Role of Conserved Arg40 and Arg117 in the Na^+/Proline Transporter of *Escherichia coli*†

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ABSTRACT: The Na^+/proline transporter of *Escherichia coli* (PutP) is a member of a large family of Na^+/solute symporters. To investigate the role of Arg residues which are conserved within this family, Arg40 at the cytoplasmic end of transmembrane domain (TM) II and Arg117 in cytoplasmic loop 4 of PutP are subjected to amino acid substitution analysis. Removal of the positive charge at position 40 (PutP−R40C, Q, E) leads to a dramatic decrease of the V_{max} of Na^+-coupled proline uptake (1−10% of PutP-wild-type). The reduced transport rates are accompanied by decreased apparent affinities of the transporter for Na^+ and Li^+ while the apparent affinity for proline is only slightly altered. Furthermore, single Cys PutP−R40C reacts with *N*-ethylmaleimide (NEM), and this reaction is partially inhibited by proline and more efficiently by Na^+ ions. Remarkably, NEM modification of Cys40 inhibits Na^+-driven proline uptake almost completely while facilitated influx of proline into deenergized cells is stimulated by this reaction, suggesting an at least partially uncoupled phenotype under these conditions. These results suggest that Arg40 is located close to the site of ion binding and is important for the coupling of ion and proline transport. The observations confirm the functional importance of TM II described in earlier studies [M. Quick and H. Jung (1997) *Biochemistry 36,* 4631−4636]. In contrast to Arg40, Arg117 is apparently not important for function of the mature protein. The low transport rates observed upon substitution of Arg117 (PutP−R117C, K, Q) can at least partially be attributed to reduced amounts of PutP in the membrane. However, once inserted into the membrane, PutP containing Arg117 replacements shows a stability comparable to the wild-type as indicated by pulse-chase experiments. These observations suggest that Arg117 plays a crucial role at a stage prior to complete functional insertion of PutP into the membrane, i.e., by stabilizing a folding intermediate.

The Na^+/proline transporter (PutP) of *Escherichia coli* is an integral protein of the cytoplasmic membrane that utilizes free energy released from downhill transport of Na^+ to drive accumulation of proline (*1, 2*; see refs *3* and *4* for review). PutP belongs to the Na^+/solute symporter family (SSF)[1], which encompasses more than 40 homologous proteins from archaea, bacteria, yeast, insects, and mammals (*5, 6*). The *putP* gene encoding the Na^+/proline transporter has been cloned and sequenced (*7, 8*), and the gene product has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for Na^+/proline symport (*9*−*11*). On the basis of gene fusion analyses, Cys accessibility studies, and site-specific proteolysis, a secondary structure model has recently been proposed according to which PutP contains 13 transmembrane domains (TM) with the N-terminus located on the periplasmic side of the membrane and the C-terminus facing the cytoplasm (*12*) (Figure 1).

On the basis of amino acid substitution analyses, Asp55 in putative TM II has been implicated in Na^+ binding (*13*). Neutral substitutions at this position impair all types of transport, whereas Glu in place of Asp55 causes a dramatic decrease of the apparent Na^+ affinity of the transporter. Furthermore, Ser57 on the opposite phase of putative TM II proved to be crucial for high-affinity proline binding (*14*). Recent studies indicate that electrostatic interactions of a conserved Asp residue, Asp187 in putative cytoplasmic loop (cL) 6, with other parts of the transporter and/or the coupling ion are crucial for active proline uptake. The findings suggest that Asp187 is located close to the pathway of the coupling ion through the membrane and may, for example, be important for the release of Na^+ on the cytoplasmic side of the membrane (*15*).

PutP contains three Arg residues, Arg40 in cL2, Arg117 in cL4, and Arg257 in cL8, that are conserved within the SSF. It has already been shown that replacement of Arg257 with Cys leads to a reduced Na^+ dependence of proline binding and to resistance against *N*-ethylmaleimide (NEM) inactivation (*16*). In this paper, we investigate the role of Arg40 and Arg117 in the symport process.

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cL, cytoplasmic loop; IPTG, isopropyl 1-thio-β,D-galactopyranoside; Mes, 2-[*N*-morpholino]ethanesulfonic acid; Mops, 3-[*N*-morpholino]-propanesulfonic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SSF, Na^+/solute symporter family; TM, transmembrane domain.

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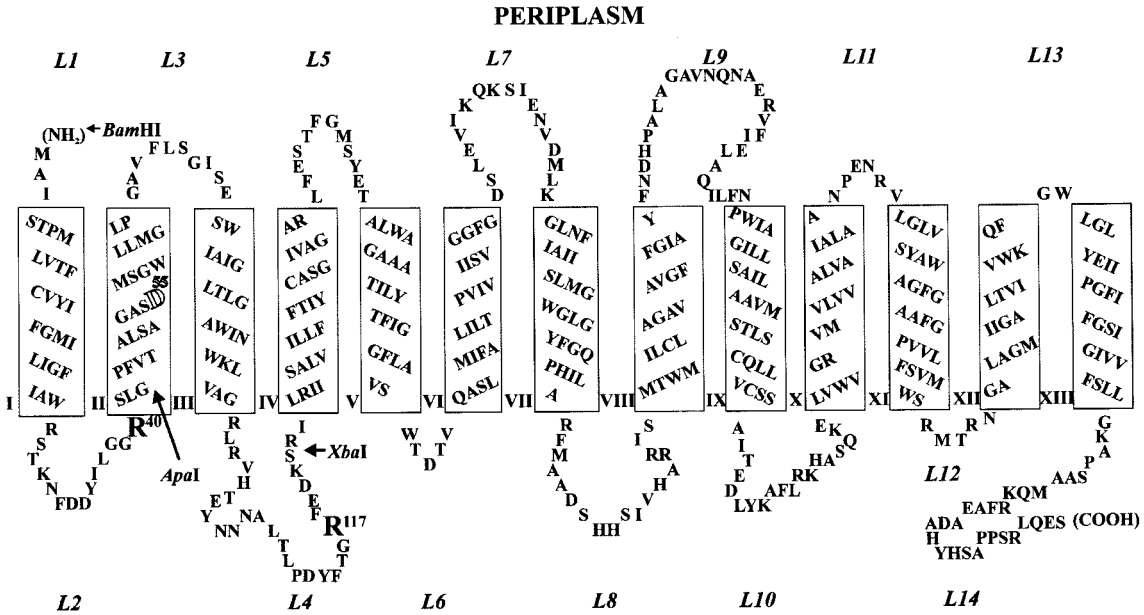

FIGURE 1: Secondary structure model of the Na^+/proline transporter of *E. coli* (according to ref *12*). Putative transmembrane domains are represented as rectangles and numbered with Roman numerals; loops are numbered with Arabic numerals starting from the N-terminus. Asp55 which is essential for function is shown as open letter. Arg40 and Arg117 which are subjects of this study are highlighted (bold letters). Positions of restriction endonuclease sites in the corresponding DNA sequence used for cloning experiments are also indicated.

EXPERIMENTAL PROCEDURES

Materials. L-[α-^{35}S]Methionine (1000 Ci/mmol) and L-[U-^{14}C]proline (257 Ci/mol) were purchased from Hartmann Analytic and ICN radiochemicals, respectively. Restriction endonucleases, alkaline phosphatase, *Taq* DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs and Gibco/BRL. Oligonucleotide primers were synthesized by Eurogentec or Gibco/BRL. Nitrocellulose membranes (0.45 μm pore size) were purchased from Schleicher & Schüll, and GF-5 filters (0.7 μm pore size) were from Machery Nagel. Mouse anti-Flag M2 IgG was from Integra Biosciences, and horseradish peroxidase-conjugated sheep anti-(mouse-IgG) antibody was obtained from Amersham Corp. The Renaissance-enhanced chemiluminescence kit was from DuPont-New England Nuclear. 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′ traD36 proAB*$^+$ *lacI*^{q} *Z*Δ*M15)*] (*17*) was used as host strain for cloning experiments and for plasmid isolation. *E. coli* WG170 [F′ *trp lacZ rpsL thi* Δ*(putPA)101 proP219*] (*18*) harboring given plasmids was used for all analyses of native or modified *putP* gene products. The following plasmids, derivatives of pT7-5 (*19*), containing the *lac* promoter/operator for expression of the *putP* gene were used for all gene manipulations: pTMH·FH and pTCl·FH, each of which harboring 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 (*12*). Seventeen codons encoding the Flag epitope and 6His-tag were attached to the 3′ end of the *putP* genes (*11*).

Site-Directed Mutagenesis and DNA Sequencing. Substitutions for Arg40 and Arg117 were created using the polymerase chain reaction (PCR) with plasmids pTMH·FH and pTCl·FH as templates, a sense primer binding upstream the 5′ end of the *putP* gene, and mutagenic antisense primers [R40C,[2] 5′-CGT CAC GAA GGG CCC AAG ACT **ACA** ACC GCC CAG-3′; R40E, 5′-CGT CAC GAA GGG CCC AAG ACT **TTC** ACC GCC CAG-3′; R40K, 5′-CGT CAC GAA GGG CCC AAG ACT **TTT** ACC GCC CAG-3′; R40Q, 5′-CGT CAC GAA GGG CCC AAG ACT **TTG** ACC GCC CAG-3′; R117C, 5′-GCG CAA AAT TCT AGA TTT ATC TTC AAA **GCA** CCC GGT GAA-3′; R117K, 5′-GCG CAA AAT TCT AGA TTT ATC TTC AAA **CTT** CCC GGT GAA-3′; R117Q, 5′-GCG CAA AAT TCT AGA TTT ATC TTC AAA **CTG** CCC GGT GAA-3′; altered codons are in bold face, the *Apa*I (Arg40 substitution) and *Xba*I (Arg117 substitution) recognition sites are underlined]. PCR fragments were digested with *Bam*HI and *Apa*I (Arg40 substitution) or *Apa*I and *Xba*I (Arg117 substitution). The resulting DNA fragments were ligated to similarly treated plasmid pTMH·FH incubated with alkaline phosphatase to avoid religation of the vector. To generate PutP with a single Cys at position 40 or 117, the corresponding PCR fragments were cloned into pTCl·FH similar as described for pTMH·FH. The resulting constructs were verified by sequencing double-stranded plasmid DNA using dideoxynucleotide chain-termination (*20*) after alkaline denaturation (*21*).

Proline Transport Assays. L-[^{14}C]Proline transport by intact cells of *E. coli* WG170 (PutP$^-$A$^-$) transformed with given plasmids was assayed as described previously (*13*). Kinetics of ion-coupled proline transport were calculated from the initial linear portion of the time courses between 0 and 10 s (0 and 3 min in the case of PutP−R40C, R40E, and R40Q) with standard deviations derived from at least three inde-

[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 second letter denotes the amino acid substitution at this position.

pendent experiments. The Na^+ concentration of the buffer used was determined to be 2−6 μM by atomic absorption spectroscopy (Na^+-free buffer). To analyze the effect of NEM on the activity of single Cys PutP−R40C or single Cys PutP−R117C, washed cells of *E. coli* WG170 (0.35 mg of protein/mL) producing the PutP variants were treated with 500 μM NEM in the presence or absence of ligand as indicated at 30 °C. The reaction was stopped after 5 min by the addition of 5 mM β-mercaptoethanol, and the cells were washed two times with 250 mM Tris/Mes, pH 6.0 and resuspended in the same buffer to yield a final protein concentration of 0.35 mg/mL. Transport was assayed as described above.

Immunological Analysis. The relative amount of PutP-wild-type or PutP with given amino acid replacements in membranes of *E. coli* WG170 was estimated by Western blot analysis. Immunoblotting was performed with mouse anti-Flag IgG against the Flag epitope at the C-terminus of each PutP variant followed by incubation with horseradish peroxidase-linked sheep anti-(mouse-IgG) antibody by the enhanced chemiluminescence method as described (*14*).

[^{35}S]Methionine Labeling. [^{35}S]Methionine labeling of PutP was performed using the T7 polymerase system similar as described (*19*, *22*). Overnight cultures of *E. coli* WG170 cotransformed with plasmid pGP1-2 encoding T7 polymerase and given pT7-5/*putP* derivatives were diluted 1:7.5 (final volume 1 L) in LB medium (*23*) containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. After growth at 30 °C for 4 h, cells were harvested and washed three times with prewarmed M9 medium (*24*) supplemented with 10 mM $MgSO_4$ and trace elements (in micromolar, 1.77 $FeCl_3$, 1.42 $MnCl_2$, 2.44 $CaCl_2$, 14.67 $ZnCl_2$, 4.69 H_3BO_3, and 8.36 $CoSO_4$), and resuspended in 5 mL of M9 medium containing 10 mM $MgSO_4$, trace elements, 0.5% glycerol, 0.001% thiamine, and all amino acids (0.05% each) except methionine and cysteine. For heat-induced expression of the *putP* gene via the T7 promoter, cultures were incubated at 42 °C for 60 min. Rifampicin (400 μg/mL) and IPTG (0.5 mM) were added, and after additional 15 min at 42 °C, cells were cultivated aerobically at 30 °C. Labeling was initiated by addition of 50 pmol of [^{35}S]methionine (50 μCi) and samples were taken at given time points and instantly supplemented with 500 μM phenylmethanesulfonyl fluoride (PMSF), shock-frozen, and stored in liquid nitrogen until use. For quantitative detection of ^{35}S-labeled PutP, cells were thawed on ice, washed, and resuspended in 100 mM KP_i, pH 8.0. An aliquot of the cell suspension (70 μg of total cell protein) was subjected to SDS−PAGE (10%) (*25*). After drying the gel, radioactivity was detected with a phosphor imager (Molecular Dynamics, model SI). For pulse-chase experiments, cells were treated and labeling was initiated as described above. After 10 min of incubation the first sample (zero time point) was removed followed by adding an excess of unlabeled methionine (2.5 mg/mL final concentration) and further samples were taken at given time points. The samples were supplemented with PMSF and frozen and stored in liquid nitrogen. After thawing on ice and washing, cells were disrupted by sonification. Cell debris were removed by low-speed centrifugation, membranes were washed and collected by ultracentrifugation at 264000*g* at 4 °C, and 20 μg of total membrane protein was subjected to SDS−PAGE (10%) (*25*). Radioactivity was detected as described above.

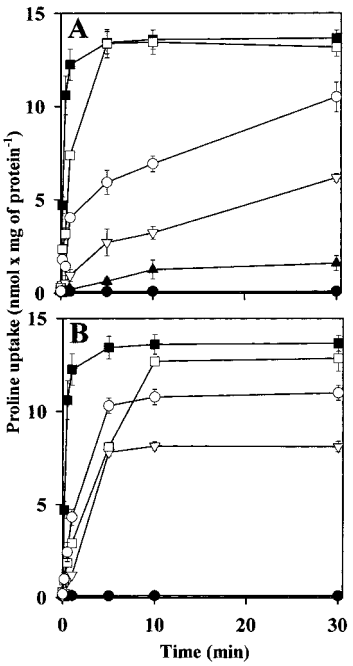

FIGURE 2: Time course of proline uptake by *E. coli* WG170 harboring PutP-wild-type or PutP with given replacements. IPTG-induced cells were harvested, washed, and resuspended in 250 mM Tris/Mes, pH 6.0, to a final protein concentration of 0.35 mg/mL. Transport of L-[U-^{14}C]proline (5 μM final concentration) 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. (A) Arg40 replacements: PutP-wild-type (■); PutP−R40C (▽); PutP−R40E (▲); PutP−R40K (□); PutP−R40Q (○); pT7-5 (●). (B) Arg117 replacements: PutP-wild-type (■); PutP−R117C (▽); PutP−R117K (□), PutP−R117Q (○); pT7-5 (●).

Protein Determination. Protein determination was performed using a modification of the method of Lowry (*26*) with bovine serum albumin as standard.

RESULTS

Generation and Verification of Mutations. The conserved Arg residues at positions 40 and 117 in PutP were individually replaced with neutral (Cys, Gln) or charged (Lys, in case of Arg40 also with Glu) amino acid residues by PCR-mediated site-directed mutagenesis. Mutagenic DNA fragments were cloned into plasmid pTMH·FH containing a cassette version of the *putP* gene (*12*) using the restriction endonuclease sites *Bam*HI and *Apa*I (Arg40 substitution) or *Apa*I and *Xba*I (Arg117 substitution) (Figure 1). In addition, the DNA fragments causing substitution of Arg40 or Arg117 by Cys were introduced into the cassette *putP* gene encoding a functional PutP version in which all five native Cys residues had been replaced with Ser or Ala (*12*) 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 desired base changes, the remainder sequences were unaffected by the genetic manipulations.

Active Proline Uptake. To analyze the effect of the Arg replacements on PutP function, active transport of proline was assayed using *E. coli* WG170 harboring given plasmids. Replacement of Arg40 with a neutral or negatively charged residue dramatically affected the time course of proline uptake (Figure 2A). Thus, PutP−R40C, −R40E, and −R40Q

Table 1: Kinetic Analysis of Ion-Coupled Proline Uptake by PutP Bearing Given Amino Acid Replacements[a]

	K_m (μM)	V_{max} (nmol/min mg of protein)	$K_{m[Na^+]}$ (μM)	$K_{m[Li^+]}$ (μM)
wild-type	2.1 ± 0.2	27 ± 1	32 ± 4	125 ± 10
PutP−R40C	6.3 ± 0.1	1.9 ± 0.5	312 ± 8	1100 ± 40
PutP−R40E	5.5 ± 0.6	0.25 ± 0.1	180 ± 15	2200 ± 125
PutP−R40K	1.6 ± 0.1	30 ± 1	220 ± 2	2350 ± 25
PutP−R40Q	0.5 ± 0.1	3 ± 0.3	100 ± 5	1800 ± 42
PutP−R117C	2 ± 0.2	5 ± 0.5	50 ± 4	-*b*
PutP−R117K	1.6 ± 0.1	25 ± 2	37 ± 1	-
PutP−R117Q	1.2 ± 0.2	8 ± 1	25 ± 2	-

[a] Initial rates of proline uptake by *E. coli* WG170 harboring either PutP-wild-type or PutP with given replacements were measured at proline concentrations from 0.2 to 50 μM in the presence of 50 mM NaCl. Apparent affinity constants for Na^+ and Li^+ ($K_{m\ [Na^+]}$; $K_{m[Li^+]}$, respectively) were determined at NaCl or LiCl concentrations varying from 0.01 to 100 mM and a proline concentration of 5 μM. The data were plotted according to Eadie−Hofstee and standard deviations were determined from at least three independent experiments. [b] Not determined.

catalyzed transport with 2.8, 0.6, and 20%, respectively, of the initial rate and 46, 12, and 80%, respectively, of the steady-state level of PutP-wild-type. The conservative substitution of Arg40 by Lys had the least impact on transport. PutP−R40K showed 40% of the initial rate and 100% of the steady-state level of proline accumulation of the wild-type. Substitution of Arg117 also affected proline uptake significantly (Figure 2B). Placement of Cys, Gln, and Lys at position 117 caused a decrease of the initial rate of proline uptake to 5, 20, and 17%, respectively, of the wild-type value. The steady-state values of proline accumulation by PutP−R117C, −R117Q, and −R117K were reduced to 64, 80, and 95%, respectively, of the PutP-wild-type value.

Kinetics of Active Transport. Determination of kinetic parameters revealed that individual replacement of Arg40 and Arg117 with a neutral amino acid or introduction of the opposite charge at position 40 caused a dramatic decrease of the V_{max} of proline transport, while Lys in place of Arg40 and Arg117 did not significantly affect the V_{max} value (Table 1). Furthermore, all substitutions tested in this study altered the K_m of the transporter for proline only slightly. However, replacement of Arg40 with Cys, Gln, Glu, and Lys resulted in about 3−20-fold enhanced apparent K_m values for Na^+ and Li^+ (Table 1). In contrast, replacement of Arg117 with Cys, Gln, and Lys had only little effect on the apparent affinity of the transporter for Na^+.

Immunological Analysis. The effect of the amino acid substitutions on the relative concentration of PutP in membranes of *E. coli* WG170 was estimated by Western blot analysis using monoclonal antibodies directed against the Flag epitope at the C-terminus of the transporter. Replacement of Arg40 with Cys, Gln, Glu, and Lys did not significantly alter the amount of PutP in the membrane (Figure 3). Therefore, the differences described between PutP-wild-type and PutP with Arg40 replacements cannot be attributed to a defective insertion of the transporter into the membrane or reduced stability of altered proteins after insertion. In contrast, Cys or Gln in place of Arg117 caused a dramatic decrease of the amount of PutP in the membrane (Figure 3). However, transporter molecules containing Lys in place of Arg117 were present in amounts comparable to

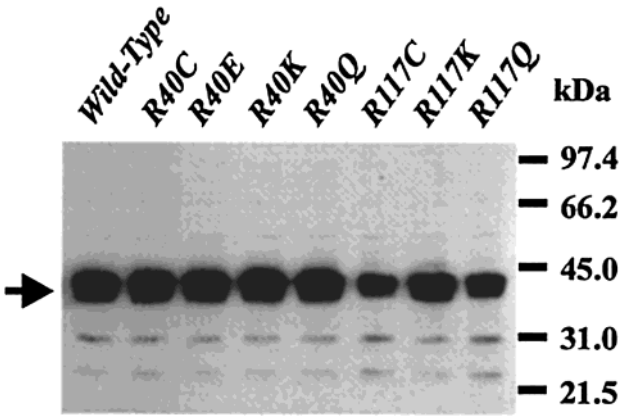

FIGURE 3: Western blot of membranes from *E. coli* WG170 cells containing PutP with given Arg replacements. Twenty micrograms of total membrane protein from IPTG-induced cultures was subjected to 10% SDS/PAGE and electroblotted onto nitrocellulose membranes. The blot was incubated with mouse monoclonal antibodies against the Flag epitope at the C-terminus of each protein, followed by incubation with sheep anti-(mouse IgG) horseradish peroxidase conjugate. Detection of PutP was assayed by the enhanced chemiluminescence method. Location of PutP is indicated by an arrow. Positions of the protein standards are shown (kDa).

PutP-wild-type. These results suggest that a positive charge at position 117 is required for optimum insertion and/or stability of PutP in the membrane.

Effect of Arg117 Replacements on PutP Stability. To analyze the effect of Arg117 replacements on the stability of PutP in membranes of *E. coli* WG170, [^{35}S]methionine labeling and pulse-chase experiments were performed. SDS−PAGE analysis of labeled proteins revealed that the amount of PutP−R117C, −R117Q, and, to a lesser extent, also −R117K in the membrane was reduced compared to PutP-wild-type (Figure 4). This observation confirms the results obtained by Western blot analysis. However, once inserted into the membrane, all PutP variants showed a similar stability over a 14 h chase period as the wild-type (Figure 4). These findings indicate that the reduced level of transporter containing Arg117 replacements in the membrane cannot be attributed to a decreased protein stability after insertion. In an attempt to find out whether the mutations affect translation or protein folding and membrane insertion, short [^{35}S]methionine pulse periods (0.5, 1, 2, 5, and 10 min) were carried out and whole cells were analyzed. ^{35}S-Labeled PutP and its derivatives were detectable after a 1 min pulse period. However, alteration of the codon encoding Arg117 resulted in amounts of the transporter which were decreased to 25−40% (PutP−R117C), 40−60% (PutP−R117K), and 40−50% (PutP−R117Q) of the corresponding wild-type value at this and later time points.

Accessibility of Cys at Position 40 or 117 to NEM. The R40C and R117C substitutions were individually placed into Cys-free PutP. The resulting single Cys PutP−R40C catalyzed active proline uptake which was inhibited to about 90% by reaction of the transporter with NEM for 5 min (Figure 5). Importantly, reaction of the Cys residue at position 40 with NEM was affected by ligand binding. Thus, incubation of single Cys PutP−R40C with NEM in the presence of 2 mM proline resulted in only about 50% inactivation of transport while 50 mM NaCl prevented NEM inhibition almost completely. The protective effect of NaCl and proline

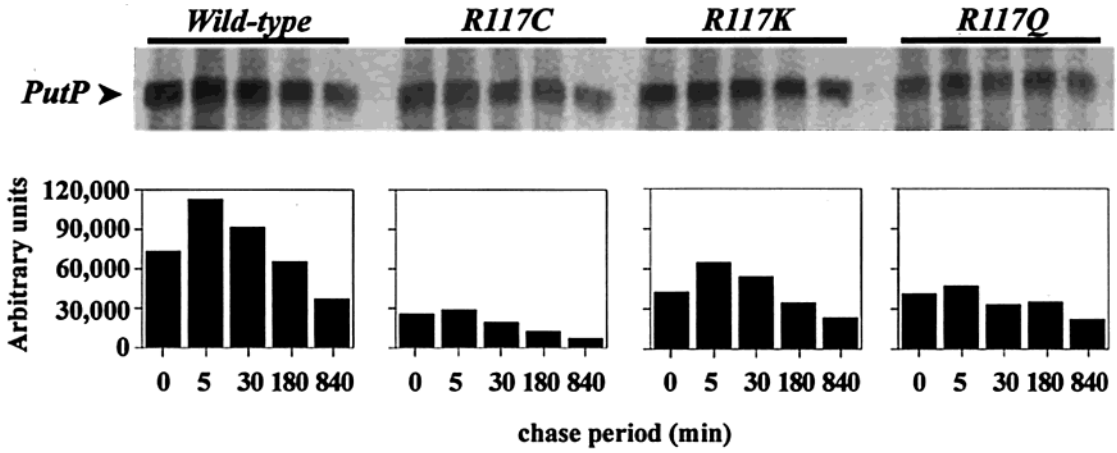

FIGURE 4: Influence of Arg117 replacements on PutP stability in membranes of *E. coli* WG170. As described in Experimental Procedures, cells were incubated with [^{35}S]methionine at 30 °C for 10 min before removal of the zero-time point aliquot and addition of an excess of unlabeled methionine (2.5 mg/mL final concentration). Further aliquots were taken at given time points of incubation. Membranes were prepared by sonication and 20 μg of total membrane protein were subjected to SDS−PAGE (10%). Radioactivity was quantitatively detected with a Phosphor imager after drying the gel. The arrow indicates the position of PutP.

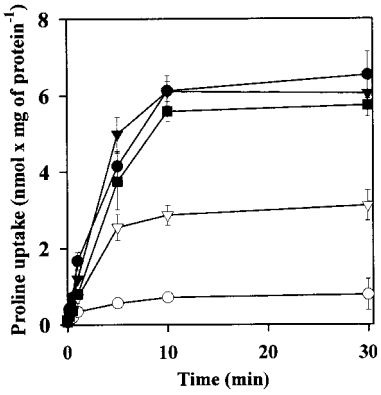

FIGURE 5: NEM inhibition of single Cys PutP−R40C. Intact cells of *E. coli* WG170 harboring single Cys PutP−R40C suspended in 250 mM Tris/Mes, pH 6.0 (total cellular protein 0.35 mg/mL), were treated with 500 μM NEM in the presence or absence of ligand as indicated. After removal of ligand and unbound NEM active proline transport was measured as described in Figure 2. Single Cys PutP−R40C unmodified (●); treated with 500 μM NEM (○); treated with 500 μM NEM in the presence of 2 mM L-proline (▽); or in the presence of 50 mM NaCl (▼); or in the presence of 2 mM L-proline and 50 mM NaCl (■).

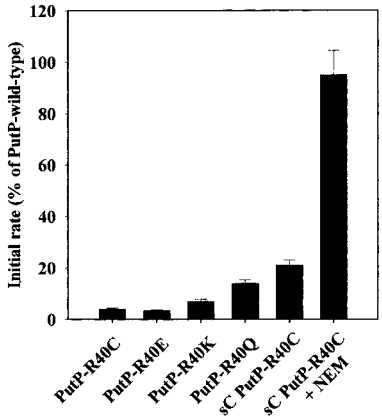

FIGURE 6: Effect of Arg40 substitutions on facilitated influx of proline. Cells of *E. coli* WG170 were reacted with 500 μM NEM as described in Figure 5. After removal of unbound NEM, cells were resuspended in 250 mM Tris/Mes, pH 6.0 (total cellular protein 0.35 mg/mL), and completely deenergized by incubation with 1 μM monensin and 5 μM CCCP. Facilitated influx of proline was measured by addition of L-[U-^{14}C] proline (50 μM final concentration). Transport was stopped and samples were rapidly filtered as described in Figure 2. Initial rates of facilitated influx are given in percent of the corresponding PutP-wild-type value [1.8 nmol min^{-1} (mg protein)$^{-1}$]. sC PutP−R40C corresponds to single Cys PutP−R40C (Cys-free background). All other substitutions were tested in the wild-type background.

is comparable to that caused by NaCl alone (Figure 5). Furthermore, NEM caused about 50% inhibition of proline transport by single Cys PutP−R117C. In contrast to single Cys PutP−R40C, the accessibility of the Cys residue at position 117 to NEM was not modified by ligand (data not shown).

Facilitated Diffusion. To study the effect of Arg substitutions on facilitated influx of proline, cells of *E. coli* WG170 harboring desired PutP molecules were completely deenergized by incubation in the presence of CCCP and monensin at 30 °C. Similarly, as observed for active transport, replacement of Arg40 in PutP-wild-type lead to highly reduced initial rates of facilitated proline diffusion (3−20% of PutP-wild-type) (Figure 6). However in contrast to active transport, reaction of single Cys PutP−R40C with NEM resulted in a ~5-fold stimulation of the facilitated transport rate, suggesting an at least partially uncoupled phenotype under these conditions (Figure 6). In a control experiment, incubation of Cys-free PutP with NEM did not affect the influx rate. Furthermore, substitution of Arg117 had only little or no effect on the facilitated influx of proline into deenergized cells (data not shown).

DISCUSSION

Arg40 and Arg117 of PutP are conserved within the members of the SSF. This study shows that removal of the positive charge at position 40 (PutP−R40C, Q, E) leads to a dramatic decrease of the V_{max} of Na^+-coupled proline uptake (1−10% of PutP-wild-type) while substitution of Arg117 (PutP−R117C, Q) has a lesser but still significant impact on the transport rate (20−30% of PutP-wild-type). The reduced transport rates observed upon substitution of Arg40 are accompanied by decreased apparent affinities of the transporter for Na^+ and Li^+ while the apparent affinity for proline is only slightly altered. These results suggest that Arg40 is located close to the site of ion binding in PutP. In

fact, Arg40 is positioned at the cytoplasmic end of TM II, a putative helix that contains amino acids which are important for function (*12*) (Figure 1). Asp55 in TM II is essential for transport and proposed to interact directly with the coupling ion (*13*). Substitution of Ser57 causes a decrease of the apparent affinity for Na^+ and proline by up to 2 orders of magnitude (*14*). Furthermore, individual replacement of Ser50 and Ala53 leads to reduced rates of active proline transport (*12*). In addition, the accessibility of a Cys in place of Ser57 in TM II or Ser71 in the periplasmic loop following TM II to sulfhydryl reagents is increased by Na^+ indicating a conformational alteration in this region of the protein induced by the coupling ion (M. Langkamp, T. Pirch, and H. Jung, unpublished information). In this context, it appears likely that the observed protection of the sulfhydryl group in single Cys PutP−R40C by Na^+ and/or proline is achieved by a structural change burying the reactive group in the protein rather than by direct steric hindering. This ligand-dependent structural alteration could involve a change in helix tilt or helix rotation similar as it has been proposed for ligand-induced helix movements in the lactose permease of *E. coli* (LacY) (*27*−*29*).

Substitution of Arg40 also leads to highly reduced initial rates of facilitated influx of proline into deenergized cells. However, in contrast to active transport, reaction of single Cys PutP−R40C with NEM does not inhibit facilitated diffusion, but even stimulates proline influx. We conclude from these observations that proline transport is at least partially uncoupled from Na^+ movement under these conditions. The findings support the idea that the amino acid side chain at position 40 is not only important for the structural flexibility of TM II but may be involved in coupling downhill transport of Na^+ with the accumulation of proline.

The Arg residue at position 117 does probably not directly participate in the transport process catalyzed by mature PutP but plays apparently a crucial role for the structure of the transporter. Thus, alteration of the amino acid side chain at the position of Arg117 affects neither the apparent affinity of the transporter for the coupling ion (Na^+) nor for proline, suggesting that Arg117 is not involved in ligand binding. Furthermore, reaction of Cys117 with NEM is not influenced by ligand binding. The reduced V_{max} values of Na^+-coupled proline uptake observed upon replacement of Arg117 can at least partially be attributed to the low amount of PutP in the membrane. Importantly, however, once inserted into the membrane, PutP containing Arg117 replacements shows a stability that is comparable to wild-type. These results leave the possibility that Arg117 plays a crucial role at a stage prior to complete functional insertion of PutP into the membrane. Thus, alteration of the codon encoding Arg117 could decrease the rate of transporter synthesis or affect protein folding and insertion into the membrane. The first idea is supported by the fact that reduced amounts of PutP containing Arg117 replacements are found in the cells already after very short [^{35}S]methionine pulse periods when the protein may not be completely inserted into the membrane yet. However, since important steps of the biosynthetic pathway of integral membrane proteins in *E. coli* are suggested to proceed simultaneously similar as described for the mammalian SRP-mediated pathway (*30*, *31*), the second possibility cannot be ruled out. Arg117 could stabilize a folding intermediate, i.e., via electrostatic interactions with other parts of the protein. In the lactose permease of *E. coli* (LacY), salt bridge formation between positively and negatively charged residues, i.e., between Asp237 and Lys358, is likely to be important for protein folding (*32*). While the salt bridge between Asp237 and Lys358 is maintained in the mature protein, removal of the positive charge at position 117 in PutP still yields functional protein. Therefore, it is unlikely that Arg117 is involved in charge pair formation in the folded protein. Alternatively, as a charged residue, Arg117 could influence a possible proton motive force-dependent organization of the transporter in the membrane (*33*, *34*).

The effects observed upon substitution of Arg40 and Arg117 clearly differ from the impact of the replacement of the Arg residues at positions 257 and 376 in PutP. Arg376 which had been proposed to be part of a Na^+ binding motif was found to be dispensable with respect to energy coupling (*35*, *36*). In agreement with our findings, the latter result supports the idea that Na^+ binding occurs in the N-terminal part of PutP similar as it has been suggested for the human Na^+/glucose transporter (SGLT1), another member of the SSF (*37*). Substitution of Arg257 in PutP yields high-affinity proline binding even at very low Na^+ concentration (*16*). The authors speculate that the reduced Na^+ dependence of proline binding might be due to an enhanced affinity of PutP for Na^+. Alternatively, the substitution could stabilize PutP in a high affinity for proline which is relatively independent from Na^+ binding.

Arg residues have been shown to be important for function also of other secondary transporters. Thus, in LacY Arg302 in TM IX is required for the coupling of H^+ and substrate translocation via formation and disruption of electrostatic interactions with acidic residues (*29*). Arg144 in TM V is involved in salt bridge formation which is important for substrate release (*38*). However, in contrast to PutP, both Arg residues are irreplaceable for active transport (*29*). Investigation of the metal-tetracycline/H^+ antiporter of *E. coli* [TetA(B)] demonstrates that substitution of three out of seven conserved Arg residues results in a drastic decrease in drug resistance and almost complete loss of transport activity (*39*). One of these residues, Arg101, which is close to the cytoplasmic end of TM IV, is suggested to participate in a substrate induced conformational change of the putative exit gate of TetA(B). In SGLT1, substitution of Arg427 or Arg499 leads to defects in trafficking of the transporter to the cytoplasmic membrane (*40*, *41*).

In conclusion, we suggest that Arg40 of PutP is located close to the ion binding site and is involved in a conformational alteration leading to the coupling between Na^+ and proline translocation without being in direct contact with one of these substrates. Arg117 on the other hand is apparently not important for the function of the mature protein but plays a crucial role at a stage prior to complete functional insertion of PutP into the membrane.

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