshift (0.6 M sucrose). Activity was also stimulated by ΔpH. Pure ProP acts, in the proteoliposome environment, as sensor, transducer, and respondent to a hyperosmotic shift. It is the first such osmosensor to be isolated.

Specific ligand−receptor interactions, used by all cells to sense changes in the biochemistry of their environment, are well understood. Cells also adapt to their physicochemical environment, including the nature of the solvent which bathes them. The mechanisms by which cells adapt to their physical environment are not well understood (*1*). For example, unlike changes in nutrient supply or the dissemination of biochemical messages, osmolality changes cannot be sensed via structure-specific ligand−receptor interactions that occur at specific subsites on receptor molecules (*2*).

The goal of our research is to understand how cells perceive and respond to changes in the osmolality of their environment. Plant, animal, and microbial physiologists share interests in osmosensory and osmoregulatory mechanisms because they are central to such diverse phenomena as salinity tolerance in plants, kidney physiology, microbial pathogenesis, and the cultivation of microorganisms for biotechnology industries. Much has been learned about cellular responses to osmotic shifts (*3*−*10*). For example, recent studies have delineated the genes and enzymes required for osmoadaptation by the bacterium *Escherichia coli* (*10*). In contrast, both osmosensory mechanisms and the cellular changes that are perceived by osmosensors remain elusive (*11*−*13*).

Modulation of cytoplasmic solvent composition is the only response that reverses the growth inhibitory effects of environmental osmolality changes. The active uptake of certain organic solutes, often denoted compatible solutes, most effectively attenuates the impact of hyperosmotic shifts on bacteria. Solutes are denoted “compatible” if, although

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* Correspondence should be addressed to this author at the Department of Microbiology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada. Telephone: 519 824 4120, Ext. 3866. FAX: 519 837 1802. Email: jwood@uoguelph.ca.

‡ Present address: Lehrstuhl Phytopathologie, Fakultät für Biologie, Universität Konstanz, Universitätsstrasse 10, 78457 Konstanz, Germany.

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Table 1: Strains and Plasmids

strain[a] or plasmid	genotype	source or reference
RM2	F$^-$ *trp lacZ rpsL thi* Δ(*putPA*)*101*	(*52*)
WG170	RM2 *proP*219	(*53*)
WG210	RM2 *srl-300*::Tn*10 proU*205	(*36*)
WG266	RM2 *proP*219 Δ(*proU*)*600*	(*54*)
WG269	RM2 Δ(*proU*)*600 zjd-351*::Tn*10*	(*54*)
WG350	WG269 Δ(*proP-melAB*)*212*	(*15*)
WG389	RM2 Δ(*proU*)*600* Δ(*proP-melAB*)*212* lac$^+$ Δ(*brnQ phoA proC*)	(*15*)
WG566	WG266 *lac*$^+$ Δ(*brnQ phoA proC*)	P1/WG389→WG266
WG571	WG566 *proP*$^+$	P1/WG269→WG566
pGEM4		Promega Corp.
pBR322		(*55*)
pDC1	5.0 kb fragment carrying gene *proP* inserted into pGEM4	(*54*)
pMTC15	pBR322 derivative carrying *galP* gene	(*38*)
pDC44	1.6 kb insert carrying gene *proP* replacing *galP* gene in pMTC15 (gene *proP* under control of *galP* promoter)	this work
pMB11	derivative of pDC44 carrying gene for ProP−(His)$_6$ under control of *galP* promoter	this work
pTrc99a/putP(FH)	*putP(FH)* (carrying Flag epitope and hexa-histadine sequence at C-terminus) cloned into plasmid pTrc99a (gene *putP(FH)* under control of tac promoter)	(*23*)

[a] All strains are derivatives of *E. coli* K-12.

they accumulate to high (up to molar) cytoplasmic concentrations, they are benign or even stabilizing for cytoplasmic macromolecules and their functions. For *E. coli*, compatible solute uptake is mediated by transporters ProP and ProU. ABC-transporter ProU is present only when the bacteria are cultivated in a hypertonic medium. Though *proP* is also inducible, its constitutive expression renders transporter ProP available as a primary respondent to hyperosmotic shifts. The substrates of ProP include proline, glycine betaine, proline betaine, ectoine, and a wide variety of structurally related molecules (*10*, *14*).

ProP is predicted to be a 500 amino acid protein that spans the cytoplasmic membrane 12 times, with cytoplasmic N- and C-termini (*15*). The membrane topology of the protein has not been determined experimentally, however. Proline transport via ProP is activated 5-fold when *E. coli* cells are subjected to a hyperosmotic shift with a membrane-impermeant osmolyte (sucrose or NaCl)(*16*). Activation by glycerol, which rapidly equilibrates across the cytoplasmic membrane, is transient. ProP was also activated in cytoplasmic membrane vesicles when hyperosmotic shifts were imposed with NaCl or sucrose, but not glycerol. Whereas the half-times for ProP activation in cells and vesicles were the same (approximately 1 min), the hyperosmotic shifts required for maximal activation of ProP differed (they were 0.2 and 0.6 M sucrose, respectively)(*16*, *17*). These observations indicated that transporter ProP of *E. coli* is an osmoregulator and that the mechanism for osmotic activation of ProP is intrinsic to the cytoplasmic membrane. They did not, however, demonstrate whether ProP, itself, is capable of sensing osmotic changes.

Here we report the overexpression and purification of transporter ProP. The transporter retained activity upon reconstitution in proteoliposomes comprised of *E. coli* phospholipid. The requirements for this activity included the imposition of *both* a membrane potential (vesicle lumen negative) *and* an osmotic upshift. A proton gradient (vesicle lumen alkaline) was also stimulatory. These results show that the ProP protein can sense hyperosmotic shifts. Thus, ProP is both an osmosensor and an osmoregulator. These results are also consistent with other data which indicate that ProP is a H^+/compatible solute cotransporter (*14*, *15*).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. Table 1 contains a description of the bacterial strains and plasmids used in this study. Bacteriophage P1 transduction was performed as described (*18*). Strain WG566 was constructed by P1 transduction of strain WG266 with a P1 lysate prepared from strain WG389. The selection was for growth on 4-morpholinopropanesulfonic acid (MOPS)[1] medium, supplemented with 0.2% (w/v) lactose as carbon source and 25 mM proline; screening was for no growth on lactose (0.2%) minimal plates not supplemented with proline. Standard molecular biological methods were as described (*19*, *20*).

The *galP* gene was first cloned by insertion of a 2.4 kb *Pvu*II fragment, carrying *galP*, into the *Pvu*II site of plasmid pBR322 (P. E. Roberts, unpublished results). To create plasmid pMTC15, this construct was modified by directed mutagenesis to create an *Nco*I restriction site in the *galP* start codon, together with the elimination of an *Eco*RI fragment containing the tetracycline resistance gene.

For construction of plasmid pDC44, a 1.6 kb DNA fragment containing the *proP* gene was amplified by the polymerase chain reaction (PCR) [conditions described by (*21*)] using plasmid pDC1 (*15*) as the template. The oligonucleotide primers used were a 28-mer (nucleotides −16 to +12 at the 5′ end of the *proP* gene) designed to change two nucleotides surrounding the ATG start codon in order to create an *Nco*I restriction endonuclease cleavage site (5′-CTATGC-3′ to 5′-CCATGG-3′) and a 34-mer (complementary to nucleotides 79−105 downstream of the *proP* gene) designed to add a *Hin*dIII restriction site. The amplified DNA was digested with *Nco*I and *Hin*dIII (GIBCO BRL Life

[1] Abbreviations: MOPS, 4-morpholinopropanesulfonic acid; PCR, polymerase chain reaction; His-tag, six consecutive histidine residues; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); Ni-NTA, nickel nitrilotriacetic acid; octyl glucoside, octyl β-D-glucopyranoside; dodecyl maltoside, dodecyl β-D-maltoside; val, valinomycin.

Technologies Inc., Burlington, Ontario), ligated into the 3.0 kb *Nco*I/*Hin*dIII fragment of pMTC15, and transformed into strain DH5α (*20*). Transformants were selected on LB plates containing 100 μg/mL ampicillin. Plasmid pDC44, which had the expected restriction endonuclease pattern, was isolated and subsequently transformed into strain WG389 using the same selective medium. A resulting transformant containing plasmid pDC44 had regained the ability to grow on MOPS medium supplemented with only 25 μM proline. Further analysis of the plasmid revealed that it had the *galP* promoter region from pMTC15, the entire *proP* gene with one base change at position +4 (C to G) resulting in one amino acid change (L2V), and the *proP* termination region (105 bp downstream of the *proP* gene). The plasmid-encoded *proP* sequence was confirmed at MOBIX (McMaster University, Hamilton, Ontario).

PCR was used to insert a sequence encoding six consecutive histidines (His-tag) at the 3′ end of the *proP* gene. Plasmid pDC44 was used as the template for PCR with two oligonucleotide primers. The forward primer was a 23-mer corresponding to nucleotides +96 to +118 (from the 5′ end) in *proP*. The reverse primer (a 59-mer) was designed to replace the stop codon and 102 bp of 3′-noncoding sequence of *proP* (in plasmid pDC44) with the His-tag sequence and another stop codon. The 1.4 kb amplified fragment was digested with restriction enzymes *Sma*I and *Hin*dIII and then subcloned into similarly digested pDC44. The resulting vector, pMB11, is 84 bp smaller than pDC44. The *proP*−*(His)*$_6$ gene was sequenced in its entirety by GenAlyTiC (University of Guelph, Guelph, Ontario).

Bacterial Growth. For genetic constructions or DNA preparation, bacterial strains were routinely grown in LB medium (*18*). For transport assays, bacteria were cultured in either LB medium or MOPS-based minimal medium (*22*), the latter supplemented with 9.5 mM NH_4Cl as nitrogen source and 0.4% (v/v) glycerol as carbon source unless otherwise indicated. To meet auxotrophic requirements, 245 μM L-tryptophan, 1 μg/mL thiamine hydrochloride, and 25 mM L-proline were added, when necessary. Osmotic stress was imposed by including 0.3 M NaCl in the growth medium, as indicated. Strains were routinely grown at 37 °C under aerobic conditions to an optical density of 1.0 at 600 nm. If necessary, antibiotics were included at the following concentrations: ampicillin, 100 μg/mL; streptomycin, 100 μg/mL; tetracycline, 15 μg/mL.

Preparation and Fractionation of Membrane Vesicles. Membrane vesicles were prepared by passing the cells, which had been harvested and washed as described (*16*), 4 times through a French pressure cell at a cell pressure of 1600 bar. Cell debris was removed by centrifugation at 12000*g* for 15 min, and membrane vesicles were collected by ultracentrifugation at 100000*g* for 90 min. The vesicles were resuspended in 100 mM KH_2PO_4, pH 6.6, and stored at −70 °C. For membrane fractionation, the final vesicle pellet was resuspended in a minimum volume of 25% (w/w) sucrose in 20 mM Tris-HCl, 0.5 mM Na-EDTA, pH 7.5. This membrane suspension was layered onto a discontinuous sucrose gradient [55%, 50%, 45%, 40%, 35%, 30% (w/w) sucrose in the same buffer] prepared in 65 mL centrifuge tubes. The tubes were centrifuged at 113000*g* overnight at 4 °C. The inner membranes were visible as a golden layer at the 35−40% sucrose interface while the outer membranes were visible as a white layer at the 50−55% sucrose interface. Both layers were drawn off using a syringe. The membrane layers were separately resuspended in 20 mM Tris-HCl, pH 7.5, and centrifuged at 131000*g* for 60 min at 4 °C. The pellets were resuspended in the same buffer and stored at −70 °C.

For the isolation of membrane vesicles containing overexpressed ProP−(His)$_6$, 4 mL of an overnight culture of pMB11/WG389 was used to inoculate each of six 2 L flasks containing 1 L of LB plus 100 μg/mL ampicillin. Cultures were grown for 18 h at 37 °C with shaking to a final OD_{600} of about 6. Cells were harvested by centrifugation and vesicles prepared as described for cultures of WG170-pTRC99a/putP(FH) (*23*).

Production and Purification of Anti-ProP Antibodies. Membrane vesicles derived from *proP* overexpressing strain WG389 pDC44 (2 mg of protein/mL) were incubated on ice in 50 mM Tris-HCl, pH 8.4, containing 6 M urea for 30 min (*24*). Urea-soluble and -insoluble fractions were separated by ultracentrifugation at 100000*g* for 1 h at 4 °C. The insoluble fraction was washed with the original volume of 50 mM Tris-HCl, pH 8.4, and resuspended in the original volume (or $^1/_5$th volume for large-scale extractions) of 50 mM Tris-HCl, pH 8.4, containing octyl β-D-glucopyranoside (octyl glucoside) [0.75% (w/v)]. Following a 30 min incubation on ice, the solution was centrifuged at 100000*g* for 1 h at 4 °C. ProP was enriched in the supernatant to about 50% of the total protein. Glycerol [20% (v/v)] was present in all buffers as a protein stabilizer (*25*). ProP protein partially purified in this manner was used as antigen to raise polyclonal anti-ProP antibodies. Two female New Zealand White rabbits were subcutaneously injected with this antigen (0.5 mg of protein per animal per immunization). Antibody boosts were performed biweekly for a period of 6 weeks. Incomplete Freund's adjuvant was used for all injections at a 1:1 volumetric ratio with the antigen solution. Rabbits were exsanguinated 2 weeks after the last boost, and sera were clarified by centrifugation at 7000*g* for 15 min at 4 °C after coagulation of the blood cells. Anti-ProP antibodies were purified by repeated adsorption of the sera with an affinity matrix bearing membrane proteins extracted from *E. coli* strain WG389 pMTC15. To prepare the matrix, membrane vesicles prepared from strain WG389 pMTC15 (Δ*proP*) were dissolved in 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, 1% (v/v) Triton X-100 and their constituents coupled to CNBr-activated Sepharose 4B (Pharmacia/LKB, Uppsala, Sweden) according to the manufacturer's instructions with the exception that all buffers contained Triton X-100 (1% v/v) to prevent aggregation of the membrane proteins.

SDS−PAGE, Western Blots, and Protein Sequencing. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to Laemmli et al. (*26*) using Bio-Rad MiniProtean II cells (BioRad Laboratories Ltd., Hercules, CA) (12% T, 2.67% C). Western Blots were performed according to Towbin et al. (*27*) using 15.6 mM Tris, 120 mM glycine, 20% (v/v) methanol, 0.02% (w/v) SDS as electrode buffer. Proteins were electrotransferred to a nitrocellulose sheet (Bio-Rad) (transfer time 3 h at 4 °C using a constant current of 60 mA), and probed with rabbit polyclonal antibodies raised against either a synthetic peptide analogue of the ProP C-terminus (*28*) (Figures 1B and 2B) or whole ProP as described above (Figure 3B). Blots were

visualized with a peroxidase-coupled goat anti-rabbit secondary antibody (Sigma Chemical Co., St. Louis, MO) and either the Bio-Rad HRP 4CN color development reagent (Figures 1B and 2B) or Amersham (Oakville, Ontario) Enhanced Chemiluminescence (ECl) reagents (Figure 3B).

The amounts of ProP in membranes derived from different overexpressing strains, relative to that in membranes from a strain expressing *proP* from the chromosome, were determined using serial dilutions of membrane vesicle preparations adjusted to 1.3 mg/mL total protein. Dilutions which yielded approximately equal intensities in band color after Western blotting were applied next to each other to minimize errors due to changes in background intensities. Band intensities were measured by densitometry using a COHU High Performance CCD camera in combination with Bio Scan Optimas 4.02 software (Optimas Corp., Bothell, WA).

For protein sequencing, electrophoresis was performed as described above except the SDS was omitted from the gel. The cytoplasmic membrane fraction, enriched in ProP (Figure 2), was used for this analysis. The PAGE gel was soaked in elution buffer [0.3% (w/v) Tris, 1.44% (w/v) glycine, 0.05% (w/v) SDS] for 30 min. The gel was blotted onto a PVDF membrane for 2.5 h at 60 mA using a semi-dry blotting apparatus. The membrane was washed for 1 h in deionized water and stored between two pieces of Whatman 3MM filter paper overnight. Following 1 h in the desiccator, the membrane was stained by quickly dipping the membrane in a 0.05% (w/v) sulforhodamine B, 30% (v/v) methanol solution several times. After rinsing with deionized water twice, the membrane was allowed to dry. The N-terminal amino acid sequencing was carried out by Dr. J. G. Keen in a SERC-funded facility at the University of Leeds, Leeds, U.K.

Purification and Reconstitution of ProP−(His)$_6$ and PutP−(His)$_6$. Membrane vesicles containing either ProP−(His)$_6$ or PutP−(His)$_6$ were solubilized and purified by Ni^{2+}-NTA affinity chromatography (Qiagen, Santa Clarita, CA) as described (*23*). Liposomes for reconstitution were prepared from Avanti (Alabaster, AL) *E. coli* phospholipids (*E. coli* polar lipid extract, acetone/ether washed). Five milliliters of 20 mg/mL lipid in chloroform was placed into a 100 mL round-bottom flask and the solvent removed on a rotary evaporator. The lipid film was dissolved in 5 mL of buffer A (100 mM potassium phosphate, pH 7.4, 2 mM β-mercaptoethanol) containing 1.5% octyl glucoside (w/v) using a magnetic stirrer. The dissolved lipids were dialyzed 3 times against buffer A to remove detergent. The liposomes were then frozen and stored in liquid nitrogen. Immediately prior to reconstitution, the liposomes were thawed at room temperature, extruded 20 times through a 400 nm polycarbonate filter, and then diluted to a final concentration of 5 mg/mL lipid in buffer A. Several detergents (at different concentrations) were initially tested for their suitability for reconstitution of ProP−(His)$_6$. Specifically, Triton X-100 was tested at 0.11 and 0.45% (w/v), octyl glucoside at 0.5 and 1.5%, and dodecyl β-D-maltoside (dodecyl maltoside) at 0.2%. The following reconstitution procedure follows that developed for PutP as described in Jung et al. (*23*). Detergent was added to the liposomes, and the sample was vortexed briefly to mix. The purified ProP−(His)$_6$ protein was added at a ratio of 100:1 (lipid:protein) (w/w), and the sample was vortexed briefly and incubated at room temperature with gentle agitation for 10 min. The detergent was removed by the addition of Bio-Beads SM-2 (BioRad Laboratories Ltd., Hercules, CA) at a wet weight bead:detergent ratio of 5:1 (w/w). The sample was incubated at room temperature for 1 h with shaking, and a second aliquot of Bio-Beads was added. Again the sample was incubated for 1 h at room temperature with shaking. One last addition of Bio-Beads was made (twice the amount of the first time), and the sample was incubated with shaking at 4 °C overnight. The proteoliposome solution was carefully removed from the Bio-Beads and the suspension dialyzed 2 times against buffer A at 4 °C. The proteoliposomes were concentrated by centrifugation at 300000*g* for 30 min at 4 °C. The pellet was resuspended in the smallest possible volume of buffer A and the sample stored in liquid nitrogen. The protein concentration of proteoliposomes was determined by the Schaffner−Weissmann protein assay (*29*). The size distribution of the resulting proteoliposomes was determined, after freeze/thaw/sonication (see Results), by dynamic light scattering spectroscopy as described (*30*). PutP−(His)$_6$ was reconstituted in the presence of 0.11% Triton X-100 as described (*23*).

Protein Determination. Protein determinations were performed by one of the following techniques: bicinchoninic acid assay (*31*) using the BCA kit from Pierce (Rockford, IL), Bradford assay (Sigma, St. Louis, MO), or the Schaffner−Weissman protein assay (*29*). In all cases, dilutions of bovine serum albumin (BSA) were used as standards.

Transport Measurements. For whole cell transport assays, bacteria were grown either with or without 0.3 M NaCl to impose osmotic stress. Cells were harvested and washed, and transport measurements were performed as described (*16*). Specific activities and final concentrations of the substrates used were as follows: L-[^{14}C]proline, 5 Ci/mol, 200 μM; L-[^{14}C]serine, 25 Ci/mol, 20 μM; D-[^{3}H]galactose, 1.8 Ci/mol, 48 μM. The assay mixture was sampled after 20, 40, and 60 s.

Proteoliposomes, prepared in 100 mM potassium phosphate, pH 7.4, were diluted 200-fold into potassium-free buffer containing valinomycin to create an outwardly directed potassium diffusion gradient. For the data shown in Figure 4A, transport assays were initiated by diluting 2 μL of ProP−(His)$_6$ proteoliposomes (0.28 mg of protein/mL) into 420 μL of either 100 mM sodium phosphate (either pH 7.4 or pH 6.4) or 100 mM potassium phosphate (pH 6.4 or 7.4) containing 2 mM β-mercaptoethanol, 5 mM $MgSO_4$, 0.2 mM L-[^{14}C]proline (5 Ci/mol) plus or minus 0.5 μM valinomycin, and 0.6 M sucrose. Proline uptake by PutP−(His)$_6$ (Figure 4B) was measured under analogous assay conditions [as described in (*23*)] except that the concentration of L-[^{14}C]-proline (5 Ci/mol) was 10 μM. The initial rate of proline uptake by ProP−(His)$_6$ proteoliposomes was also measured in the presence of concentrations of NaCl and glycerol that gave osmotic upshifts equivalent to 0.6 M sucrose. In this case, 3 μL of the ProP−(His)$_6$ proteoliposomes (0.21 mg of protein/mL) was diluted into 630 μL of 100 mM sodium phosphate (pH 6.4) containing 2 mM β-mercaptoethanol, 5 mM $MgSO_4$, 0.2 mM L-[^{14}C]proline (5 Ci/mol), and 0.5 μM valinomycin plus either 0.6 M sucrose (1003 mmol/kg), 0.41 M NaCl (1010 mmol/kg), 0.98 M glycerol (1019 mmol/kg), or no added osmolyte (219 mmol/kg). In all cases, reactions were stopped at various time points by the addition of 2.5 mL of ice-cold quench buffer (100 mM potassium phosphate,

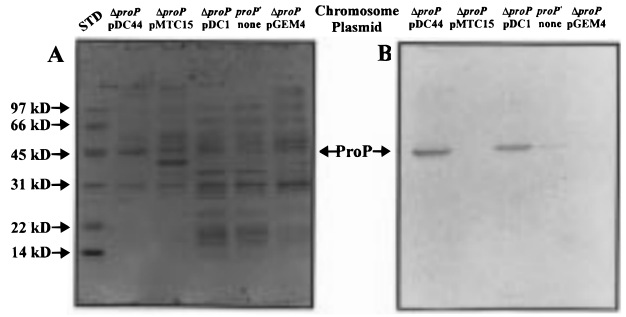

FIGURE 1: ProP overproduction. Membrane proteins were separated by SDS−PAGE and detected with Coomassie Brilliant Blue (A) or by Western blot analysis using the anti-C-terminal antibody (B). Samples analyzed included: STD, Bio-Rad SDS−PAGE standards (low range); and membrane proteins prepared from bacteria with the indicated genotypes. The notation *proP*$^+$, none indicates membrane proteins prepared from *E. coli* strain WG571. The notation Δ*proP* indicates *E. coli* strain WG389 (Table 2) containing the indicated plasmid (pGEM4, pDC1, pMTC15, or pDC44). Strains WG389 pDC44 and WG389 pMTC15 were cultivated in MOPS minimal medium supplemented with 25 mM proline. All other strains were grown in LB medium supplemented with 25 mM proline and 0.3 M NaCl. Membrane vesicles were prepared according to Experimental Procedures.

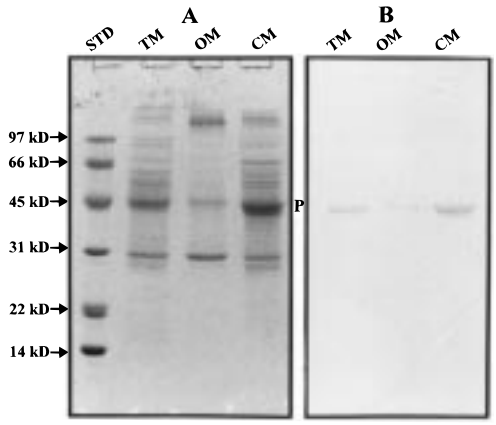

FIGURE 2: ProP is localized in the cytoplasmic membrane. Proteins extracted from membranes fractionated by sucrose density gradient centrifugation were separated by SDS−PAGE and detected with Coomassie Brilliant Blue (A) or by Western blot analysis using the anti-C-terminal antibody (B). STD, Bio-Rad SDS−PAGE standards (low range); TM, total membranes; OM, outer membrane fraction; CM, cytoplasmic membrane fraction; P, ProP. Strain WG389 pDC44 was grown in proline-supplemented MOPS minimal medium. Membrane vesicles were prepared, and sucrose density gradient centrifugation was performed according to Experimental Procedures.

pH 6.0, 100 mM LiCl) and the reaction mixtures filtered through a 0.2 μm nitrocellulose filter. The filters were washed with an additional 2.5 mL of quench buffer, then dried, and counted.

The ability of ProP−$(His)_6$ proteoliposomes to maintain a membrane potential under the various assay conditions was measured using the membrane-potential-sensitive fluorescent dye diS-C_3-(5) (*32*, *33*). Proteoliposomes were diluted 200-fold into 100 mM sodium phosphate (pH 6.4) containing 2 mM β-mercaptoethanol, 5 mM $MgSO_4$, 0.2 mM proline, and 0.6 μM diS-C_3-(5) (Molecular Probes, Eugene, OR), plus or minus 0.6 M sucrose. Fluorescence was measured over time in an Hitachi F-2000 Fluorescence Spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 690 nm. Addition of valinomycin (at 0.2 μM) to the sample caused the fluorescence of the dye to be quenched by 48%.

RESULTS

Overproduction and Localization of ProP. Insertion of gene *proP* into plasmid vector pGEM4, functional complementation of a *proP* defect with the resulting plasmid pDC1, and production of a *proP*-related polypeptide were reported previously (*15*). Polyclonal antibodies, raised against a synthetic peptide corresponding to the predicted ProP C-terminal pentadecapeptide (anti-C-terminal antibody), reacted with membrane vesicles derived from *proP*$^+$ but not *proP*$^-$ bacteria (*28*). Electrophoretic analysis of membranes derived from a pDC1-containing *E. coli* strain revealed a polypeptide with an apparent molecular mass of 45 kDa that was detectable both by staining with Coomassie Brilliant Blue and by immunolabeling (Figure 1).

Galactose transporter GalP (apparent molecular mass 41 kDa) is dramatically overproduced when *galP* is expressed from its own promoter with multicopy plasmid pMTC15 (Figure 1A, Δ*proP* pMTC15). ProP constituted approximately 30% of total membrane protein in cells expressing *proP* from the *galP* promoter in plasmid pDC44 (Figure 1A, Δ*proP* pDC44). In contrast, ProP was not detectable by staining, and it was barely detectable by Western blotting when it was expressed from the bacterial chromosome (Figure 1B, *proP*$^+$ none).

On the basis of its amino acid sequence, ProP was expected to be integral to the cytoplasmic membrane of *E. coli* (*15*). Cytoplasmic and outer membrane fractions from bacteria overexpressing *proP* were separated by sucrose density gradient centrifugation. The cytoplasmic membrane fraction was enriched and the outer membrane fraction depleted of a protein with the electrophoretic mobility and immunoreactivity expected of ProP (Figure 2). Determination of the amino-terminal sequence of the amplified protein confirmed that its first 20 amino acids were those predicted by the *proP* sequence, including the L2V modification introduced during the construction of plasmid pDC44. The protein with an apparent molecular mass of 45 kDa seen by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was therefore ProP, and ProP is a cytoplasmic membrane protein. The predicted molecular mass of ProP is 55 kDa (*15*), but anomalously rapid migration during SDS−PAGE is a widely observed characteristic of integral membrane proteins (*34*, *35*). ProP was partially purified from strain WG389 pDC44 and used to generate polyclonal antibodies (anti-ProP antibody, see Experimental Procedures).

ProP Activity and ProP Expression in Whole Cells. The ProP activity in intact cells of *E. coli* is modulated at the genetic and biochemical levels in response to medium composition, medium osmolality, and bacterial growth phase (*36*, *37*). The proline uptake activities of strains WG571 (*proP*$^+$), WG389 pDC1, and WG389 pDC44 were assessed to determine whether the ProP protein in *proP* overexpressing *E. coli* strains was functional. The relative levels of ProP protein were determined by using scanning densitometry to identify protein bands of comparable intensity in Western blots derived from serial dilutions of the pertinent membrane preparations. Significant uncertainty is associated with the

Table 2: Kinetics of Proline Uptake via ProP[a]

E. coli strain	genotype: chromosome	genotype: plasmid	K_M for proline (μM)	V_{max} [nmol of proline min^{-1} (mg of protein)$^{-1}$]
WG210	*proP*$^+$	Nil	175 ± 17	83.6 ± 3.4
WG350 pDC44	Δ*proP*	*proP*	29.1 ± 5.7	230 ± 14.2
WG350 pMB11	Δ*proP*	*proP*−(*His*)$_6$	26.0 ± 3.8	219 ± 9.6

[a] Strain WG210 (*proP*$^+$ *proU*$^-$) or strain WG350 (*proP*$^-$ *proU*$^-$) containing plasmid pDC44 or pMB11 was grown in MOPS minimal medium supplemented with 0.3 M NaCl. Samples were taken when the cultures reached an optical density of 1.0, cells were washed, and transport activity was measured as described under *Experimental Procedures* using proline concentrations in the range 25−500 μM for strain WG210 and 6−200 μM for strains WG350 pDC44 and WG350 pMB11.

latter values, however, since the ProP protein level in wild-type bacteria was at the limit of detection.

Expression of ProP activity by bacteria harboring plasmid pDC1, which were cultivated in LB medium containing supplementary NaCl, was approximately 6-fold higher than that of bacteria retaining only the chromosomal *proP* locus [271 and 44 nmol min^{-1} (mg of protein)$^{-1}$, respectively]. The corresponding amplification of the ProP protein was 20-fold. Proline transport activity was amplified approximately 12-fold over the same wild-type level [44 nmol min^{-1} (mg of protein)$^{-1}$] when *proP* was expressed from the *galP* promoter (plasmid pDC44) in bacteria cultivated in MOPS-based minimal medium at low osmolality [530 nmol min^{-1} (mg of protein)$^{-1}$]. The corresponding amplification of the ProP protein was 37-fold. [Similar amplification of GalP activity (20-fold) was attained when this system (WG389 pMTC15) was compared with a strain expressing *galP* from the chromosome only (WG389).] No proline uptake activity was detectable [detection limit of 1 nmol min^{-1} (mg of protein)$^{-1}$] in strains from which the *proP* locus had been deleted and which contained the plasmid vectors used to construct plasmids pDC1 and pDC44. Although some of the *E. coli* strains examined during these experiments retained the *proU* locus, the ProU system did not contribute measurably to the detected proline uptake activity (data not shown). To determine whether the overproduction of ProP had nonspecific effects on active transport processes, serine uptake was measured in parallel with proline uptake. As expected, serine uptake activity varied in response to growth medium composition but not to the genetic constitution of the *E. coli* strains tested (data not shown).

Transporter ProP is activated by a hyperosmotic shift in both *E. coli* cells and cytoplasmic membrane vesicles (*16*). To determine whether the overexpressed ProP protein would undergo a similar activation, bacteria containing plasmid pDC44 were cultivated in MOPS minimal medium (of low osmolality), and the proline uptake activity of the resulting cells was determined in the same medium with and without supplementary osmolyte (0.3 M NaCl). The observed proline uptake rates were 351 and 64 nmol min^{-1} (mg of protein)$^{-1}$, respectively. This activation was similar to that observed when the same treatment was applied to bacteria which had expressed *proP* from the chromosome (*36*).

Creation and Characterization of ProP(His)$_6$. To facilitate the purification of ProP for reconstitution, the *proP* gene (in plasmid pDC44) was altered to encode six consecutive histidines at the C-terminal end of ProP, yielding variant *proP*−*(His)$_6$*. Vector pMB11 encodes the variant gene under the control of the *galP* promoter as in vector pDC44. Table 2 compares the K_M and V_{max} values of ProP−(His)$_6$ and wild-type ProP for bacteria cultivated in minimal medium of high osmolality (MOPS medium plus 0.3 M NaCl). The kinetic parameters of the overexpressed ProP and ProP−(His)$_6$ are the same within error, indicating that the hexa-histidine tag does not affect the activity of ProP. The K_M values of the overexpressed proteins were almost 6 times lower than that for ProP expressed from a single chromosomal copy of the gene, however.

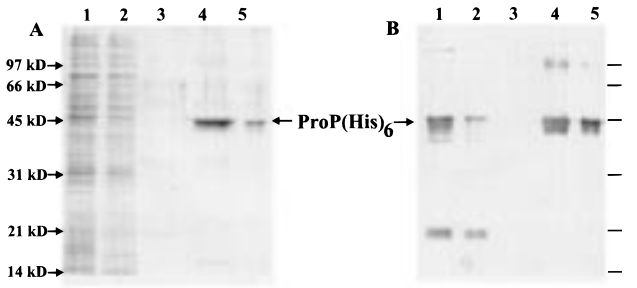

FIGURE 3: Affinity purification of ProP−(His)$_6$ from WG389/pMB11 membranes solubilized in 1% dodecyl maltoside. Column fractions were analyzed by SDS−PAGE, and proteins were detected with Coomassie Brilliant Blue (A) or by Western blot analysis with anti-ProP antibody (B). Lane 1, total soluble membrane protein; lane 2, flow-through from the Ni^{2+}-NTA column in 10 mM imidazole; lane 3, wash of column with 30 mM imidazole; lanes 4 and 5, different quantities [2 or 0.5 μg (A) or 0.5 and 0.12 μg (B), respectively] of pure ProP−(His)$_6$ eluted with 200 mM imidazole. Molecular weight (MW): Bio-Rad SDS−PAGE standards (low range).

Purification and Reconstitution of ProP−(His)$_6$. The Coomassie Brilliant Blue-stained SDS−polyacrylamide gel in Figure 3A documents the purification of ProP−(His)$_6$ by nickel chelate affinity chromatography. The identity of the purified protein was confirmed by Western blot using the anti-ProP antibody as shown in Figure 3B. When purified ProP−(His)$_6$ is deliberately overloaded as in lane 4 of Figure 3A, only small amounts of contaminating proteins can be visualized. Note that bands at approximately 97 kDa are ProP-specific as deduced from the diffuse band in the Western blot (Figure 3B). These high molecular mass bands are most likely aggregates of ProP−(His)$_6$ as their relative intensity can be increased by heating the protein samples in SDS−PAGE loading buffer prior to electrophoresis. Approximately 0.5 mg of purified ProP−(His)$_6$ was obtained from 50 mg of membrane protein.

Purified ProP−(His)$_6$ was reconstituted into preformed liposomes that were prepared from *E. coli* phospholipid and partially solubilized with detergent. Three detergents were tested in the reconstitution procedure (see Experimental Procedures). Proteoliposomes prepared using Triton X-100 (0.11% or 0.45%, w/v) or octyl glucoside (0.5%, w/v) showed high and approximately equal proline uptake activity

(see below). Although the efficiency of protein incorporation was equivalent to that achieved with the other detergents, no ProP−$(His)_6$ activity was observed in proteoliposomes made in the presence of 0.2% (w/v) dodecyl maltoside. After these initial experiments, we chose to continue with the reconstitution procedure using 0.11% Triton X-100. This was also the optimal condition for the reconstitution of the *E. coli* proline transporter PutP (*23*) and the *Streptococcus thermophilus* lactose transporter LacS (*38*). Immediately prior to activity assays, the proteoliposomes were subjected to three cycles of freezing in liquid N_2, thawing at room temperature, and bath sonication. The mean diameter of the resulting vesicles was estimated by dynamic light scattering spectroscopy to be 150 nm. The number of lipid molecules per vesicle could be calculated from the total surface area of a 150 nm vesicle and the average surface area of a phospholipid headgroup (50 Å^2) (*39*). Using an average molecular mass of 800 g/mol for phospholipid molecules and knowing the weight of lipid and protein per volume of sample, we estimated an average of 20 molecules of ProP−$(His)_6$ per proteoliposome.

The Isolated ProP Protein Is an Osmosensor. Initially we attempted to measure the activity of ProP−$(His)_6$ in the proteoliposomes by counterflow assay in the presence or absence of an osmotic upshift; however, the results were erratic and inconclusive (data not shown). Proline uptake activity was then tested using a valinomycin-induced potassium diffusion gradient to generate a membrane potential (interior negative) in the proteoliposomes. As shown in Figure 4A, proline uptake was detected only if the proteoliposomes were subjected to an osmotic upshift. Sucrose (0.6 M) was selected as osmolyte since it produced the optimal activation of ProP in membrane vesicles (*17*). Importantly, when the same conditions were applied to proteoliposomes containing PutP−$(His)_6$, proline uptake was inhibited by the osmotic upshift (Figure 4B). The maximum initial rate of proline uptake [1.4 μmol min^{-1} (mg of protein)$^{-1}$] and the steady-state level of proline accumulation (2.4 μmol/mg of protein) were observed when the ProP−$(His)_6$ proteoliposomes were subjected to a pH downshift as well as an osmotic upshift, in the presence of a membrane potential. This proline uptake rate exceeds that previously observed in cytoplasmic membrane vesicles derived from *proP*$^+$ bacteria (*16*, *17*) more than 1400-fold. Based on the vesicle diameter and estimated number of proteoliposomes per unit volume discussed above, this steady-state level represents an intravesicular proline concentration of 2.1 mM and indicates that ProP−$(His)_6$ mediated more than 10-fold accumulation of proline.

The membrane potential-sensitive fluorescent dye diS-C_3-(5) was used to assess the integrity of the proteoliposomes during the transport assays. Under the assay conditions, the degree of fluorescence quenching of the dye, which is directly proportional to the membrane potential across the proteoliposomal membrane, was independent of the presence or absence of sucrose in the dilution medium. Furthermore, the proteoliposomes maintained a constant membrane potential during the time course of the assay (data not shown).

It has been previously observed that ProP, in whole bacteria and membrane vesicles, is activated by hyperosmotic shifts imposed with sucrose and NaCl, but not with membrane-permeant solute glycerol (*16*, *17*). Similarly, ProP−$(His)_6$ in proteoliposomes, subjected to hyperosmotic shifts (of the same magnitude) imposed with sucrose, NaCl, and glycerol, gave initial proline uptake rates of 557 ± 114, 286 ± 64, and 21 ± 47 nmol min^{-1} mg^{-1}, respectively. The initial proline uptake rate without added osmolyte was 41 ± 28 nmol min^{-1} mg^{-1}.

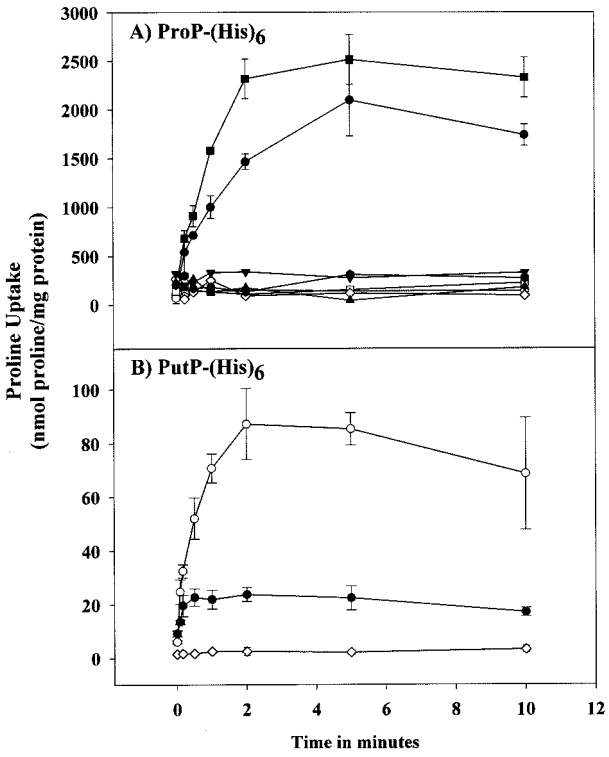

FIGURE 4: Time course of proline uptake into proteoliposomes containing either ProP−$(His)_6$ (A) or PutP−$(His)_6$ (B). Proline uptake was measured as described under Experimental Procedures in the presence (closed symbols) or absence (open symbols) of an osmotic upshift (0.6 M sucrose). Proteoliposomes in potassium phosphate (pH 7.4) were diluted into: K^+-free buffer (pH 7.4) plus valinomycin, circles; K^+-free buffer (pH 6.4) plus valinomycin, squares; K^+-free buffer (pH 7.4), triangles; K^+-free buffer (pH 6.4) inverted triangles; potassium phosphate (pH 7.4) plus valinomycin, diamonds; potassium phosphate (pH 6.4), hexagons. Samples were incubated at room temperature for the indicated times and then quenched and filtered as described under Experimental Procedures. Points represented by closed circles and closed squares are averages of triplicate assays with standard errors indicated by error bars. Other data points represent averages of duplicate determinations where no range of values exceeded 52% of the average.

DISCUSSION

Overexpression of the ProP protein has been achieved by expressing gene *proP* from multicopy plasmids under the control of the *proP* and the *galP* promoter (Figure 1). Despite its anomalous migration during SDS−PAGE, the ProP protein was identified conclusively via both its cross reaction with antibodies raised against a synthetic analogue of its C-terminal peptide (Figures 1B and 2B) and its amino-terminal sequence analysis. Overexpression of ProP also allowed us to determine that ProP is enriched in the cytoplasmic membrane fraction (Figure 2).

The measured apparent K_M value of ProP for proline depended on the level of *proP* expression in whole cells at high osmolality (Table 2). The K_M values for ProP expressed at wild-type and elevated levels were 175 and 29 μM,

respectively. Thus, the K_M decreased almost 6 times while the V_{max} increased 3-fold (Table 2). Initial characterization of ProP overexpression using the *galP* promoter revealed that the fold amplification of ProP activity (12-fold, estimated using 200 μM proline) fell short of the fold amplification of the ProP protein (37-fold). In the context of current K_M estimates, the corresponding amplification of V_{max} would have been only 6-fold. It is not clear why it would be advantageous to the cell for the activity of ProP to decrease when the level of ProP protein increases, and this phenomenon may be merely an artifact of the very high expression levels we have obtained. The growth rates were identical in strains expressing *proP* from the chromosome or from a plasmid (data not shown); however, the presence of large quantities of ProP in cells harboring pDC44 may alter the properties of the cytoplasmic membrane, in turn affecting the activity and apparent proline affinity of ProP. Marshall (*17*) observed a pleiotrophic transport defect in cytoplasmic membrane vesicles derived from *E. coli* WG389 pDC44. Conversely, the difference in the activity/protein ratios between cells that do and do not overexpress ProP might indicate the involvement of an additional limiting component, such as ProQ (*40*). In any case, we observed that ProP is activated by an osmotic upshift to a similar extent (approximately 5-fold) whether expressed from the chromosome or plasmid pDC44. Therefore, no cellular factor appears to limit biochemical activation of ProP upon osmotic upshift.

Reconstitution of functional ProP−$(His)_6$ was achieved by incorporation of the purified protein into preformed liposomes, prepared with *E. coli* lipid and partially destabilized with detergent. The purification and reconstitution of ProP−$(His)_6$ were optimized following protocols developed by Jung et al. (*23*) for the *E. coli* proline transporter PutP. A detailed analysis and discussion of detergent-mediated reconstitution can be found in a review by Rigaud et al. (*41*). ProP−$(His)_6$ proteoliposomes with high proline uptake activities (see below) were obtained using either Triton X-100 or octyl glucoside at detergent-to-phospholipid ratios corresponding to the onset of liposome solubilization (as measured by sample turbidity). Experiments were continued with proteoliposomes prepared using Triton X-100 since this was also optimal for PutP and allows us to directly compare data collected on the two transporters.

The activation of ProP in whole cells and membrane vesicles after an osmotic upshift has been well documented (*16*). In *E. coli*, an increase in medium osmolality increases ProP activity at two levels: a 3-fold enhancement of *proP* transcription and a 5-fold activation of the ProP protein. Our goal is to understand the latter, biochemical activation of the ProP protein. However, at the outset of these experiments we could not be certain that ProP was, itself, capable of both sensing and responding to an osmotic upshift. Figure 4A shows that proline uptake mediated by isolated ProP−$(His)_6$ was detected only when proteoliposomes were subjected to an osmotic upshift (0.6 M sucrose) in the presence of a membrane potential. Maximum proline uptake into ProP−$(His)_6$ proteoliposomes was observed in the presence of a pH downshift in agreement with independent evidence that ProP is a solute/H^+ symporter (*14*, *15*). The possibility that contaminating protein contributed to the osmotic activation of ProP cannot be ruled out. However, no protein copurified with ProP (Figure 3A), and it is difficult to imagine how a contaminating protein could act substoichiometrically or catalytically to elicit the high ProP activities observed in this system.

We therefore conclude that purified ProP, reconstituted in proteoliposomes, is both an osmosensor, sensing the osmotic gradient imposed on the vesicles, and an osmoregulator, increasing the osmolality of the vesicle lumen through the uptake of proline. By what mechanism does increased osmolality activate ProP? Candidates include (1) modulation of α-helical coiled-coil formation by the ProP carboxyl terminus, (2) modulation of ProP activity by K^+, and (3) osmotic effects on the membrane and/or other aspects of ProP protein structure.

Analysis of the *proP* sequence placed ProP in a superfamily of transporters that are integral to the cytoplasmic membranes of eukaryotes and prokaryotes (*15*). These enzymes are predicted to exist as multiple (usually 12) membrane-spanning α-helices linked by hydrophilic loops. Still, ProP differs from its homologues in possessing an extended central loop and carboxyl terminus, the latter strongly predicted to form an α-helical coiled-coil. Since coiled-coils are well-known as mediators of regulatory protein−protein interactions (*42*), this unique C-terminal structure is an obvious candidate site for formation of regulatory homo- or heteromeric association(s) (*15*). The addition of a hexa-histidine affinity tag to the C-terminal resulted in a protein, ProP−$(His)_6$, with equivalent kinetic properties (Table 1) and osmotic activation (Figure 4A) to wild-type ProP. Thus, the C-terminal His-tag appears not to interfere with ProP function.

It is possible that osmolality modulates the stability of a coiled-coil incorporating the ProP C-terminus, thereby effecting a conformational change that alters ProP activity. Stability of the coiled-coil structure, with its extensive helix−helix interface, may be modulated in response to varying water activity or cytoplasmic solvent composition (*43*, *44*). Changes in crowding or confinement (*45*) within the cytoplasm or proteoliposome lumen might also contribute to changes in coiled-coil stability. Osmotic activation of the purified protein, shown here (Figure 4A), rules out a requirement for heteromeric coiled-coil formation in ProP activation and implies that, if macromolecular crowding is implicated in ProP activation in vivo, crowding can be replaced by confinement due to proteoliposome shrinkage in vitro. In addition, the carboxyl-terminal domain of ProP may act as an output domain, transmitting a signal sensed by ProP to cellular machinery that is disrupted when ProP is purified. It is now particularly important to explore the potential for coiled-coil formation by ProP.

A specific K^+ requirement for ProP activity has been identified, but its basis remains ill-defined (*17*, *46*). Potassium glutamate, accumulated during the primary response of *E. coli* to an osmotic upshift, has been proposed to act as a second signal of osmotic stress (*47*). The concentration of lumenal K^+ is expected to be a common feature of the whole cell (*36*, *48*), cytoplasmic membrane vesicle (*16*), and proteoliposome (this report) systems within which ProP activation has been demonstrated. K^+ accumulates in cells through the action of osmoregulatory K^+ transporters (*10*) whereas in vesicles and liposomes osmotically induced shrinkage is expected to concentrate lumenal K^+. Since K^+ is also required to support respiration (*49*) and formation of

a membrane potential in proteoliposomes (this report), the K^+ requirement for energization of ProP has not yet been disentangled from a putative K^+ requirement for ProP activation. Further experimentation will therefore be required to determine whether K^+ plays a direct role in the osmotic activation of ProP.

If neither K^+ nor altered coiled-coil stability is responsible for the osmotic activation of ProP, attention will become focused on osmotically induced structural changes in membrane lipid, the protein−lipid interaction, and/or the protein itself. Candidate changes in the lipid/ProP assembly would include changes in lateral strain, bending, or hydration. Since activation of ProP requires an osmotic upshift imposed with a membrane-impermeant osmolyte (e.g., NaCl or sucrose, but not glycerol), a change in transmembrane osmotic gradient, not in osmolality per se, appears to be necessary. The purification and reconstitution of ProP−$(His)_6$, reported here, will allow osmotic effects on liposome systems (*30*, *50*, *51*) to be correlated with osmotic modulation of ProP activity in the absence of other membrane-based phenomena.

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