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The ion coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*

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Abstract

Transporter ProP of *Escherichia coli*, a member of the major facilitator superfamily, mediates osmoprotective proline or glycine betaine accumulation by bacteria exposed to high osmolality environments. Morpholinopropane sulfonic acid, a common constituent of microbiological media, accumulates in osmoadapting *E. coli* cells but it is not osmoprotective and it did not influence *proP* transcription or ProP activity. The apparent K_m for proline uptake via ProP increased with decreasing pH in the range 7.5–4. ProP-dependent proline uptake by de-energized bacteria was associated with alkalinization of the external medium. Thus ProP mediates cotransport of H^+ and zwitterionic proline and a transporter functional group with a pK_a of 5–6 is implicated in catalysis. Exogenous proline or glycine betaine elicits K^+ release from osmoadapting *E. coli* cells and ProP activity is stimulated by exogenous K^+. However, uptake of proline or glycine betaine stimulated K^+ efflux from K^+-loaded bacteria which expressed either ProP or alternative, osmoregulatory transporter ProU. This indicated that ProP was unlikely to mediate K^+ efflux. Zwitterions ectoine, pipecolate, proline betaine, *N*,*N*-dimethylglycine, carnitine and 1-carboxymethylpyridinium were identified as alternative ProP substrates. Choline, a cation and a structural analogue of glycine betaine, was a low affinity inhibitor but not a substrate of ProP.

Keywords: Osmoregulation; *Escherichia coli*; Transporter ProP; Proton symport; Substrate specificity

1. Introduction

When extracellular osmolality is increasing or continuously high, cells remain hydrated by accumulating certain solutes to high cytoplasmic levels. In bacteria these osmoregulatory solutes include K^+, amino acids (e.g. glutamate, proline), other zwitterionic organic solutes (e.g. glycine betaine, choline-*O*-sulfate, ectoine) and polyols (e.g. trehalose) [1–4]. Compatible solutes are organic solutes that accumulate to high levels, without compromising metabolic functions, in cells exposed to hypertonic conditions. Osmoregulatory solute profiles are species- and environment-specific, depending upon factors such as nutrient availability, compatible solute availability, growth phase and the magnitude and duration of the imposed osmotic stress.

Accumulation of K^+ and glutamate is the primary

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response to an osmotic upshift for *Escherichia coli* K-12 cells in minimal salts media. If available, however, proline or glycine betaine can attenuate K^+ accumulation. Addition of proline or glycine betaine to K^+-loaded cells elicits K^+ efflux and glutamate depletion. Both cellular rehydration and restoration of growth are more complete if these compatible solutes are available exogenously (summarized in [3]). The regulatory mechanisms that implement the preference for compatible solutes over K^+ and its counterions are not fully understood, however.

Compatible solute accumulation by *E. coli* K-12 is mediated by secondary transporters ProP and BetT and by ABC transporter ProU [3]. Transporters ProP and ProU have overlapping substrate specificities, their organic substrates including proline and glycine betaine. Gene *proP* is expressed by bacteria cultivated in standard, low osmolality laboratory media (e.g. LB [5], MOPS medium [6]). Transcription of *proP* is also induced during bacterial growth in hypertonic media and during the transition from logarithmic to stationary growth phase. Bacteria grown in low osmolality media do not express *proU*, but *proU* transcription is dramatically induced by an osmotic upshift. BetT is a choline transporter encoded in operon *betTIBA*. Enzymes BetB and BetA catalyze the cytoplasmic oxidation of choline to glycine betaine. Transcription of *betT* responds positively to increased osmolality, choline and O_2. ProP is thus more likely than ProU or BetT to catalyze immediate compatible solute uptake in response to an osmotic shift.

ProP has been classed as a compatible solute/H^+ co-transporter ([7] as cited in [8]) and as a compatible solute/Na^+ co-transporter [9]. Although the ion coupling mechanism of ProP has not been analyzed in detail, ProP does mediate active, uncoupler-sensitive solute uptake in cells and cytoplasmic membrane vesicles [10,11] and it is most closely related in sequence to H^+ co-transporters [12]. K^+ dependence distinguishes the ProP system of *Salmonella typhimurium* from other bacterial organic solute transporters, however [8]. The research reported here further defines the ion coupling specificity of ProP and the role of ProP in the K^+ efflux observed when bacteria loaded with K^+ at high osmolality are offered proline or glycine betaine.

Accumulating evidence suggests that the organic substrate specificity of transporter ProP is broad. To demonstrate that a compound is a ProP substrate using intact bacteria, the compound must be shown to accumulate, unmodified, in the cytoplasm of cells which retain (and not in those which do not retain) ProP. According to this criterion, only L-proline, glycine betaine and taurine are clearly ProP substrates. ProP was also reported to mediate accumulation of ectoine and pipecolic acid, but participation of related transporter PutP in the observed uptake was not ruled out [13,14]. Since L-azetidine-2-carboxylate and 3,4-dehydro-D,L-proline exert ProP-dependent cytotoxic effects at targets internal to *E. coli* they are also believed to be ProP substrates [15]. This paper reports further definition of the substrate specificity of ProP obtained through direct measurements of transporter activity and of its inhibition by substrate analogues.

The results reported below demonstrate that in *E. coli* (1) ProP is a compatible solute/H^+ symporter, (2) ProP activity is K^+ dependent whereas the activities of other transporters are not, (3) the K^+ efflux associated with proline or glycine betaine accumulation by osmoregulating *E. coli* cells is not specifically ProP-dependent, (4) structurally diverse, zwitterionic organic compounds serve as substrates and/or competitive inhibitors of ProP and (5) choline acts as a low affinity inhibitor (though not a substrate) for ProP.

2. Materials and methods

2.1. Bacterial strains and culture media

The *E. coli* strains and plasmids used in this study are listed in Table 1. Strain WG621 was isolated by streaking strain RM2 onto Lactose-MacConkey indicator plates and incubating at 37°C for 48 h. A single red colony, which indicated a *lac*$^+$ revertant of strain RM2, was selected and restreaked twice more on the same medium to obtain a pure culture. Strain WG693 (Δ(*putPA*)*101* Δ(*proU*)*600 srl*$^+$) was prepared by bacteriophage P1-mediated transduction of *E. coli* strain WG443 (Δ(*putPA*)*101* Δ(*proU*)*600 srl-300*::Tn*10*) from *E. coli* K-12 as described by

Table 1
E. coli strains and plasmids

Strain/plasmid	Genotype	Source or Ref.
CSH4	F$^-$ *trp lacZ rpsL thi*	Cold Spring Harbor Laboratory
EF046	MC4100 Δ(*putPA*)*101* Δ(*proU*)*600*	[48]
EF047	MC4100 Δ(*putPA*)*101 proP* Δ(*proU*)*600*	[48]
K-12	Wild type	*E. coli* Genetic Stock Center
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsH150 relA1 deoC1 ptsF25 rbsR flbB5301*	[49]
RM2	F$^-$ *trp lacZ rpsL thi* Δ(*putPA*)*101*	[50]
WG389	RM2 *lac*$^+$ Δ(*brnQ phoA proC*) Δ(*proU*)*600* Δ(*proP-melAB*)*212*	[12]
WG439	K-12 Δ(*putPA*)*101* Δ(*proU*)*600 srl-300*::Tn*10* Δ(*proP-melAB*)*212*	[51]
WG441	K-12 Δ(*proP-melAB*)*212* Δ(*putPA*)*101* Δ(*proU*)*600*	[51]
WG443	K-12 Δ(*putPA*)*101* Δ(*proU*)*600 srl-300*::Tn*10*	[51]
WG445	K-12 Δ(*putPA*)*101* Δ(*proP-melAB*)*212 srl-300*::Tn*10*	[51]
WG527	WG389 pDC44	[31]
WG528	WG389 pMTC15	[31]
WG621	RM2 *lac*$^+$	This work
WG662	WG439 pDC44	This work
WG663	WG439 pMTC15	This work
WG693	K-12 Δ(*putPA*)*101* Δ(*proU*)*600*	This work
pMTC15	pBR322 containing the *galP* gene	[31]
pDC44	*proP* insert replacing gene *galP* in pMTC15	[31]

Miller [5] with selection for growth on sorbitol (2 mg/ml) as sole carbon source.

Bacteria were cultivated in LB [5], MOPS (3-[*N*-morpholino]propanesulfonic acid) medium [6] or K5 medium [16] as indicated. All media were supplemented with ampicillin (100 μg/ml) as required to maintain plasmid pDC44 or pMTC15. MOPS medium was supplemented with 9.5 mM NH_4Cl as nitrogen source and 43.5 mM glycerol as carbon source with 25 mM proline (strain WG389 and its derivatives only), 245 μM L-tryptophan and 1 μg/ml thiamine hydrochloride (strain RM2 and its derivatives only) to meet auxotrophic requirements. Where appropriate, nutritional stress was imposed by providing L-tryptophan at a growth rate limiting concentration (24.5 μM) during the final subculture. K5 medium was supplemented with 8 mM $(NH_4)SO_4$ as nitrogen source and 43.5 mM glycerol as carbon source. High osmolality media were those supplemented with 0.3 M NaCl. Unless otherwise stated, bacteria were initially grown in LB medium overnight at 37°C in a rotary shaker at 200 rpm, subcultured into appropriately supplemented minimal medium and again incubated overnight at 37°C in a rotary shaker at 200 rpm. Subsequent subcultures were prepared as specified below.

2.2. Transport assays

Bacteria were cultivated in MOPS medium and initial rates of proline uptake by whole cells were measured with a filtration assay essentially as described by Grothe et al. [15] and Milner et al. [11]. Bacteria were harvested, washed twice and resuspended with unsupplemented MOPS minimal medium (MOPS medium (pH 7.4) without carbon source, nitrogen source or other organic supplements). In a typical assay, the cells (25 μl) were added to an assay mixture which consisted of unsupplemented MOPS minimal medium (450 μl) plus 20% (w/v) glucose (5 μl) as energy source and incubated at 25°C for 3 min. Transport was initiated by adding a radiolabeled substrate (20 μl) to the indicated final concentration and specific radioactivity: L-[^{14}C]proline, 200 μM, 5 Ci/mol, L-[^{14}C]serine, L-[^{14}C]glutamine, 20 μM, 25 Ci/mol or [^{14}C]choline, 50 mM, 0.16 Ci/mol (Dupont-New England Nuclear, Mississauga, ON). Samples of the assay mixture (150 μl) were applied to filters after 20, 40, and 60 s and immediately washed with 5 ml of unsupplemented MOPS minimal medium. The densities of the cell suspensions and the specific radioactivities of the substrates were adjusted to ensure that filtration

was sufficiently rapid to quench uptake effectively, no more than 10% of the available substrate was taken up during any assay period and quantities of radioactivity incorporated were sufficient to yield statistically reliable uptake estimates.

Measurements of the ion dependence of proline uptake via ProP were performed as described by Chen et al. [17] using cells grown under nutritional stress. The pH dependence of proline transport by ProP in cells grown under osmotic stress was determined as described by van der Rest et al. [18] using the indicated buffers, supplemented with NaCl (0.3 M), in place of the standard transport assay medium. Initial rates of proline uptake were measured at proline concentrations of 25, 100, 200, and 400 μM and data were analyzed with Eadie-Hofstee plots to determine K_m and V_{max}.

For other measurements of proline uptake kinetics and inhibition, bacteria were preincubated for 3 min (25°C) in a 1.25× concentrated assay buffer (composition described above). To initiate uptake, preincubated cells (400 μl) were added to a tube containing ^{14}C-proline or ^{14}C-choline in 100 μl of water. To determine the K_m for proline, it was provided at 29 μM (35 Ci/mol), 54 μM (19 Ci/mol), 79 μM (13 Ci/mol), 104 μM (10 Ci/mol), 129 μM (8 Ci/mol), 204 μM (5 Ci/mol), 304 μM (2.5 Ci/mol) and 404 μM (2 Ci/mol). For inhibition assays, the proline concentration was held constant (204 μM, 5 Ci/mol) and the putative inhibitor concentration was titrated upwards to 200 mM. For K_i determinations both proline and inhibitor concentrations were varied as described (see Section 3). Data were fit to the mixed, competitive, non-competitive and uncompetitive inhibition models and estimates of K_i were obtained for each model using SigmaPlot (Jandel Scientific). The most appropriate model was selected by evaluating the error associated with the fitted estimates.

2.3. Analysis of intracellular solute accumulation

Bacteria were grown overnight in MOPS medium and subcultured into the same medium without or with 0.2 mM proline or 100 mM choline. NaCl was added to impose osmotic stress (0.3 M NaCl when no other additive or proline (0.2 mM) was present, 0.2 M NaCl when choline (100 mM) was present). Bacteria were harvested by centrifugation and lyophilized. Samples to be analyzed by HPLC or HPLC-MS were extracted as described by Kunte et al. [19]. The extracts were lyophilized and resuspended in distilled water. For NMR analyses, solutes were extracted as described by Smith and Smith [20] and resuspended in phosphate buffered ^2H_2O (50 mM, pH 7.4).

Analysis of MOPS accumulation by HPLC was performed with the following variation of the technique reported by Frings et al. [21]. Samples were mixed 1:2 with the HPLC mobile phase (acetonitrile, 50 mM sodium acetate, 80:20 v/v) and separated with a reverse phase isocratic system (GROM-Sil amino PR 3 μM column) at a flow rate of 1 ml/min. The refractive index of the eluate was monitored. MOPS and trehalose were identified by comparison with the retention times of standards and solutes were quantified using a standard curve.

When samples were analyzed by HPLC-MS, the extracted solutes were mixed (1:3) with the HPLC mobile phase (acetonitrile:water, 1:1) supplemented with 0.2% formic acid and separated by HPLC using a Prodigy 5 ODS-2 column (150×3.2 mm ID with 5 μm particle size) at a flow rate of 0.1 ml/min. (Acetonitrile was Lichrosolv from Merck (Darmstadt, Germany).) Eluate was introduced into a VG Quattro II mass spectrometer (Fision, UK) by an electrospray ion source operating in positive ion mode (ion source temperature, 95°C, capillary needle and cone voltage 41 kV and 21 V, respectively). Species with the mass to charge (m/z) ratio of the desired protonated molecular ion (choline 104; proline 116) were transmitted through a second quadrupole containing ultrapure argon gas (2×10^4 mbar pressure) and fragment ions were monitored (choline 45; proline 70) by simultaneous multiple reaction monitoring.

For samples analyzed by NMR, extracts were passed through a Chelex-100 resin (Sigma, St. Louis, MO), lyophilized, and the solutes were resuspended in 0.5 ml of phosphate-buffered ^2H_2O (50 mM, pH 7.4). When necessary, the pH of samples or chemical standards was re-adjusted to 7.4 with NaOH or HCl. Carbon spectra were obtained at 100.62 MHz on a Bruker Avance 400 MHz NMR spectrometer, equipped with a 5 mm dual broadband probe head. Data points (64 000) were collected with broadband proton decoupling at a sweep width of 21 645 Hz using a pulse width of 4 μs. A recycle delay of 1 s

was used, and spectra were processed with a line broadening of 3 Hz. Chemical shifts were recorded relative to that of 3-(trimethylsilyl)tetradeuterosodium propionate (TSP). Compounds were identified in cytoplasmic extracts by comparing the chemical shifts in the cytoplasmic extract spectra with those of trehalose, glutamic acid, MOPS, choline and proline standards (20 mg ml^{-1} in phosphate-buffered ^2H_2O, pH 7.4).

2.4. K^+ estimation using fluorescent dye CD222

The intensity of the fluorescence of coumarin diacid cryptand [2.2.2] (CD222, Molecular Probes, Eugene, OR) was recently shown to depend on K^+ concentration in the sub-millimolar range [22]. A K^+ assay based on CD222 fluorescence was therefore devised to facilitate K^+ efflux experiments (see Section 2.5). CD222 was prepared as a stock solution in DMSO. The concentration of this solution was determined by absorption spectroscopy (360 nm) after dilution in the efflux assay buffer (0.1 M Tris-MES, pH 7.2, containing 0.60 M. sucrose, see Section 2.5) and assuming an extinction coefficient of 31 000 dm^3 mol^{-1} cm^{-1} [22]. K^+ standards were prepared in the efflux assay buffer and the stock solution was added to achieve a final CD222 concentration of 1 μM. The intensity of the fluorescence emitted by each sample was measured with a Hitachi Model F2000 fluorimeter (Mississauga, ON) (excitation wavelength 364 nm; emission wavelength 465 nm; bandpass 10 nm (excitation and emission) and photomultiplier voltage, 700 V). A standard curve was obtained by fitting the data to the empirical equation:

$$F = bC + [(a-b)K_dC]/([K^+] + K_d)$$

where F was the observed fluorescence intensity, C was the total CD222 concentration, K_d was the dissociation constant for the CD222/K^+ complex and [K^+] was the potassium concentration. The equation was based on the assumption that, at 1 μM CD222 and within the limited K^+ concentration range tested, the intensities of the fluorescence emitted by free and K^+-bound dye were direct functions of dye concentration with proportionality constants a and b, respectively. The fit yielded the regression line illustrated in Fig. 1A and parameter estimates of: K_d, 0.4432 mM; a, 1037 mM^{-1}; b, 5045 mM^{-1}.

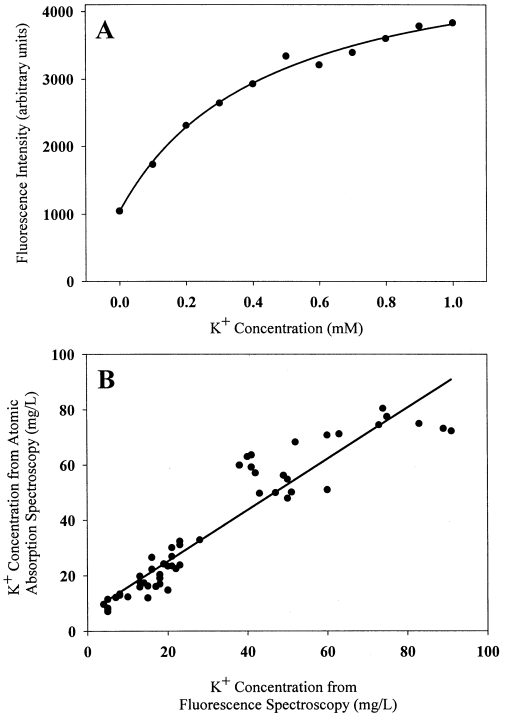

Fig. 1. Estimation of K^+ concentration with fluorescent dye CD222. (A) Standard curve for K^+ estimation. Standard assay mixtures contained CD222 (1.0 μM) and K^+ at the indicated concentrations in Tris-MES buffer (0.1 M, pH 7.2) containing 0.60 M sucrose. Fluorescence intensities were measured and data were fit by non-linear regression analysis to yield the illustrated regression line (see Section 2.4). (B) Comparison of K^+ concentration estimated by fluorescence and atomic absorption spectroscopies. Representative K^+ efflux assay samples (see Section 2.5) were prepared and analyzed by both fluorescence and atomic absorption spectroscopy (see Section 2.4). Each data point corresponds to an individual sample. Linear regression analysis of these data yielded a slope of 0.93, an intercept of 6.7 mg/l and a correlation coefficient of 0.89.

2.5. K^+ efflux experiments

Bacterial cultures were cultivated for K^+ efflux experiments essentially as described by Dinnbier et al. [16]. Bacteria were initially grown in LB medium overnight at 37°C in a rotary shaker at 200 rpm, then subcultured (0.5 ml of LB culture in 100 ml of K5 medium in a 250 ml flask) and again incubated overnight at 37°C with rotary shaking at 200 rpm. This culture was used to inoculate 1 l of K5 medium in a 4 l Fernbach flask to an optical density (600 nm) of 0.1 and incubated until the culture reached an optical density of 0.5. Chloramphenicol was added to a final concentration of 0.1 mg/ml. After a further

5 min incubation, solid sucrose was added to bring the sucrose concentration to 0.65 M and the culture was incubated for 30 min before the cells were harvested by centrifugation in a Sorvall GSA rotor (20 min, 9000 rpm, room temperature). The supernatant was discarded after reserving a sample for osmolality measurement, adhering medium was removed from the interior of the tube and the cells were resuspended in 3 ml of K^+-free, isotonic buffer (0.1 M Tris-MES, pH 7.2, containing 0.60 M sucrose). (Tris-MES is buffered by tris(hydroxymethyl)aminomethane (Tris) and 2-[*N*-morpholino]ethanesulfonic acid (MES).) Aliquots (75 μl) of this cell suspension were diluted 20-fold, in 13×100 mm test tubes, in the same buffer supplemented with proline (10 mM), glycine betaine (10 mM), galactose (10 mM) or SDS (0.001%, w/v) and chloroform (1%, v/v) or no addition. The resulting suspensions were incubated horizontally, with shaking for 30 min at 200 rpm in a 37°C incubator. The K^+ efflux period was terminated by transferring each suspension to a 1.5 ml microcentrifuge tube and sedimenting the bacteria by centrifugation for 5 min in a Fisher Scientific Model 235C microcentrifuge. A control sample was treated in the same manner with no addition and no 30 min incubation. Each treatment was performed in triplicate with bacteria cultivated on two occasions. Supernatants were stored frozen (−40°C) before appropriate dilution with the same buffer in the presence and absence of CD222 (1 μM). The intensity of the fluorescence emitted by each sample was measured as described above (see Section 2.4). The K^+ content of each reaction mixture was determined from its CD222-dependent fluorescence intensity using the standard curve of fluorescence intensity versus K^+ concentration (Fig. 1A) and corrected for the K^+ content of the control sample.

For selected samples, the K^+ content was also determined by flame atomic absorption spectroscopy (766.5 nm) in the presence of $LaCl_3 \cdot 7H_2O$ (0.1%, w/v) using standard solutions prepared in the efflux assay buffer (measurements performed with a Model 5100ZL Atomic Absorption Spectrometer (Perkin Elmer, Norwalk, CT) at the Laboratory Services Division, University of Guelph). The two analytical methods yielded similar estimates of K^+ content (Fig. 1B) and showed similar precision (data not shown).

For the efflux experiments illustrated in Fig. 4B, the conditions were modified to allow expression of transporter ProU. To increase their osmolalities, sucrose was present in all K5 media at a concentration of 0.3 M. When the cells reached an optical density of 0.5 (600 nm), solid sucrose was added to increase its concentration to 0.65 M. Following this osmotic upshock, the cells were incubated for 55 min at 37°C with rotary shaking at 200 rpm. Solid chloramphenicol was added to a final concentration of 0.1 mg/ml and allowed to incubate for an additional 5 min at 37°C with shaking at 200 rpm. The cells were harvested, resuspended, treated and assayed for potassium as described above. The intensity of the fluorescence emitted by each sample was measured with a Spex 1681 0.22 M spectrometer (excitation wavelength, 370 nm; emission wavelength, 470 nm; bandpass, 10.8 nm (excitation and emission) and photomultiplier voltage 900 V).

2.6. H^+ uptake measurements

H^+ uptake was measured as described [23,24] with some modifications. *E. coli* strains WG527 and WG528 were cultivated in MOPS medium as described by Grothe et al. [15] and the harvested bacteria were de-energized as described by Henderson and MacPherson [23]. Bacteria were harvested, washed, resuspended in anoxic solution A (sodium chloride (150 mM), glycylglycine (2 mM), sodium thiocyanate (20 mM), sodium iodoacetate (1 mM), pH 7.8) and diluted to the desired density in solution A within an anoxic reaction vessel maintained at 25°C. The pH of the resulting suspension was adjusted to 7.3. Five to 10 min prior to initiation of H^+ uptake either an osmotic upshift was imposed by adding NaCl in solution A (to achieve 0.3 M NaCl) or the same volume of solution A, alone, was added and the pH was re-adjusted as necessary. H^+ uptake was initiated by adding proline (5 mM in solution B), galactose (3 mM in solution B) or solution B alone (solution B included NaCl (150 mM) and glycylglycine (2 mM) at pH 7.2). The pH of the resulting cell suspension was monitored with a pH electrode (Fisher Model 13-620-92) interfaced to a pH meter (Radiometer, Model PMH92) and the data were captured on a chart recorder (Sargent Welch, Model 57220-21). The magnitude of the resulting flux was

determined by titrating the cell suspension back to the baseline pH with dilute standardized HCl.

2.7. Osmoprotection assays

Bacteria were inoculated into 2 ml LB medium and grown overnight (shaking) at 37°C. Serial dilutions of the overnight culture were prepared and 100 μl aliquots containing approx. 100 viable cells were spread on low and high osmolality minimal medium plates with glucose as carbon source. K5 medium-based plates were used to assess osmoprotection by MOPS buffer while MOPS medium-based minimal plates were used for all other osmoprotection assays. The high osmolality plates were used without and with putative osmoprotectants at the specified concentrations. The sizes and numbers of colonies formed over 72 h were monitored. All strains grew well on low osmolality media and did not grow on high osmolality media in the absence of osmoprotectant.

2.8. Other analytical techniques

Solution osmolalities were measured using a Wescor vapor pressure elevation osmometer (Mandel Scientific, Guelph, ON) according to the manufacturer's instructions. The protein content of cell and membrane suspensions was determined in microtiter plates using the BCA kit from Pierce (Rockford, IL) with dilutions of BSA as standards [25,26].

3. Results

3.1. The experimental system

Neidhardt et al. selected MOPS (3-[*N*-morpholino]propanesulfonic acid) as a metabolically inert buffer species for incorporation in MOPS medium [6]. MOPS accumulated in the cytoplasm when *E. coli* K-12 was cultivated in hypertonic MOPS medium, however [27,28]. Although MOPS did not stimulate bacterial growth in hypertonic media (it was not an osmoprotectant), MOPS accumulation was suppressed when glycine betaine or proline accumulated as a compatible solute. This interaction raised the possibility that MOPS accumulation might alter the expression or activity of osmoregulatory transporter ProP.

E. coli strain RM2 (*proP*$^+$ *proU*$^+$), cultivated with amino acid limitation to enhance ProP activity, was selected for these experiments because it lacks proline transporter PutP (it is Δ(*putPA*)*101*) and it shows negligible ProU activity under these conditions [15]. *E. coli* RM2 grew at the same rates (0.077 ± 0.003 and 0.074 ± 0.002 generations/h, respectively) and to similar optical densities in MOPS medium and in phosphate-buffered K5 medium at high osmolality. MOPS was not detectable in extracts of cells grown in K5 medium (high or low osmolality) or in MOPS medium at low osmolality, but it was detected if bacteria were grown in high osmolality MOPS medium (58 ± 15 nmol MOPS/mg dry weight). The accumulation of MOPS did not influence the accumulation of endogenous compatible solute trehalose. Trehalose accumulated in bacteria grown in high but not low osmolality media, reaching levels of 97 ± 5 and 84 ± 8 nmol trehalose/mg dry weight for high osmolality K5 and MOPS medium, respectively. Thus MOPS accumulation is not osmoregulatory.

Since MOPS accumulated under conditions that also elicit *proP* induction and ProP activation it was necessary to further analyze the effects of MOPS on those responses. The ProP activity of *E. coli* RM2 was similar after growth in high osmolality MOPS and K5 media (proline uptake rates of 46 ± 3 and 41 ± 3 nmol/min/mg protein, respectively). MOPS did not affect proline uptake via ProP in cells of *E. coli* WG693 (*proP*$^+$ *proU*$^-$ *putPA*$^-$) cultivated (in the absence of MOPS) in high osmolality K5 medium (Fig. 2). Thus MOPS did not influence ProP activity at a biochemical or a genetic level. In light of these results, the use of MOPS minimal medium during past and future research on ProP is justified.

3.2. pH dependence of ProP activity

The apparent K_m values of most amino acid transporters in *E. coli* for their organic substrates are less than 10 μM [29]. The relatively high apparent K_m of ProP for its organic substrates (more than 100 μM, see further discussion below) could, in principle, arise from utilization of minor ionic species as the true transporter substrates (cf. [18]). The kinetics of pro-

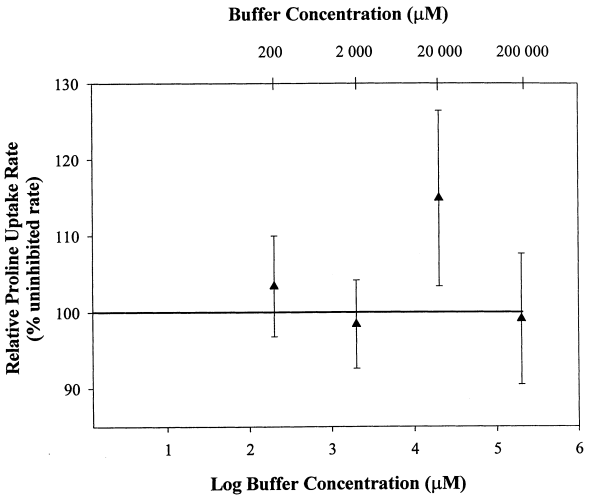

Fig. 2. Influence of MOPS on proline uptake via ProP. *E. coli* strain WG693 (*proP*$^+$ *proU*$^-$ *putPA*$^-$) was grown and initial rates of proline (204 μM) uptake were measured in high osmolality K5 medium in the presence of MOPS (3-[*N*-morpholino]-propanesulfonic acid) at the indicated concentrations. Uptake is expressed relative to the uninhibited proline uptake rate, which was 44 ± 2 nmol min^{-1} (mg protein)$^{-1}$. The horizontal line indicates the predicted relative proline uptake rate for a compound that has no effect on ProP. Data shown are means ± S.E. for three replicate assays during one of two experiments which yielded similar results.

line uptake were measured using whole cells washed and suspended in media at a number of pH values. Uptake was negligible and hence no kinetic parameters could be determined at pH 3. The V_{max} for proline uptake via ProP showed no strong, systematic dependence on pH over the range 7.5–4 (Fig. 3, inset). The apparent K_m for proline increased steadily in this range (Fig. 3). The pH dependence of V_{max}/K_m was therefore dominated by that of K_m (data not shown). If doubly protonated (cationic) proline were the true ProP substrate and the effect of pH on the K_m for proline were manifest only via the substrate ionization state, the apparent K_m would be expected to decrease as the medium pH decreased towards the pK_a of the proline carboxyl, which is 2. Thus the predominant, zwitterionic species of proline is most likely to be the true ProP substrate, the systematic dependence of apparent K_m (but not V_{max}) on pH implicated an enzyme functional group (or groups) with an apparent pK_a of 5–6 in catalysis and pH 7.4 is appropriate for measurements of ProP activity. Since transporter function requires respiratory energy in vivo, further analysis of the purified transporter [30] will be required to demonstrate that the critical functional groups reside in the transporter, not required respiratory enzymes.

3.3. Ion coupling specificity of ProP

Koo et al. showed that exogenous K^+ stimulated glycine betaine uptake via transporter ProP in *S. typhimurium* [8]. By measuring proline uptake in the presence of different cations in *E. coli* RM2, this ion dependence was confirmed and its specificity was tested (Table 2). An increase in the proline uptake rate in the presence of K^+, as compared to Na^+, Li^+ or choline$^+$, was observed as expected. To determine whether this K^+ dependence was ProP-specific, proline, serine and glutamine transport rates were estimated in the presence of Na^+ or K^+ (Table 2). Only proline uptake via ProP was stimulated by K^+.

Dinnbier et al. demonstrated that the uptake of proline or glycine betaine attenuated K^+ uptake or caused release of previously accumulated K^+ by osmoadapting cells of *E. coli* [16]. The dependence of that phenomenon on ProP was tested. Bacteria

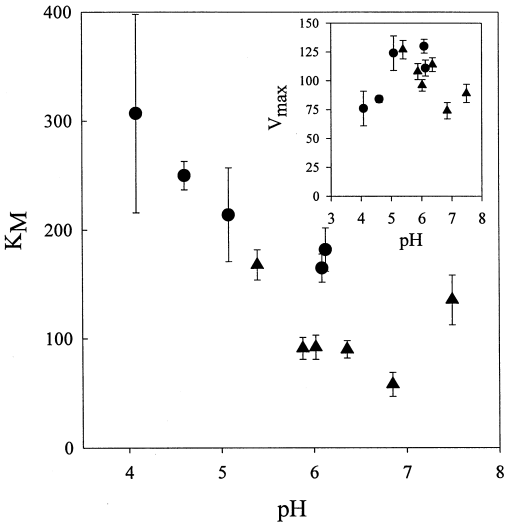

Fig. 3. pH dependence of proline uptake via ProP. *E. coli* RM2 was grown in MOPS minimal medium under osmotic stress, and washed twice in 60 mM potassium citrate, 0.3 M NaCl (●) or 20 mM potassium PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonate]), 20 mM potassium MES, 20 mM potassium phosphate, 0.3 M NaCl (▲), at the indicated pH. The proline uptake activity of the cells was determined using the same buffer as described in Section 2. V_{max} and apparent K_m values were obtained by linear regression analysis using Eadie-Hofstee plots.

Table 2
ProP is specifically K^+ dependent

Cation (as chloride salt)	Amino acid uptake rate (nmol/min/mg protein)		
	Proline	Serine	Glutamine
K^+	4.6 ± 0.46	1.8 ± 0.13	1.5 ± 0.30
Na^+	1.3 ± 0.05	1.8 ± 0.10	1.5 ± 0.07
Li^+	0.28 ± 0.07	NT	NT
Choline$^+$	0.34 ± 0.12	NT	NT

Strain RM2 (*proP*$^+$ *proU*$^+$ *putPA*$^-$) was grown under nutritional stress in MOPS medium, the cells were washed with 250 mM Tris-MES buffer, pH 6.0 and initial rates of amino acid uptake were measured in the presence of the indicated salts at a final concentration of 10 mM as described in Section 2. The potassium concentration in the 250 mM Tris-MES, pH 6.0 buffer was found by atomic absorption spectrometry to be 0.96 μM. NT, not tested.

loaded with K^+ by osmotic upshock as described by Dinnbier et al. [16] were offered no addition, proline, glycine betaine or galactose. K^+ released by the cells was estimated by atomic absorption spectroscopy and/or with K^+ dependent fluorescent dye CD222. These analytical techniques yielded similar estimates of K^+ efflux (Fig. 1), validating the use of this newly described fluorescent dye [22] for K^+ estimation.

Proline and glycine betaine elicited K^+ efflux from *E. coli* strain WG662 (*proP*^{O}, over-expressing compatible solute transporter ProP) and from *E. coli* strain WG443 (*proP*$^+$, expressing a single, chromosomal copy of *proP*) but not from *E. coli* strain WG663 (which over-expresses H^+/galactose symporter GalP and is ProP deficient) (Fig. 4A). Galactose did not elicit K^+ efflux, regardless of the GalP level. The proportion of cellular K^+ released by these bacteria was comparable to that observed by Dinnbier et al. [16] and consistent with the proportion of K^+ releasable by cold hypo-osmotic shock [30]. These data demonstrated that ProP activity could elicit K^+ release from osmoadapting cells, but they did not fully define the K^+ efflux pathway.

To determine whether the proline- and glycine betaine-dependent release of K^+ from osmoadapting *E. coli* cells was ProP-dependent, the experiment described above was replicated using bacteria that expressed ProP, ProU or neither transporter. The experimental protocol was modified to allow expression of *proU* prior to K^+ loading (see Section 2). Proline and glycine betaine were both effective in stimulating K^+ release from bacteria expressing either ProP or ProU (Fig. 4B) but not from bacteria lacking both transporters. Thus, although compatible solute uptake (via either transporter) stimulated K^+ release,

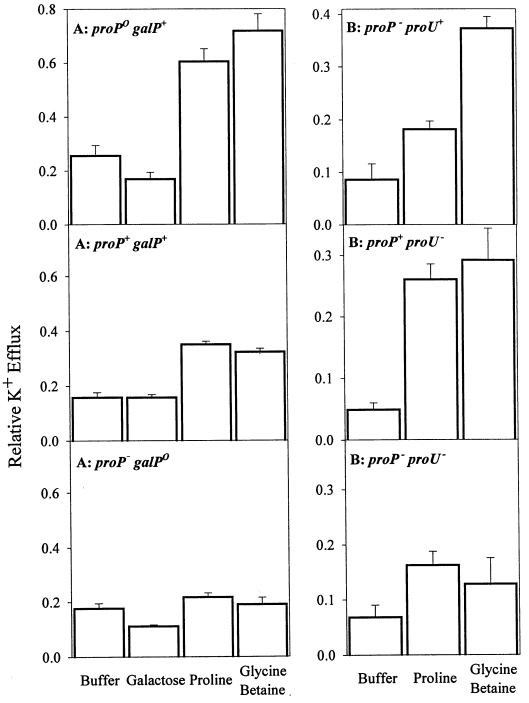

Fig. 4. Compatible solute uptake stimulates K^+ release from osmoadapting *E. coli* cells. K^+ efflux from osmoadapting *E. coli* cells (see Section 2) was estimated by fluorescence spectroscopy using CD222 (1 μM) (see Fig. 2). Data are derived from representative experiments and error bars represent standard errors ($n = 3$). K^+ release is expressed relative to the quantity of K^+ extracted from the same cells with SDS/chloroform (see Section 2). Those quantities (in μmol K^+/mg cell protein) were: (A) *E. coli* WG662 (*proP*^{O} *galP*$^+$), 0.98 ± 0.17; *E. coli* WG443 (*proP*$^+$ *galP*$^+$), 0.95 ± 0.04; *E. coli* WG663 (*proP*$^-$ *galP*^{O}), 1.01 ± 0.09. (B) *E. coli* WG445 (*proP*$^-$ *proU*$^+$), 1.67 ± 0.22; *E. coli* WG443 (*proP*$^+$ *proU*$^-$), 1.73 ± 0.17; *E. coli* WG439 (*proP*$^-$ *proU*$^-$), 1.97 ± 0.31.

that release was unlikely to be mediated by ProP or ProU and may have occurred via one or more channels [3]. These results suggest that K^+ is not a ProP substrate. Rather it influences ProP activity indirectly (for example, through effects on cellular energy metabolism) or it interacts with a regulatory site on transporter ProP.

The amino acid sequence-based prediction that ProP is a compatible solute/H^+ symporter [12] was confirmed by monitoring extracellular pH changes in suspensions of bacteria overexpressing or not expressing ProP (*proP*^{O} or *proP*$^-$) (Fig. 5). The *E. coli* strains retained the chromosomal *galP* locus (*galP*$^+$) but lacked a chromosomal *proP* locus (Δ*proP*). Strain WG528 overexpressed transporter GalP by expressing a plasmid-borne *galP* gene and strain WG527 overexpressed transporter ProP by expressing a plasmid-borne *proP* gene. Cells expressing galactose/H^+ symporter GalP at wild type or amplified levels (*galP*$^+$ or *galP*^{O}) were used as controls. Introduction of galactose to osmotically upshocked cell suspensions of *E. coli* strain WG528 (*galP*^{O}, *proP*$^-$) caused alkalinization of the external medium

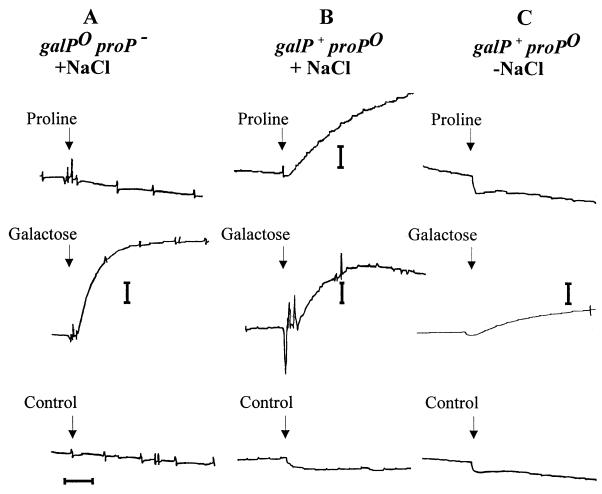

Fig. 5. Galactose/GalP- and proline/ProP-mediated proton uptake by *E. coli* cells. Bacteria were cultivated and galactose- or proline-dependent proton uptake was measured with a pH electrode as described in Section 2. All reaction mixtures contained 15–20 mg cell protein/ml. For A and B, osmotic upshifts were imposed by adding NaCl (to 0.3 M) 5 min before substrate addition. Arrows indicate the addition of proline (to 5 mM) (Proline), galactose (to 3 mM) (Galactose) or unsupplemented solution B (see Section 2) (Control). Horizontal and vertical markers indicate an elapsed time of 12 s and a 25 nmole proton flux, respectively.

while addition of solvent or proline produced a slight acidification (Fig. 5A). Alkalinization of the extracellular medium was observed when either galactose or proline, but not the solvent alone, was introduced to osmotically upshocked suspensions of strain WG527 (*galP*$^+$ *proP*^{O}) (Fig. 5B). Galactose-dependent alkalinization of the extracellular medium was observed in strain WG527 regardless of an osmotic upshift, while proline-induced alkalinization was osmotic upshift dependent (Fig. 5B and C). Subsequent addition of galactose to proline supplemented suspensions of *E. coli* strain WG527 (no upshift) resulted in medium alkalinization in all cases (data not shown). These results demonstrate that measurable ProP-dependent alkalinization was contingent on imposition of an osmotic upshock prior to ProP substrate addition and indicate that ProP is a compatible solute/H^+ symporter. This conclusion is consistent with the observations that a membrane potential, alone, is sufficient to power proline uptake via purified ProP in proteoliposomes and that an imposed pH gradient further stimulates proline uptake in that system [31].

3.4. Organic substrate specificity of ProP

In preparation for analyses of the organic substrate specificity of ProP, the kinetics of proline uptake were determined for cells of *E. coli* strains WG443 and WG693 (both *proP*$^+$ Δ*putP* Δ*proU*) which had been cultivated and resuspended in high osmolality MOPS medium (pH 7.4). Transport activity was measured at the following proline concentrations (μM): 29, 54, 79, 104, 129, 204, 304, 404 and 504. The resulting apparent K_m values (119 ± 10 μM and 111 ± 13 μM, respectively) were consistent with those previously determined using bacteria suspended in other media (Fig. 3).

Glycine betaine and its structural analogues inhibited proline uptake in a manner that depended systematically on the degree of *N*-methylation and the presence of the carboxyl function (Fig. 6A). Glycine betaine and proline betaine were similarly powerful inhibitors of proline uptake. The dependence of proline uptake rate on proline and inhibitor concentration was determined (see, for example, representative glycine betaine inhibition data illustrated in Fig. 7). With the exception of glycine, which was not signifi-

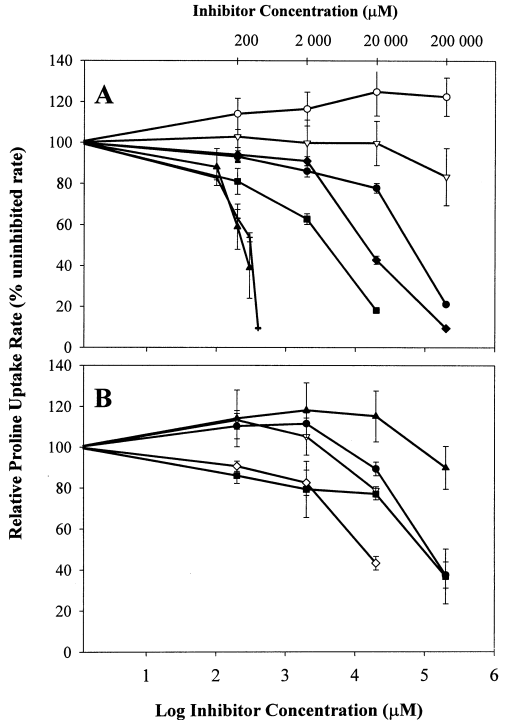

Fig. 6. Inhibition of proline uptake via ProP. Strain WG693 (*proP*$^+$ *proU*$^-$ *putPA*$^-$) was grown and initial rates of proline uptake were measured in high osmolality MOPS medium. Values shown are the mean ± S.E. for triplicate measurements performed during one of two replicate experiments that gave similar results. (A) Proline was provided at 204 μM. The uninhibited rates (in nmoles/min/mg protein) were: 74 ± 2 for glycine betaine (▲); 30 ± 2 for proline betaine (+); 36 ± 1 for *N*,*N*-dimethylglycine (■); 20 ± 1 for *N*-methylglycine (♦); 30 ± 1 for choline (●); 30 ± 1 for glycine (▽) and 36 ± 1 for trimethylamine oxide (○). (B) Proline was provided at 204 μM. The uninhibited proline uptake rates (in nmoles/min/mg protein) were: 36 ± 1 for 1-carboxymethylpyridinium (◇); 20 ± 2 for carnitine (■); 43 ± 6 for trigonelline (▽); 23 ± 1 for alanine (●); and 18 ± 2 for *O*-acetylcarnitine (▲).

cantly inhibitory, data obtained with each of these compounds best fit the competitive inhibition model (see Fig. 7 and apparent K_i values summarized in Table 3). It is important to note that the kinetic models which form the basis for distinctions among competitive, non-competitive and mixed inhibition do not encompass such special features of transport mechanisms as exposure of transporter surfaces (and hence active sites) to different solution environments. These apparent K_i values are thus empirical descriptors of relative inhibitory potency that cannot readily be interpreted in terms of transport mechanism. With the exception of choline, these compounds were all predominantly zwitterionic under the assay conditions.

It was important to determine whether a contaminant was responsible for the observed inhibition of proline uptake by high levels of choline. Glycine betaine was not detected when the choline used for these experiments was analyzed by HPLC-MS/MS. Glycine betaine was readily detected, however, when it was added at a level sufficient to cause the observed inhibition, 0.2 mole% (data not shown). Cytoplasmic extracts of *proP*$^+$ bacteria grown under osmotic stress, with and without choline, were analyzed by NMR spectroscopy and the spectra were compared. Except for resonances attributed to chol-

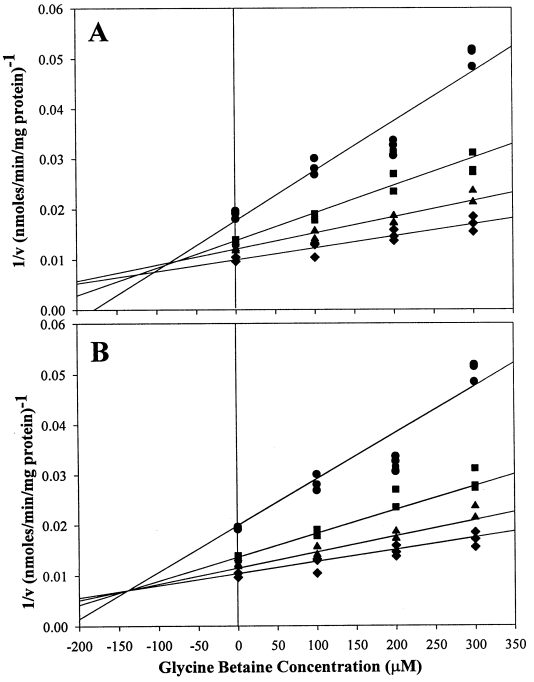

Fig. 7. Inhibition kinetics: glycine betaine as inhibitor of proline uptake via ProP. Strain WG693 (*proP*$^+$ *proU*$^-$ *putPA*$^-$) was grown and initial rates of proline uptake were measured in high osmolality MOPS medium. The initial velocity of proline uptake (v) was measured at 104 μM, 204 μM, 304 μM and 404 μM proline, without an inhibitor or with 100 μM, 200 μM or 300 μM glycine betaine as described in Section 2. Values shown are for triplicate measurements performed during one of two replicate experiments that gave similar results. Best fit lines were obtained by linear regression of data obtained at each proline concentration (A) or non-linear regression of all data (B) using the competitive inhibition model ($v = (V_{max}\ s)/[K_m(1+i/K_i)+s]$). For non-linear regression, the apparent K_i and V_{max} (but not the apparent K_m, 114 μM) were allowed to vary (see discussion in text).

Table 3
Competitive inhibitors of proline uptake via ProP

Inhibitor	V_{max} (nmol/min/mg protein)	K_i (mM)
Glycine betaine	35 ± 1	0.091 ± 0.013
Proline betaine	30 ± 1	0.208 ± 0.017
N,*N*-Dimethylglycine	52 ± 3	1.4 ± 0.2
Methylglycine	55 ± 1	5.0 ± 0.5
Choline	28 ± 1	62 ± 12

E. coli WG693 (*proP*$^+$ Δ*putPA* Δ*proU*) was cultivated in high osmolality MOPS medium and initial rates of proline uptake were measured in reaction mixtures containing ^{14}C-L-proline (at a series of four concentrations between 54 μM and 304 μM (*N*-methylglycine) or 104 μM and 304 μM (other compounds)) and no inhibitor or unlabeled inhibitor (at a series of four concentrations appropriate for the determination of each K_i). Data were fit to the competitive model for enzyme inhibition (see text and legend to Fig. 7). In each case the cited value is mean ± S.E. for one of two representative experiments which yielded similar apparent K_i values.

ine, all chemical shifts were identical in the two spectra (data not shown). Thus choline was not contaminated with a physiologically significant level of a high affinity ProP substrate, as ProP substrate accumulation is readily detectable by NMR spectroscopy ([27,32] and this study, data not shown).

Lamark et al. reported that choline is a substrate for transporter ProU, but not ProP [33]. However, the choline concentration used by Lamark et al. (0.5 mM) was too low to support choline uptake via ProP if it occurred with an apparent K_m similar to the measured apparent K_i (59 mM). No ProP-dependent choline uptake activity was detected when *E. coli* strains EF046 (*betT*$^-$ *proP*$^+$) and EF047 (*betT*$^-$ *proP*$^-$) were offered radiolabeled choline at 50 mM but the expected proline uptake was observed (proline at 0.2 mM) (data not shown). These results demonstrate that choline is a ProP inhibitor of very low affinity, but it is not a ProP substrate.

The inhibition of proline uptake via ProP effected by alanine, carnitine, *O*-acetyl carnitine, 1-carboxymethylpyridinium and trigonelline was also explored (Fig. 6B). These compounds all contain amino and carboxylic acid groups, but differ in the extent of amino-methylation, charge separation and the nature of the carbon backbone (straight chain vs. ring). *O*-Acetylcarnitine was not an inhibitor. The relative potency of the other compounds was: 1-carboxymethylpyridinium > alanine ≈ trigonelline ≈ carnitine.

Compounds that act as competitive inhibitors of

Table 4
ProP inhibition and ProP-dependent osmoprotection

Compound	ProP inhibition (see text)	Osmoprotectant concentration (mM)	Osmoprotection
Alanine	+	25	−
1-Carboxymethylpyridinium	+	18	+
Carnitine	+	50	+
Choline	+	50	−
Ectoine	NT	1	+
Glycine	−	50	−
Glycine betaine	+	1	+
N-Methylglycine	+	6	−
N,*N*-Dimethylglycine	+	1.5	+
O-Acetylcarnitine	−	50	+
Pipecolic acid	NT	1	+
Proline	NT	1	+
Proline betaine	+	0.8	+
Trigonelline	+		NT

ProP inhibition was detected (during this and other work) as discussed in the text. Osmoprotection was assessed using *E. coli* strains EF046 (*proP*$^+$ *proU*$^-$ *putPA*$^-$ *bet*$^-$) and EF047 (*proP*$^-$ *proU*$^-$ *putPA*$^-$ *bet*$^-$) (choline osmoprotection assay) or WG693 (*proP*$^+$ *proU*$^-$ *putPA*$^-$ *bet*$^+$) and WG441 (*proP*$^-$ *proU*$^-$ *putPA*$^-$ *bet*$^+$) (osmoprotection by other compounds) as described in Section 2.

ProP are likely to bind at the transporter active site and may be transporter substrates. Osmoprotection assays were used to further assess whether these compounds undergo ProP-dependent cytoplasmic accumulation when offered at a concentration approximating the apparent K_i for proline uptake via ProP (inhibitors) or at 50 mM (compounds that did not inhibit proline uptake via ProP) (Table 4). 1-Carboxymethylpyridinium, carnitine, *N*,*N*-dimethylglycine and proline betaine, all inhibitors of proline uptake via ProP, were also osmoprotectants. These results provided further support for previous indications that carnitine [34] and proline betaine [35] are ProP substrates. The fact that *O*-acetylcarnitine was an osmoprotectant but not a ProP inhibitor may indicate that it is metabolized to carnitine extracellularly, as in *Bacillus subtilis* [36]. Choline, alanine, trigonelline and *N*-methylglycine (sarcosine) were weak inhibitors of proline uptake via ProP (Fig. 6) but not osmoprotectants (Table 4 and [37]). This behavior may indicate that, like choline, these compounds were not accumulated via ProP or that, as for taurine, they did not afford sufficient cellular rehydration, without inhibiting cellular functions, to stimulate bacterial growth. Ectoine [14] and pipecolic acid [13] were previously shown to be accumulated by *proP*$^+$ *putP*$^+$ but not by *proP*$^-$ *putP*$^-$ bacteria. Since these compounds also provided ProP-dependent osmoprotection in *putP*$^-$ bacteria, they are ProP substrates.

4. Discussion

Data summarized above ([10,11], Fig. 6) and those reported recently by Racher et al. [31] substantiate claims that ProP catalyzes electrogenic co-transport of protons with various zwitterionic organic substrates [7,8,12]. ProP activity is stimulated by exogenous K^+ in intact cells of *S. typhimurium* [8] and *E. coli* (Table 2). In *E. coli* at least, that stimulation is transporter-specific (Table 2). Proline- or glycine betaine-stimulated release of K^+ from osmoadapting *E. coli* cells was not ProP-specific, however (Fig. 4). It is therefore unlikely that K^+ is a ProP substrate and likely that K^+ exerts regulatory effects directly, by interacting with ProP, or indirectly, through other physiological effects. Since ProP(His)$_6$ has been purified and reconstituted in proteoliposomes [31], the impact of K^+ on ProP activity can now be explored in the absence of other cellular constituents.

This research has extended the list of organic compounds that are ProP substrates (Fig. 8). The compounds illustrated in Fig. 8 are ProP substrates according to the following criteria: (1) they are accumulated by *proP*$^+$ and not by otherwise isogenic *proP*$^-$ bacteria (proline, glycine betaine, taurine) [11,38,39], (2) they are taken up by *proP*$^+$ *putP*$^+$ and not by otherwise isogenic *proP*$^-$ *putP*$^-$ bacteria and they provide osmoprotection to *proP*$^+$ but not to otherwise isogenic *proP*$^-$ bacteria (ectoine, pipecolic acid) ([13,14] and Table 4), (3) they competitively inhibit proline uptake via ProP and they provide ProP-dependent osmoprotection (proline betaine, *N*,*N*-dimethylglycine, carnitine, 1-carboxymethylpyridinium) (Tables 3 and 4, Figs. 6 and 7) [13,35,40,52] or (4) they show ProP-dependent cytotoxicity at intracellular targets (3,4-dehydroproline, L-azetidine-2-carboxylate) [10]. (Some of these compounds are also known by alternate names: glycine betaine (*N*,*N*,*N*-trimethylglycine); taurine (2-aminoethanesulfonate); carnitine (2-hydroxy-*N*,*N*,*N*-trimethyl-4-aminobutyrate); proline betaine (*N*,*N*-dimethylproline or stachydrine); pipecolate (2-piperidinecarboxylate or pipecolinate); ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylate).

All of the ProP substrates illustrated in Fig. 8 are zwitterions, present fully or predominantly in forms with no net charge at or near neutral pH. In addition, choline has been shown to act as an inhibitor but not a substrate for ProP. Since choline is otherwise similar in structure to compounds with much lower K_i values (e.g. glycine betaine, proline betaine), zwitterionic character appears to be an important

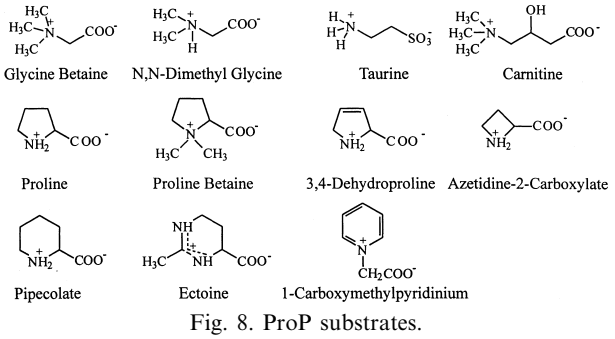

Fig. 8. ProP substrates.

feature of ProP substrates. A variety of other structurally related compounds are known to accumulate to varying degrees in *E. coli* cells in hypertonic media. They include L-alanine betaine, arsenobetaine, betonicine, butyrobetaine, α,α-dimethylglycine betaine, *N*,*N*-dimethylpipecolic acid, dimethylsulfoniopropionate, dimethylthetin, hormane, *N*-methyl-L-proline, L-phenylalanine betaine, α-(phenyl)glycine betaine, *N*-(phenyl)glycine betaine, pipecolic betaine, D-proline betaine, pyridinium, propionobetaine, β-selena-L-proline, γ-selena-L-proline, L-serine betaine, sulfobetaine, β-thia-L-proline, γ-thia-L-proline, thiolanium betaine, *N*,*N*,*N*-triethylglycine betaine and L-valine betaine [13,37,40–44]. Since transporters ProP, ProU, BetT and PutP of *E. coli* have overlapping substrate specificities, it is not yet evident which of these compounds are ProP substrates.

Like *E. coli*, cells of the mammalian renal medulla accumulate compatible solutes, including glycine betaine, in response to hypertonic media [45]. The levels of glycine betaine and choline in urine are controlled by homeostatic mechanisms. Glycine betaine, choline and other urine constituents with dietary origins (e.g. proline betaine) stimulate growth of *E. coli*, the primary causative agent of ascending urinary tract infections, in hypertonic media [46]. Introduction of a *proP* defect to a pyelonephritis isolate of *E. coli* impaired its ability to grow in hypertonic human urine and to colonize the murine bladder [47]. On the basis of these observations, bacterial betaine transporters have been identified as potential targets for new antibacterial agents [46]. The development of agents which would directly impair or exploit osmoregulatory betaine uptake by bacteria requires identification of the bacterial and host betaine transporters effective in vivo and of differences in substrate specificity between them. The data reported and summarized here further define the substrate specificity of ProP, the transporter that is expected to respond first to increases in the osmolality of the bacterial environment.

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References

[1] E.A. Galinski, Adv. Microb. Physiol. 37 (1995) 273–328.
[2] B. Poolman, E. Glaasker, Mol. Microbiol. 29 (1998) 397–407.
[3] J.M. Wood, Microbiol. Mol. Biol. Rev. 63 (1999) 230–262.
[4] B. Kempf, E. Bremer, Arch. Microbiol. 170 (1998) 319–330.
[5] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972.
[6] F.C. Neidhardt, P.L. Bloch, D.F. Smith, J. Bacteriol. 119 (1974) 736–747.
[7] J. Cairney, I.R. Booth, C.F. Higgins, J. Bacteriol. 164 (1985) 1218–1223.
[8] S.-P. Koo, C.F. Higgins, I.R. Booth, J. Gen. Microbiol. 137 (1991) 2617–2625.
[9] M. Farwick, R.M. Siewe, R. Krämer, J. Bacteriol. 177 (1995) 4690–4695.
[10] R.R. Anderson, J. Wood, R.M. Menzel, J. Bacteriol. 141 (1980) 1071–1076.
[11] J.L. Milner, S. Grothe, J.M. Wood, J. Biol. Chem. 263 (1988) 14900–14905.
[12] D.E. Culham, B. Lasby, A.G. Marangoni, J.L. Milner, B.A. Steer, R.W. van Nues, J.M. Wood, J. Mol. Biol. 229 (1993) 268–276.
[13] G. Gouesbet, M. Jebbar, R. Talibart, T. Bernard, C. Blanco, Microbiology 140 (1994) 2415–2422.
[14] M. Jebbar, R. Talibart, K. Gloux, T. Bernard, C. Blanco, J. Bacteriol. 174 (1992) 5027–5035.
[15] S. Grothe, R.L. Krogsrud, D.J. McClellan, J.L. Milner, J.M. Wood, J. Bacteriol. 166 (1986) 253–259.
[16] U. Dinnbier, E. Limpinsel, R. Schmid, E.P. Bakker, Arch. Microbiol. 150 (1988) 348–357.
[17] C.-C. Chen, T. Tsuchiya, Y. Yamane, J.M. Wood, T.H. Wilson, J. Membr. Biol. 84 (1985) 157–164.
[18] M.E. van der Rest, T. Abee, D. Molenaar, W.N. Konings, Eur. J. Biochem. 195 (1991) 71–77.
[19] H.J. Kunte, E.A. Galinski, H.G. Trüper, J. Microbiol. Methods 17 (1993) 129–136.
[20] L.T. Smith, G.M. Smith, J. Bacteriol. 171 (1989) 4714–4717.
[21] E. Frings, H.J. Kunte, E.A. Galinski, FEMS Microbiol. Lett. 109 (1993) 25–32.
[22] R. Crossley, Z. Goolamali, P.G. Sammes, J. Chem. Soc. Perkin Trans. 2 (1994) 1615–1623.

[23] P.J.F. Henderson, A.J.S. MacPherson, Methods Enzymol. 125 (1986) 387–429.
[24] R.T. Voegele, E.V. Marshall, J.M. Wood, in: G.C. Brown, C.E. Cooper (Eds.), Bioenergetics: a Practical Approach, Oxford University Press, Oxford, 1995, pp. 17–37.
[25] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[26] K.J. Wiechelman, R.D. Braun, J.D. Fitzpatrick, Anal. Biochem. 175 (1988) 231–237.
[27] S. Cayley, M.T. Record Jr., B.A. Lewis, J. Bacteriol. 171 (1989) 3597–3602.
[28] S. Cayley, B.A. Lewis, M.T. Record Jr., J. Bacteriol. 174 (1992) 1586–1595.
[29] J.L. Milner, B. Vink, J.M. Wood, CRC Crit. Rev. Biotechnol. 5 (1987) 1–47.
[30] D. McLaggan, J. Naprstek, E.T. Buurman, W. Epstein, J. Biol. Chem. 269 (1994) 1911–1917.
[31] K.I. Racher, R.T. Voegele, E.V. Marshall, D.E. Culham, J.M. Wood, H. Jung, M. Bacon, M.T. Cairns, S.M. Ferguson, W.-J. Liang, P.J.F. Henderson, G. White, F.R. Hallett, Biochemistry 38 (1999) 1676–1684.
[32] S. Cayley, B.A. Lewis, H.J. Guttman, M.T. Record Jr., J. Mol. Biol. 222 (1991) 281–300.
[33] T. Lamark, O.B. Styrvold, A.R. Strom, FEMS Microbiol. Lett. 96 (1992) 149–154.
[34] H. Jung, K. Jung, H.P. Kleber, J. Basic Microbiol. 30 (1990) 409–413.
[35] M. Haardt, B. Kempf, E. Faatz, E. Bremer, Mol. Gen. Genet. 246 (1995) 783–786.
[36] R.M. Kappes, E. Bremer, Microbiology 144 (1998) 83–90.
[37] K. Randall, M. Lever, B.A. Peddie, S.T. Chambers, Biochim. Biophys. Acta 1291 (1996) 189–194.
[38] G. May, E. Faatz, M. Villarejo, E. Bremer, Mol. Gen. Genet. 205 (1986) 225–233.
[39] D. McLaggan, W. Epstein, FEMS Microbiol. Lett. 81 (1991) 209–214.
[40] B.A. Peddie, M. Lever, C.M. Hayman, K. Randall, S.T. Chambers, FEMS Microbiol. Lett. 120 (1994) 125–132.
[41] M. di Girolamo, C. Cini, V. Busiello, R. Coccia, C. de Marco, Physiol. Chem. Phys. Med. NMR 16 (1984) 75–81.
[42] B. Perroud, D. Le Rudulier, J. Bacteriol. 161 (1985) 393–401.
[43] S.T. Chambers, C.M. Kunin, D. Miller, A. Hamada, J. Bacteriol. 169 (1987) 4845–4847.
[44] Y.S. Abdel-Ghany, M.A. Ihnat, D.D. Miller, C.M. Kunin, H.M. Tong, J. Med. Chem. 36 (1993) 784–789.
[45] M.B. Burg, in: G.N. Somero, C.B. Osmond, C.L. Bolis (Eds.), Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and Molecular Levels, Springer-Verlag, Berlin, 1992, pp. 33–51.
[46] S.T. Chambers, M. Lever, Nephron 74 (1996) 1–10.
[47] D.E. Culham, C. Dalgado, C.L. Gyles, D. Mamelak, S. MacLellan, J.M. Wood, Microbiology 144 (1998) 91–102.
[48] A. Barron, G. May, E. Bremer, M. Villarejo, J. Bacteriol. 167 (1986) 433–438.
[49] M.J. Casadaban, J. Mol. Biol. 104 (1976) 541–555.
[50] J.M. Wood, J. Bacteriol. 146 (1981) 895–901.
[51] D.E. Culham, K.S. Emmerson, B. Lasby, D. Mamelak, B.A. Steer, C.L. Gyles, M. Villarejo, J.M. Wood, Can. J. Microbiol. 40 (1994) 397–402.
[52] A. Verheul, J.A. Wouters, F.M. Rombouts, T. Abee, J. Appl. Microbiol. 85 (1998) 1036–1046.