A Conserved Aspartate Residue, Asp187, Is Important for Na^+-Dependent Proline Binding and Transport by the Na^+/Proline Transporter of *Escherichia coli*†

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ABSTRACT: Asp187 in the Na^+/proline transporter of *Escherichia coli* (PutP) is conserved within the Na^+/solute cotransporter family. Information on the role of this residue has been gained by amino acid substitution analysis. PutP with Glu, Asn, or Cys in place of Asp187 catalyzed Na^+-coupled proline uptake at 75%, 25%, and 1.5%, respectively, of the V_{max} of PutP-wild-type while the apparent K_m for proline was only slightly altered. Importantly, acetylation or amidoacetylation of an engineered transporter containing a single Cys at position 187 stimulated proline uptake. Strikingly, PutP-D187C exhibited high-affinity proline binding even at very low Na^+ concentrations (2 μM) while proline binding to PutP-wild-type, -D187E, and -D187N was strictly dependent on the Na^+ concentration. The apparent independence of proline binding from the Na^+ concentration can at least partially be attributed to an enhanced Na^+ affinity of PutP-D187C. In addition, reaction of PutP containing a single Cys at position 187 with *N*-ethylmaleimide was inhibited by Na^+ but not by Li^+ or proline. The results indicate that electrostatic interactions of the amino acid side chain at position 187 in PutP with other parts of the transporter and/or the coupling ion are crucial for active proline transport. It is suggested that Asp187 is located close to the pathway of the coupling ion through the membrane and may be involved in the release of Na^+ on the cytoplasmic side of the membrane.

The Na^+/proline transporter of *Escherichia coli* (PutP) is a hydrophobic, integral protein of the cytoplasmic membrane that catalyzes the coupled translocation of proline and Na^+ (Li^+) ions according to a symport mechanism (*1*−*3*; see *4*, *5* for review). PutP is a member of the Na^+/solute cotransporter family (SCF) (*6*, *7*). The protein has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for Na^+/proline transport (*8*, *9*). On the basis of hydropathy profile analysis of the primary amino acid sequence, a secondary structure model is proposed according to which PutP has a short hydrophilic N-terminus, 12 α-helical transmembrane domains that traverse the membrane in a zigzag fashion connected by hydrophilic loops, and a 27-residue hydrophilic C-terminal tail (*10*). Evidence confirming the location of the C-terminus on the cytoplasmic side of the membrane has been obtained from immunological analysis (*11*).

Spontaneous and site-directed mutagenesis have been utilized in an attempt to identify amino acid residues in PutP involved in proline and/or ion binding and translocation. Thus, substrate specificity mutants of *Salmonella typhimurium* were isolated, but the precise sites of mutations in the *putP* gene have not yet been determined (*12*−*14*). Furthermore, spontaneous mutations resulting in substitution of Gly22 and Cys141 by Glu and Tyr, respectively, reduced the affinity of PutP to Na^+ ions with no effect on proline binding (*15*). Site-specific alteration of Arg376 implied that this positively charged amino acid residue of a proposed Na^+ binding motif of several Na^+ symporters (*16*) does not reside at the postulated Na^+ binding site (*17*). Furthermore, it was demonstrated that Ser57 is critical for high-affinity proline uptake by PutP (*18*). In addition, analysis of the role of four acidic amino acid residues in the N-terminal part of PutP revealed that a carboxylate at posion 55 is essential for Na^+-coupled proline uptake (*19*). Thus, replacement of the native Asp55 with a neutral amino acid (Cys or Asn) completely impaired all types of transport, whereas Glu at this position reduced the apparent Na^+ affinity dramatically, suggesting an involvement of Asp55 in Na^+ binding.

A sequence alignment revealed that Asp187 of PutP is conserved in 37 members of the SCF. In this paper, we have investigated the functional consequences of replacing this residue with Asn, Cys, and Glu. Analyses of transport kinetic and ligand binding together with site-directed labeling studies indicate that electrostatic interactions of the amino acid side chain at position 187 in PutP with other parts of the transporter and/or the coupling ion are crucial for active proline transport.

EXPERIMENTAL PROCEDURES

Materials. L-[U-^{14}C]Proline (246 μCi/μmol) and *N*-ethyl-1-^{14}C]maleimide (NEM)[1] (40 μCi/μmol) were purchased

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DM, *n*-dodecyl β,D-maltoside; IPTG, isopropyl 1-thio-β,D-galactopyranoside; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; Ni-NTA, nickel nitrilotriacetic acid; PMSF, phenylmethanesulfonyl fluoride; TMA-OH, tetramethylammonium hydroxide.

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from Hartmann Analytic and DuPont−New England Nuclear, respectively. Restriction endonucleases, alkaline phosphatase, T4 DNA ligase, and *Taq* DNA polymerase were obtained from New England Biolabs and from Gibco/BRL. Synthetic oligonucleotide primers were purchased from Eurogentec, and small-DNA agarose was from Biozym. Mouse anti-Flag M2 IgG was from Integra Biosciences. Horseradish peroxidase-conjugated sheep anti-(mouse-IgG) antibody and the enhanced chemiluminiscence kit were obtained from Amersham Corp. Nitrocellulose membranes (0.45 μm pore size) were purchased from Schleicher & Schüll, and GF/F filters (0.7 μm pore size) were from Whatman. *n*-Dodecyl β,D-maltoside (DM) was obtained from Anatrace, and nickel nitriloacetic acid (Ni-NTA) spin columns were from Quiagen, Inc. All other chemicals used were of analytical grade and purchased from commercial sources.

Bacterial Strains and Plasmids. *E. coli* JM109 [*endA1 recA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *tra*Δ*36 proAB*$^+$ *lacI*^{q} ZΔ*M*15)] (*20*) served as a host strain for cloning and plasmid isolation. *E. coli* WG170 [F′ *trp lacZ rpsL thi* Δ(*putPA*)*101 proP219*] (*21*) harboring given plasmids was used for in vitro proline transport assays, proline-driven Na^+ uptake measurements, and immunoblots. The following plasmids, derivatives of pT7-5 (*22*), containing the *lac* promotor/operator for expression of the *putP* gene were used for all gene manipulations: pTMH·FH and pTCl·FH, each of which harbored a cassette version of the *putP* gene encoding PutP-wild-type and an engineered transporter devoid of all five native Cys residues (Cys-free PutP), respectively (*23*). Proximal to the TAA stop codon of each cassette gene successive triplets were inserted, encoding a Flag epitope (*18*) and a 6·His-tag at the C-terminus of PutP (*24*). For overproduction of PutP via the *trc* promoter, the mutated entire cassette *putP* gene flanked by unique *Nco*I and *Hin*dIII sites at its 5′ and 3′ ends, respectively, was cloned into the multicloning site of plasmid pTrc99a (Pharmacia) using the corresponding restriction enzymes.

Site-Directed Mutagenesis and DNA Sequencing. The cassette *putP* gene in plasmid pTMH·FH was used as template for mutagenesis. Substitutions of Asp187 by Asn, Cys, and Glu were created using the polymerase chain reaction (PCR) with synthetic mutagenic antisense primers (D187C:[2] 5′-GGC AAA AAT CAT CAG GCT AGC CTG TAC AGT **GCA** AGT CCA-3′; D187E: 5′-GGC AAA AAT CAT CAG GCT AGC CTG TAC AGT **TTC** AGT CCA-3′; D187N: 5′-GGC AAA AAT CAT CAG GCT AGC CTG TAC AGT **GTT** AGT CCA-3′; altered codons are in bold phase, the *Nhe*I recognition site is underlined) and a sense primer binding upstream of the *Bss*HII endonuclease recognition site. PCR fragments were isolated from small DNA agarose and digested with *Bss*HII and *Nhe*I restriction endonucleases. The resulting 128 bp DNA fragments were ligated to similarly treated pTMH·FH incubated with alkaline phosphatase to avoid religation of the vector. In addition, to create a *putP* gene encoding transporter with a single-Cys at position 187 (single-Cys PutP-D187C), the isolated 128 bp *Bss*HII/*Nhe*I fragment was ligated into pTCl·FH incubated with the same restriction enzymes and alkaline phosphatase. The integrity of all inserted fragments carrying the desired mutation was confirmed by sequencing double-stranded plasmid DNA using dideoxynucleotide chain-termination (*25*) after alkaline denaturation (*26*).

Proline Transport Assays. Cells of *E. coli* WG170 ($PutP^-A^-$), transformed with each plasmid described, were grown aerobically at 37 °C in Luria−Bertani medium (LB medium) (*27*) supplemented with ampicillin (100 μg/mL). Overnight cultures were diluted 25-fold and allowed to grow up to an optical density at 420 nm (OD_{420}) of 1.0 before gene expression via the *lac* promoter was induced with 0.5 mM IPTG. After further growth for 2 h, cells were harvested by centrifugation, washed with 250 mM Tris/Mes buffer, pH 6.0, and resuspended in the same buffer to yield a final protein concentration of 0.35 mg/mL. Proline transport assays were performed as described (*19*). Kinetics of transport were calculated from the initial linear portion of the time courses between 0 and 10 s (0 and 2 min in the case of PutP-D187C). Data represent the mean of at least three independent experiments with standard deviations of less than 25%. The Na^+ contamination of the buffer used (Na^+-free buffer) was determined to be 2 μM by atomic absorption spectroscopy (Eppendorf, Elex6361).

Proline-Induced Na^+ Transport. Cells of *E. coli* WG170 were prepared as described for proline transport except that a protein concentration of 25 mg/mL was adjusted. Uptake of Na^+-coupled proline transport was measured as described previously (*19*) using a Na^+-selective electrode (ISEC221Na, Radiometer) and the PHM95 ion/pH meter (Radiometer). Na^+ uptake was initiated by addition of 2 mM L-proline.

Immunological Analysis. Membrane fractions of *E. coli* WG170 cells containing PutP with given substitutions were prepared by sonication as described (*18*). The amount of each transporter molecule present in the membrane was estimated by immunoblotting with mouse anti-Flag-IgG against the Flag epitope at the C-terminus of PutP followed by incubation with horseradish peroxidase-linked sheep anti-(mouse-IgG) antibody by the enhanced chemiluminiscence method.

Proline Binding. Overexpression of the *putP* gene in *E. coli* WG170 via the *trc* promoter in plasmid pTrc99a was achieved by induction of exponentially growing cells with 0.5 mM IPTG and continuing incubation for additional 3 h. After harvesting, cells were washed with ice-cold 250 mM Tris/Mes, pH 7.0, and resuspended in the same buffer. Right-side-out membrane vesicles were prepared by lysozyme/EDTA treatment of cells as described (*28*). The vesicles were washed 2 times with 100 mM Tris/Mes, pH 6.0 (Na^+ free), resuspended in the same buffer to yield a final protein concentration of 0.5 mg/mL, and deenergized by preincubation with 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 1 μM monensin for 15 min. Binding of L-[^{14}C]-proline to the vesicles was assayed in the presence of various concentrations of NaCl or LiCl at 30 °C for 30 min. Membranes were collected by ultracentrifugation at 105000*g* and 4 °C for 30 min, and membrane-bound radioactivity was determined by scintillation counting. The data were corrected for the amount of radioactivity bound to membrane vesicles of WG170 harboring pTrc99a without *putP* gene (negative control). The k_d values were determined by plotting the data according to Scatchard.

[2] Amino acid replacements are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the native residue in wild-type PutP. The sequence is followed by a second letter denoting the substitution at this position.

[^{14}C]-NEM Labeling. Inverted membrane vesicles containing single-Cys PutP-D187C were obtained from a cell suspension prepared as described for proline binding. The suspension was supplemented with 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and DNase I and passed through a Ribi pressure cell (Sorvall, RF-1, 20 000 psi). After low-speed centrifugation to remove cell debris, membrane vesicles were collected by ultracentrifugation at 150000*g* and resuspended in buffer containing 250 mM Tris/Mes, pH 7.0, 2 mM β-mercaptoethanol, and 0.5 mM PMSF. Samples of inverted membrane vesicles (5 mg of total membrane protein) were washed 3 times by suspension in Na^+-free 250 mM Tris/Mes, pH 7.0, and centifugation at 95000*g* and 4 °C to remove β-mercaptoethanol and Na^+. Membrane pellets were resuspended in 150 μL of the same buffer and incubated with 500 μM [^{14}C]-NEM (8 μCi/μmol) in the absence or presence of ligand as indicated at 25 °C. The reaction was stopped by the addition of 2 mM β-mercaptoethanol after 5 min, and labeled transporter molecules were solubilized from the membrane by addition of 1.5% DM followed by permanent stirring on ice for 30 min. Samples were then centrifuged at 95000*g*, and the supernatants were used for purification of labeled protein using the Ni-NTA spin column procedure according to the manufacturer's protocol. An aliquot of the purified protein was subjected to SDS/PAGE followed by silver staining (*29*). [^{14}C]-NEM-labeled PutP was detected on the gel with a Phosphor-Imager (Molecular Dynamics, Model SI). In addition, the radioactivity of another aliquot of purified PutP was determined quantitatively by scintillation counting. The obtained values were corrected for unspecific labeling using Cys-free PutP as a negative control.

Protein Determination. The amount of protein was determined using a modified Lowry method (*30*) with bovine serum albumin as standard.

Computer-Generated Sequence Comparison. For computer-assisted multiple protein sequence analysis, the Human Genome Center (Baylor College of Medicine, Houston, TX) pattern-induced-multiple-sequence alignment (PIMA) databank program (*31*, *32*) was used.

RESULTS

Sequence Comparison. A multiple sequence alignment of 40 members of the SCF was generated using the PIMA databank program (*31*, *32*; see also *6*, *7* for comparison). Asp187 in the putative cytoplasmic loop between transmembrane domains IV and V in PutP of *E. coli* (Figure 1) is conserved in 37 family members of known (Na^+/solute cotransport) or hypothetical function. The hypothetical proteins of unknown function, HypBac of *Bacillus subtilis*, HypE59 of *E. coli*, and HypAeu of *Alcaligenes eutrophus*, contain the polar amino acid residue Gln at the position corresponding to Asp187 in PutP. The latter proteins show the highest degree of sequence divergence.

Generation and Verification of Mutations. PCR-mediated site-directed mutagenesis was performed to replace Asp187 in PutP with Asn, Cys, or Glu. Mutagenic DNA fragments were cloned into plasmid pTMH·FH containing a cassette version of the *putP* gene (*23*) using the restriction endonuclease sites *Bss*HII and *Nhe*I. In addition, the DNA fragment causing substitution of Asp187 by Cys was introduced into

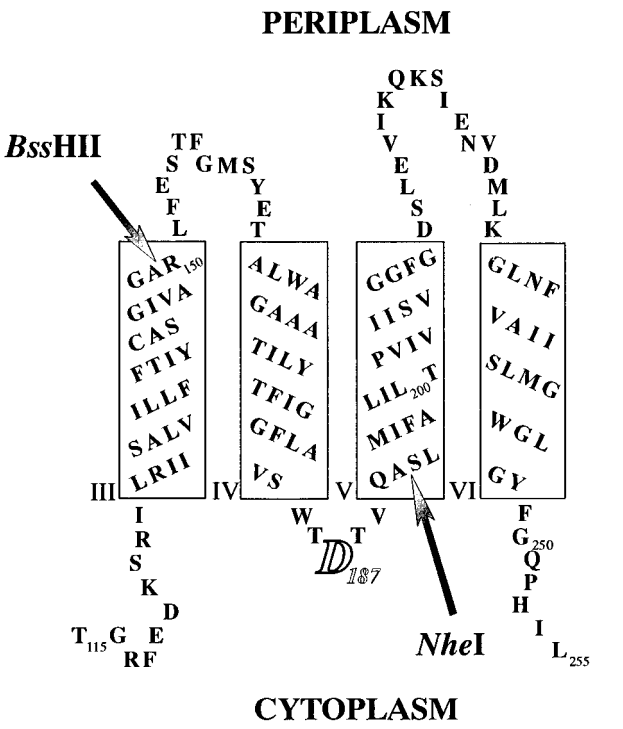

FIGURE 1: Secondary structure model of the flanking regions of Asp187 in the Na^+/proline transporter of *E. coli*. The model is based on hydropathy analysis of the primary amino acid sequence of the transporter (10). Putative transmembrane domains are represented as rectangles and numbered with Roman numerals. Asp187 is highlighted. The positions of relevant restriction endonuclease sites in the corresponding DNA sequence are also indicated.

the cassette *putP* gene encoding a functional PutP version in which all five native Cys residues had been replaced with Ser or Ala (*23*) using plasmid pTCl·FH and the same restriction endonuclease sites as above. All mutations were verified by sequencing of double-stranded plasmid DNA, and, except for the alterations introduced by synthetic mutagenic oligonucleotide primers, the remainder of the *putP* sequence was identical with that in pTMH·FH or pTCl·FH.

Immunological Analysis. The relative concentration of PutP molecules bearing replacements of Asp187 in membranes of *E. coli* WG170 was approximated by Western blot analysis using a monoclonal antibody against the Flag epitope at the C-terminus of the protein (*18*). Transporter molecules with Asn, Cys, or Glu in place of Asp187 were present in amounts comparable to the wild-type (Figure 2). Therefore, mutations resulting in substitution of Asp187 did not have a significant effect on the insertion or stability of the transporter in the membrane.

Transport Properties of Modified PutP. Active proline transport was measured in *E. coli* WG170 ($PutP^-A^-$) transformed with each plasmid described. Analysis of Na^+-coupled proline uptake revealed that the nature of the amino acid side chain at position 187 is critical for PutP activity. Thus, PutP with Glu or Asn in place of Asp187 catalyzed proline uptake at about 35% and 15%, respectively, of the initial rate to 60% and 15%, respectively, of the steady-state level of proline accumulation of PutP-wild-type (Figure 3). Furthermore, cells producing PutP with Cys in place of Asp187 showed only marginal transport activity (0.6% of the initial rate of the wild-type). In addition, the steady-state level of proline accumulation was reached after about

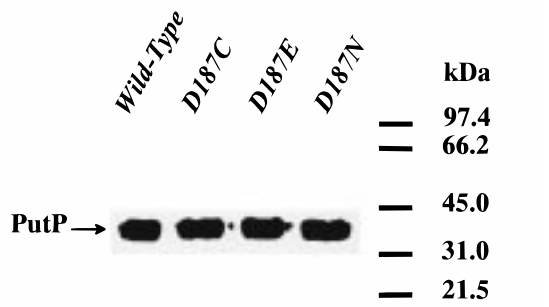

FIGURE 2: Western blot of membranes from *E. coli* WG170 cells containing PutP with given Asp187 replacements. Membranes of IPTG-induced cultures were subjected to 10% SDS−PAGE (40 μg of protein/lane) and electroblotted. The blot was incubated with mouse monoclonal antibody directed against the Flag epitope at the C-terminus of PutP. After incubation with sheep anti-(mouse-IgG) horseradish peroxidase conjugate, PutP was detected by the enhanced chemiluminiscence method. Positions of the protein standards are indicated (kDa).

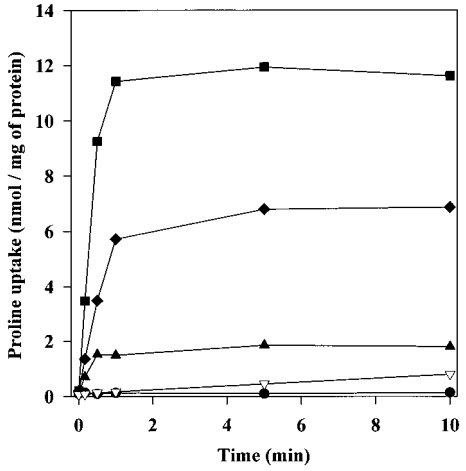

FIGURE 3: Time course of proline uptake by *E. coli* WG170 containing PutP with given amino acid replacements. Cells were grown and treated as described under Experimental Procedures. Transport of L-[U-^{14}C]proline (5 μM final concentration) was assayed in the presence of 50 mM NaCl and 20 mM D-lactate (Na^+ salt) as the electron donor at 25 °C under aerobic conditions. PutP-wild-type (■); PutP-D187C (▽); PutP-D187E (◆); PutP-D187N (▲); pT7−5 (●).

30 min and corresponded to 11% of the PutP-wild-type value. Interestingly, when Li^+ was used as a coupling ion instead of Na^+, a 3−4-fold stimulation of the initial rate of proline uptake catalyzed by PutP-D187C was observed. A similar stimulation was not seen with PutP-wild-type, -D187E, or -D187N.

Proline-induced Na^+ uptake was assayed using a Na^+-selective electrode. Addition of 2 mM proline to a suspension of *E. coli* WG170 cells producing either PutP-wild-type, -D187E, or -D187N caused an immediate decrease of the external Na^+ concentration as a result of Na^+/proline symport (Figure 4). In contrast, a significant alteration of the external Na^+ concentration was not observed with cells containing PutP-D187C or cells transformed with plasmid pT7-5 without *putP*.

Finally, to analyze facilitated diffusion of proline, cells of *E. coli* WG170 producing Na^+/proline transporter were completely deenergized by preincubation with CCCP and monensin. Measurement of time courses of facilitated diffusion did not yield significant differences between

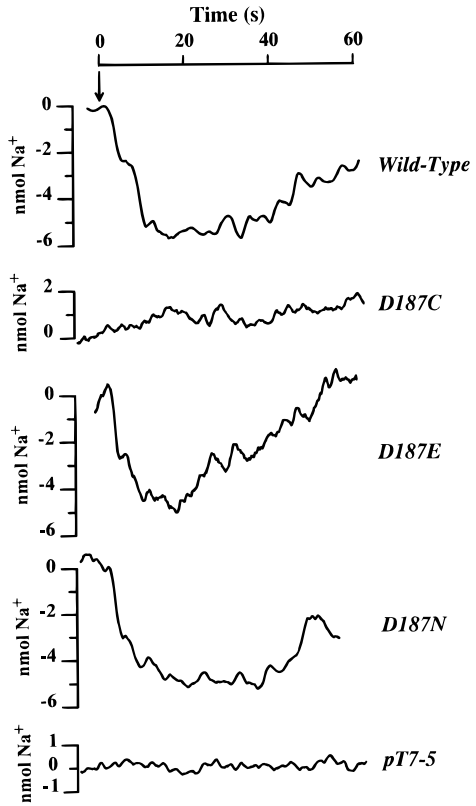

FIGURE 4: Proline-induced Na^+ uptake by *E. coli* WG170 containing PutP with the given amino acid replacements. Cells were prepared as described under Experimental Procedures and diluted to a final protein concentration of 0.1 mg/mL in 10 mL Tricine/TMA-OH, pH 8.0. For Na^+ uptake measurements, cells were incubated in the presence of 20 μM 5-(*N*,*N*-hexamethylene)-amiloride and 25 μM NaCl under a constant stream of N_2. 100 μL of a 200 mM L-proline solution was added as indicated by the arrow ($t = 0$). Negative ordinate values reflect a decrease of the Na^+ concentration in the external medium.

Table 1: Kinetic Analysis of Na^+- and Li^+-Coupled Proline Uptake by PutP Containing Given Replacements of Asp187[a]

	$K_{m(Pro)}$ (μM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	$K_{m(Na+)}$ (μM)	$K_{m(Li+)}$ (μM)
PutP-wild-type	2.1	27	32	125
PutP-D187C	8.1	0.4	4	50
PutP-D187E	6.8	21	67	500
PutP-D187N	6.2	6.8	51	130

[a] Initial rates of proline uptake by *E. coli* WG170 producing either wild-type or PutP with given replacements were measured at proline concentrations from 0.2 to 200 μM in the presence of 50 mM NaCl [determination of apparent $K_{m(Pro)}$]. Apparent affinity constants for Na^+ and for Li^+ [$K_{m(Na+)}$ and $K_{m(Li+)}$] were determined at ion concentrations varying from 0.002 (0.005 for Li^+) to 1 mM at a proline concentration of 5 μM. The data were plotted according to Eadie−Hofstee.

PutP-wild-type, -D187C, -D187E, and -D187N (data not shown).

Kinetics of Active Transport. Further kinetic characterization of Na^+-coupled proline transport by PutP-D187C, -D187E, and -D187N revealed only little alteration of the apparent K_m for proline while V_{max} was reduced to 1.5%, 78%, and 25%, respectively, of the wild-type value (Table 1). Furthermore, apparent K_m values for Na^+ and Li^+ were determined by measuring the rate of proline uptake at various ion concentrations (Na^+: 0.002−1 mM; Li^+: 0.005−1 mM). Analysis of the data according to Eadie−Hofstee revealed

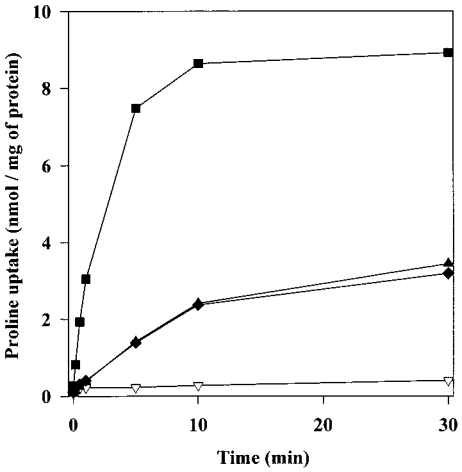

FIGURE 5: Effect of chemical modification of Cys187 in PutP on transport properties. *E. coli* WG170 cells harboring Cys-free PutP or single-Cys PutP-D187C were prepared as described in Figure 3. Subsequently, cells were preincubated with 1 mM either iodoacetic acid or iodoacetamide at 25 °C for 30 min as described (43). Transport of L-[^{14}C]proline (5 μM final concentration) was assayed in the presence of 50 mM NaCl and 20 mM D-lactate at 25 °C under aerobic conditions. Cys-free PutP (■); single-Cys PutP-D187C in the absence (▽) or presence of iodoacetic acid (◆) or iodoacetamide (▲).

that substitution of Asp187 by Asn or Glu had only a small effect on the apparent K_m values for Na^+ and Li^+. However, Cys in place of Asp187 caused an 8- and 2-fold decrease of the apparent K_m values for Na^+ and Li^+, respectively (Table 1).

Effect of Chemical Modification of Cys187 on PutP Activity. The D187C substitution was placed into a functional PutP devoid of all five native Cys residues, and the effect of iodoacetic acid and iodoacetamide on protein activity was tested (Figure 5). Single-Cys PutP-D187C was unable to catalyze accumulation of proline against a concentration gradient, but reaction of Cys187 with either iodoacetic acid or iodoacetamide restored low but significant Na^+-dependent proline uptake (1.5% and 23% of the initial rate and steady-state level of proline accumulation, respectively, of Cys-free PutP). The activity of a Cys-free PutP containing the native Asp at position 187 was unaffected by either reagent. The results provide a strong indication that a polar or negatively charged side chain at position 187 is important for active transport.

Ligand Binding. Proline binding to right-side-out membrane vesicles of *E. coli* WG170 containing PutP with given substitutions was analyzed using a centrifugation assay. In the case of PutP-wild-type, -D187E, and -D187N, shifting the Na^+ concentration from 0.5 to 50 mM led to an increase in the proline affinity of the transporter molecules while significant binding of proline was not observed in Na^+-free buffer (the latter buffer contained about 2 μM Na^+) (Figure 6, Table 2). Strikingly however, an increase of the Na^+ concentration from 0.5 to 50 mM did not cause any significant effect on proline binding to PutP-D187C. Instead, high-affinity proline binding (k_d values of about 2 μM) was determined even in Na^+-free buffer. Furthermore, proline binding to PutP-wild-type, -D187E, and -D187N was dependent on the Li^+ concentration similar to as observed for Na^+ (Figure 6, Table 2). Although the Li^+ concentration altered the proline affinity of PutP-D187C to some extent,

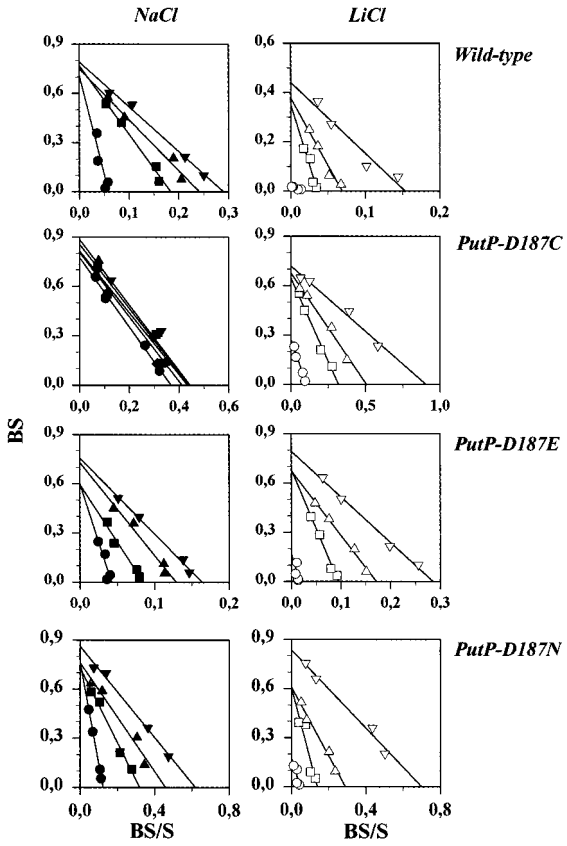

FIGURE 6: Scatchard plot analysis of ion-dependent L-proline binding to right-side-out membrane vesicles of *E. coli* WG170 containing PutP with given amino acid replacements. Proline binding was analyzed using a centrifugation assay as described under Experimental Procedures. L-[^{14}C]proline concentrations were varied from 0.4 to 10 μM at the following salt concentrations: no addition of NaCl (◆); 0.5 mM NaCl (●); 5 mM NaCl (■); 10 mM NaCl (▲); 50 mM NaCl (▼); 0.5 M LiCl (○); 5 mM LiCl (□); 10 mM LiCl (△); 75 mM LiCl (▽). BS corresponds to the amount of L-proline bound to membrane vesicles normalized to the protein concentration (nmol of L-proline/mg of total membrane protein), and S represents the total L-proline concentration (μM) in the assay. The data were corrected for the amount of proline bound to membrane vesicles without PutP.

the Li^+ dependency of proline binding was clearly reduced compared to the other PutP molecules.

Ligand Protection of Cys187. To test the influence of ligand binding on the accessibility of Cys187 to the sulfhydryl reagent NEM, inverted membrane vesicles of *E. coli* WG170 overproducing single-Cys PutP-D187C were reacted with [^{14}C]-NEM as described under Experimental Procedures. In the absence of ligand, 0.8 nmol of [^{14}C]-NEM was bound per nanomole of PutP (Figure 7). Remarkably, the labeling ratio was decreased dramatically in the presence of 2 mM L-proline and 50 mM NaCl, indicating that the physiological substrates protected Cys187 from reaction with [^{14}C]-NEM. Based on these findings, the ligands Na^+, Li^+, and proline were tested individually. Interestingly, it was shown that Na^+ alone caused significant protection of Cys187 while Li^+ and proline did not yield an efficient inhibition of the reaction with NEM.

DISCUSSION

In this study, we have investigated the role of a conserved acidic amino acid residue, Asp187, in PutP of *E. coli*.

Table 2: Ion Dependence of the Proline Affinity of PutP with Given Replacements of Asp187[a]

ion concentration (mM)	PutP-wild-type		PutP-D187C		PutP-D187E		PutP-D187N	
	Na^+	Li^+	Na^+	Li^+	Na^+	Li^+	Na^+	Li^+
no addition[b]	−[c]		2.0 ± 0.3		−		−	
0.5	12.5 ± 0.4[d]	nd[e]	2.1 ± 0.1	3.0 ± 0.1	14.1 ± 0.4	9.6 ± 0.1	6.8 ± 0.4	7.9 ± 0.1
5	4.5 ± 0.3	11.0 ± 0.8	2.1 ± 0.1	2.0 ± 0.1	5.4 ± 0.4	7.3 ± 0.6	2.3 ± 0.1	4.5 ± 0.4
10	3.4 ± 0.5	5.5 ± 0.1	2.1 ± 0.2	1.5 ± 0.1	5.1 ± 0.3	3.9 ± 0.2	1.8 ± 0.1	2.3 ± 0.2
50/75[f]	2.8 ± 0.1	2.6 ± 0.2	2.1 ± 0.1	1.0 ± 0.1	4.4 ± 0.2	2.7 ± 0.1	1.4 ± 0.1	1.3 ± 0.1

[a] Proline binding to right-side-out membrane vesicles of *E. coli* WG170 containing PutP with given amino acid replacements was analyzed using a centrifugation assay as described under Experimental Procedures. The proline concentration was varied from 0.4 to 10 μM at the ion concentrations indicated. The k_d values were taken from Scatchard plot analysis (Figure 6). [b] No addition of NaCl or LiCl. The Na^+ concentration in the buffer was 2 μM. [c] Proline binding was not detectable. [d] k_d values for proline binding are given in micromolar. [e] k_d value could not be determined. [f] The concentrations of NaCl and LiCl were 50 mM and 75 mM, respectively.

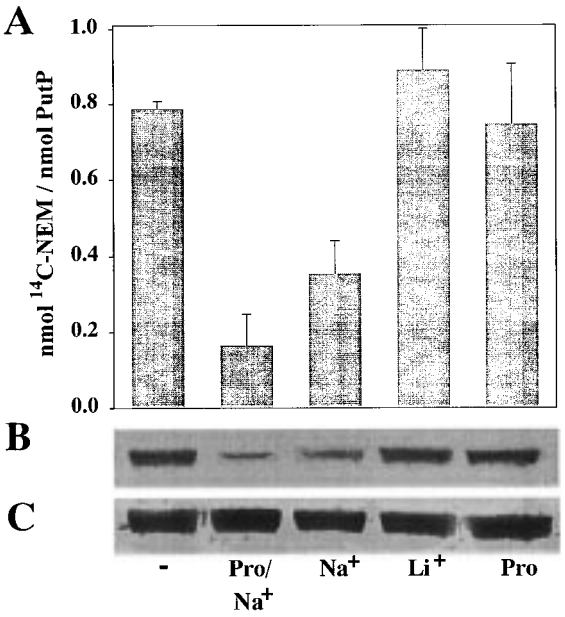

FIGURE 7: Ligand protection of Cys187 in PutP. Inverted membrane vesicles of *E. coli* WG170 cells containing single-Cys PutP-D187C were labeled with 500 μM [^{14}C]-NEM (8 μCi/μmol) at 25 °C in the absence (−) or presence of 2 mM proline/50 mM NaCl (Pro/Na^+), 50 mM NaCl (Na^+), 50 mM LiCl (Li^+), or 2 mM L-proline (Pro). The reaction was stopped by addition of 2 mM β-mercaptoethanol after 5 min. Subsequently, PutP was solubilized from the membrane and purified as described under Experimental Procedures. The radioactivity of an aliquot of the purified protein was quantified by scintillation counting. The obtained values were corrected for unspecific labeling using Cys-free PutP as a negative control. The labeling ratio is given as nanomoles of [^{14}C]-NEM per nanomole of PutP (A). Another aliquot of the purified transporter was subjected to SDS−PAGE, and the [^{14}C]-NEM-labeled protein was detected with a Phosphor Imager (B). In addition, silver staining of the gel was performed (C).

Alteration of the polarity of the amino acid side chain at position 187 by amino acid substitution or chemical modification of single-Cys PutP-D187C indicates that a highly polar or charged amino acid (i.e., Asp, Asn, Glu) at this position is crucial for active proline transport. Thus, replacement of Asp187 with Cys leads to only marginal transport activity (1.5% of the wild-type V_{max} of proline uptake) while the same alteration in a functional transporter devoid of all five native Cys residues abolishes proline accumulation completely. Importantly, the activity of the latter transporter molecule is regained by acetylation or amidoacetylation of Cys187.

In the presence of saturating Na^+ concentrations, substitution of Asp187 results in only slight alteration of the proline affinity of PutP, suggesting that the residue is not directly involved in proline binding. Strikingly, however, replacement of Asp187 with Cys causes high-affinity proline binding even at very low Na^+ concentrations (2 μM) while proline binding to PutP-wild-type, -D187E, and -D187N is strictly dependent on the Na^+ concentration. Thus, replacement of Asp187 with Cys seems to stabilize the protein in a high affinity conformation for proline. Alternatively, the transporter could have acquired a very high affinity for Na^+, leading to a high affinity for proline even at low Na^+ concentrations.

The characteristics of Na^+- and Li^+-dependent proline binding and transport indicate that the properties of PutP-D187C can at least partially be attributed to an enhanced Na^+ affinity of the transporter. Thus, the apparent K_m of PutP-D187C for Na^+ is about 8-fold reduced compared to PutP-wild-type. The same substitution causes only little alteration of the apparent K_m for Li^+ (about 2-fold reduction). Furthermore, the Li^+ dependence of proline binding to PutP-D187C was clearly reduced compared to PutP containing Asp (wild-type), Asn, or Glu at position 187 (Table 2). Nonetheless, shifting the Li^+ concentration from 0.5 to 75 mM leads to a slight increase of the proline affinity of PutP-D187C. The latter effect was not observed with Na^+. The different results obtained with Na^+ or Li^+ as coupling ion may be explained by differences in cation coordination. Analyses of crown ether complexes and ionophores indicate that the number of ligands necessary for cation binding depends on the size and solvation energy of the ion (*33*−*36*). In agreement with this observation, replacement of Asp51 in the melibiose permease of *E. coli* (MelB) changes the ion selectivity of the transporter in a way that indicates a participation of Asp51 in Na^+ but not in H^+ (H_3O^+) binding (*37*, *38*). Thus, substitution of Asp187 in PutP may alter the properties of a coordination site(s) contributing to Na^+ binding which is not important for Li^+ complexation.

In addition, the inhibition of the reaction of single-Cys PutP-D187C with NEM by Na^+ and not by proline supports the idea that this position is located at or near a cation binding site. Li^+ appears to be too small to protect Cys187 efficiently. Asp187 is located in a short cytoplasmic loop, and the topology has been confirmed by *putP-phoA* and *putP-lacZ* fusion analysis (Figure 1) (*23*). Thus, it is unlikely that the acidic residue participates in Na^+ binding from the outside. Therefore, it is suggested that Asp187 is located at or close to the site of Na^+ release on the cytoplasmic phase of the membrane. An altered release of Na^+ could also account for the impact of the substitution of Asp187 on the

rate of Na^+-coupled proline uptake. In this context, it is interesting to note that an acidic residue, Glu126, in the putative cytoplasmic loop between helices IV and V has recently been suggested to be involved in the release of H^+ and substrate in the lactose permease of *E. coli* (LacY) (*39*). Clearly, Asp187 differs from Glu126 in LacY in that the carboxylate in PutP is not essential for activity. Nonetheless, electrostatic interactions of Asp187 with the ion and/or other parts of the protein might play a crucial role in Na^+ release into the cytoplasm. It cannot be excluded that protection of Cys187 is achieved by a long-range conformational alteration induced by ion binding elsewhere. However, in the latter case, one would rather expect that binding of Na^+ or Li^+ induces the same change in PutP conformation.

Electrostatic interactions between amino acid residues have also been shown to be important for the function of the Na^+/glucose transporter (SGLT1) (*40*). Thus, replacement of Asp176 by Ala altered the kinetics of charge transfer while Asn at this position had no effect on charge movement. It is suggested that removal of the polar side chain causes a change in the rate constants for a partial step in the reaction cycle, e.g., the conformational changes of the unloaded transporter (*41*). Furthermore, a reduced Na^+ dependence of proline binding was observed upon replacement of Arg257 in PutP with Cys (*42*). The authors speculate that removal of the positive charge leads to an enhanced affinity of PutP for Na^+. However, the effect of the substitution of Arg257 on proline uptake was much less dramatic as in the case of Asp187.

Taken together, the results indicate that electrostatic interactions of the amino acid side chain at position 187 in PutP with other parts of the transporter and/or the coupling ion are crucial for active proline transport. The findings suggest that Asp187 is located close to the pathway of the coupling ion through the membrane and may be important for the release of Na^+ on the cytoplasmic side of the membrane. Clearly, a more precise assignation of the role of Asp187 requires more information on the tertiary structure and dynamics of PutP.

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