Unidirectional Reconstitution and Characterization of Purified Na^+/Proline Transporter of *Escherichia coli*†

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ABSTRACT: A simple approach for large-scale purification and unidirectional reconstitution of the Na^+/proline transporter of *Escherichia coli* (PutP) is described. The procedure is based on the insertion of a highly polar peptide composed of 17 amino acids including a 6His tag at the C-terminus of the transporter. Purification of the hybrid protein is achieved by Ni^+−NTA affinity (purity >95%) and ion exchange chromatography (purity >99%). The purified transporter is reconstituted into preformed, detergent-destabilized liposomes. Detergent is removed slowly by adsorption to polystyrene beads. The highest activities [$V_{max} = 1.1 \times 10^3$ nmol min^{-1} (mg of protein)$^{-1}$] are measured when Triton X100 is used for liposomes destabilization at a concentration corresponding to the onset of lipid solubilization. Site-directed labeling of PutP and site-specific proteolytic cleavage indicate that the transporter is inserted into proteoliposomes in an inside-out orientation. Reconstituted PutP is able to accumulate proline against a concentration gradient in the presence of an inwardly directed electrochemical Na^+ or Li^+ gradient, while a pH gradient does not affect transport. The apparent proline affinity of PutP in proteoliposomes is similar to the value determined with intact cells. Interestingly however, the apparent Na^+ affinity of reconstituted PutP is reduced by a factor of about 25 compared to cells, suggesting a lower cation affinity on the cytosolic side of PutP relative to the outside.

The Na^+/proline transporter of *Escherichia coli* (PutP) is an integral protein of the cytoplasmic membrane and catalyzes the coupled translocation of proline and Na^+ (Li^+) (*1*, *2*). The *putP* gene encoding the transporter has been cloned and sequenced (*3*, *4*). Based on hydropathy profile analysis of the primary amino acid sequence, a secondary structure model is proposed according to which the protein consists of a short N-terminal tail, 12 transmembrane domains in α-helical conformation that traverse the membrane in a zigzag fashion connected by hydrophilic loops, and a hydrophilic C-terminal tail (*3*). Immunological analysis reveals that the C-terminus of PutP is located on the cytoplasmic side of the membrane (*5*). Analysis of the effect of amino acid substitutions on protein function indicates that a carboxylate at position 55 (putative loop 2) is essential for Na^+-coupled proline transport. Alteration of the Na^+ affinity and other results suggest that the native Asp55 is involved in binding of the coupling ion (*6*). Furthermore, based on a kinetic analysis, it is shown that Ser57 (putative loop 2) is required for high-affinity proline uptake (*7*).

PutP has been purified as a β-galactosidase hybrid protein by anti-β-galactosidase IgG−Sepharose chromatography and reconstituted into proteoliposomes in a functional state after removal of the β-galactosidase moiety (*8*, *9*). However, a simpler procedure allowing large-scale purification of PutP is needed for protein chemical and spectroscopic studies as well as attempts at crystallization. In addition, a method for the unidirectional reconstitution of the protein is desirable for topology analysis and kinetic studies. Therefore, we report here the large-scale purification and oriented reconstitution of PutP. The method is based on a PutP molecule to which a highly polar peptide composed of 17 amino acids including a 6His tag has been attached at the C-terminus. After purification by affinity and ion exchange chromatography, the transporter is functionally reconstituted into proteoliposomes in an inside-out orientation using preformed, detergent-destabilized liposomes. Kinetics of proline transport are analyzed.

EXPERIMENTAL PROCEDURES

Materials. L-[^{14}C]Proline (261 μCi/μmol) and sheep anti-(mouse-IgG)−horseradish peroxidase and streptavidin−horseradish peroxidase conjugates were purchased from Amersham Buchler, Braunschweig, Germany. Mouse anti-FLAG-M2 antibody was from Integra Biosciences, Fernwald, Germany. Bio-Beads SM-2 were obtained from Biorad, Munich, Germany. Ni−NTA resin was from Qiagen GmbH,

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Hilden, Germany; DEAE-Sepharose CL-6B and HiTrap desalting columns were from Pharmacia Biotech, Uppsala, Sweden. *E. coli* phospholipid extract was from Avanti Polar Lipids, Inc., Alabaster, AL. The endoproteinase AspN was purchased from Sigma, Deisenhofen, Germany.

Bacterial Strains and Plasmids. *E. coli* JM109 (*endA1 recA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB*$^+$ *lac*^{q}*ZΔM15*)) (*10*) served as carrier for the plasmids described. *E. coli* WG170 (F$^-$ *trp lacZ rpsL thi* Δ(putPA)101 proP219) (*11*) harboring the given plasmids was used for overexpression of the *putP* gene, transport assays, and immunoblots. Plasmid pT7-5/*putP* (*7*), a derivative of plasmid pT7-5 (*12*) containing the *lac* promoter/operator region as well as the *putP* gene, was used for genetic manipulations and expression of the *putP* gene. Plasmid pTrc99a (*13*) was used for overexpression of the *putP* gene.

Site-Directed Mutagenesis and DNA Sequencing. Eight codons encoding the amino acid sequence DYKDDDDK (Flag epitope) were introduced at the 3′ end of *putP*, yielding plasmid pT7-5/*putP(Flag)* (*7*). Subsequently, an additional nine codons encoding the amino acid sequence ASSHHHHHH were attached to the 3′ end of *putP(Flag)* by oligonucleotide-directed site-specific mutagenesis. Furthermore, in a gene encoding functional PutP(FH) devoid of all five native Cys residues (H. Jung and S. Tebbe, manuscript in preparation), a single Cys was placed at the C-terminus (position 502), yielding pT7-5/*putP-S502C(FH)*.[1] For overexpression, *putP(FH)* and *putP-S502C(FH)* were cloned into plasmid pTrc99a using restriction endonucleases *Nco*I and *Hin*dIII. The resulting constructs were verified by DNA sequencing of double-stranded DNA using the dideoxynucleotide chain-termination method after alkaline denaturation (*14*).

Proline Transport in Cells. Active transport was measured in *E. coli* WG170 harboring given plasmids with 5 μM L-[^{14}C]proline in the presence of 20 mM D-lactate and 50 mM NaCl at 25 °C using the rapid filtration method as described (*6*).

Preparation of Inverted Membrane Vesicles. *E. coli* WG170 harboring plasmid pTrc99a/*putP(FH)* or pTrc99a/*putP-S502C(FH)* was grown aerobically in LB medium (*15*) containing 100 μg/mL ampicillin at 37 °C, and expression was initiated by addition of 0.3 mM isopropyl thio-β-D-galactoside (IPTG)[2] at the middle of the exponential growth phase. After further growth for 3 h, cells were harvested by centrifugation, washed with 100 mM KP$_i$, pH 7.5/2 mM β-mercaptoethanol, and resuspended in the same buffer to give 0.4 g of cells (wet weight)/mL. Inverted membrane vesicles were prepared by passage of the cell suspension through a Sorvall RF1 Ribi refrigerated cell fractionator followed by low-speed centrifugation at 12000*g* for 30 min at 4 °C to remove unbroken cells. Membranes were collected by centrifugation at 230000*g* for 90 min at 4 °C, washed with 50 mM KP$_i$, pH 7.5/2 mM β-mercaptoethanol, resuspended in the same buffer, and stored at −80 °C.

Purification of PutP(FH). For solubilization of PutP(FH), inverted membrane vesicles were diluted into 50 mM KP$_i$, pH 8.0, to yield a protein concentration of 5 mg/mL. The membrane suspension was supplemented with 2 mM β-mercaptoethanol and 10% glycerol. β-D-Dodecylmaltoside was added stepwise to yield a final concentration of 1.5% (w/v) while stirring on ice. After additional stirring for 30 min, the sample was centrifuged at 230000*g* for 20 min. The supernatant was supplemented with 10 mM imidazole and 300 mM NaCl and incubated with Ni^{2+}−NTA (1 mL of resin/45 mg of membrane protein) for 45 min with gentle shaking at 4 °C. The resin had been preequilibrated with 25 bed volumes of 50 mM KP$_i$, pH 8.0/300 mM NaCl/10 mM imidazole/2 mM β-mercaptoethanol/10% glycerol (v/v)/0.04% β-D-dodecylmaltoside (w/v) (buffer E). The protein−resin complex was then packed into a column, and unbound protein was removed by washing with buffer E until the absorbance at 280 nm returned to the base line. Subsequently, the resin was washed with 20 bed volumes of buffer E containing 30 mM imidazole to remove loosely bound protein. PutP(FH) was eluted from the column with 200 mM imidazole in buffer E. The ionic strength of the Ni^{2+}−NTA column fraction was lowered by passage through a HiTrap desalting column. The protein was loaded onto a DEAE-Sepharose CL-6B column preequilibrated with 10 mM KP$_i$, pH 8.0/2 mM β-mercaptoethanol/10% glycerol (v/v)/0.04% β-D-dodecylmaltoside (w/v). After application of a linear gradient of 0−100 mM NaCl, the transporter was eluted from the column at a salt concentration of about 55 mM.

Reconstitution of PutP(FH). Acetone/ether-washed *E. coli* polar lipid extract in chloroform/methanol (20 mg/mL) was transferred into a beaker, and solvent was removed under a stream of argon to obtain a thin layer of dry lipids. The lipids were suspended in argon-saturated buffer containing 100 mM KP$_i$, pH 7.5/2 mM β-mercaptoethanol/1.5% β-D-octylglucoside (w/v) to yield a lipid concentration of 10 mg/mL. Subsequently, β-D-octylglucoside was removed by dialysis against 100 mM KP$_i$, pH 7.5/2 mM β-mercaptoethanol, and the resulting liposomes were stored in liquid nitrogen. For reconstitution, the liposomes were thawed and extruded through a 400 nm filter to obtain unilamellar vesicles of relatively homogeneous size (*16*). Detergent was added at concentrations corresponding to the onset or total solubilization of lipids as determined by measuring changes in absorbance at 540 nm. The following detergent concentrations were used (onset/total solubilization in %, w/v): β-D-octylglucoside 0.5/1.5, β-D-dodecylmaltoside 0.2/0.5, and Triton X100 0.12/0.45. Detergent-destabilized liposomes were mixed with purified protein in a 100:1 ratio (w/w) and incubated at room temperature under gentle agitation for 10 min. Detergent was removed by adding Bio-Beads SM-2 pretreated according to (*17*) at a wet weight bead:detergent ratio of 5:1 (w/w) (for β-D-dodecylmaltoside, the ratio was 10:1). After 1 h of incubation at room temperature, fresh Bio-Beads were added, and incubation was continued for an additional hour. After the third addition of Bio-Beads, incubation was continued overnight at 4 °C. Bio-Beads were removed by filtration on glass silk, and the turbid proteoliposome suspension was dialyzed 2 times against 100 mM

[1] 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 Na$^+$/proline transporter. The sequence is followed by a second letter denoting the substitution at this position.

[2] Abbreviations: biotin maleimide, 3-(*N*-maleimidylpropionyl)biocytin; IPTG, isopropyl 1-thio-β,D-galactopyranoside; Mes, 2-(*N*-morpholino)ethanesulfonic acid; stilbene disulfonate, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate.

KP_i (pH 7.5)/2 mM β-mercaptoethanol at 4 °C. Proteoliposomes were concentrated by centrifugation at 300000*g* for 45 min and stored in liquid N_2.

Site-Directed Labeling of PutP(FH). A proteoliposome suspension containing purified single Cys PutP-S502C(FH) (~0.25 mg of protein/mL) was supplemented with 200 μM 3-(*N*-maleimidylpropionyl)biocytin (biotin maleimide), subjected to three cycles of freeze/thaw/sonication, and incubated at room temperature for 30 min. Where indicated, proteoliposomes were preincubated with 200 μM of the membrane-impermeant thiol reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (stilbene disulfonate) for 10 min. The reaction was stopped by addition of 2 mM β-mercaptoethanol. For comparison, intact cells of *E. coli* WG170 producing single Cys PutP-S502C(FH) were labeled with stilbene disulfonate and biotin maleimide as described for proteoliposomes. Subsequently, the cells were disrupted by sonication, membranes were prepared, and the transporter was purified by Ni−NTA chromatography as described above. The labeled protein from proteoliposomes and cells was subjected to SDS−PAGE. Reaction with biotin maleimide and the amount of protein were estimated by Western blot analysis using streptavidin−horseradish peroxidase and anti-Flag-M2 antibody, respectively.

Proteolysis. Proteoliposomes containing PutP(FH) were diluted in 10 mM Tris/HCl, pH 8.0, to a final protein concentration of 0.25 mg/mL. The endoproteinase AspN was added at a PutP(FH):AspN ratio of 200:1 (w/w), and proteolysis was carried out at 37 °C. The reaction was stopped by addition of 25 mM EDTA after 0.5, 2, 6, or 17 h of incubation. Subsequently, the protein was solubilized in 1% SDS, subjected to SDS−PAGE, and stained with silver. N-Terminal sequencing was performed as described (*18*).

Transport Measurements with Proteoliposomes. [^{14}C]-Proline uptake was measured with proteoliposomes containing PutP(FH) (~3 mg of protein/mL) preloaded with 100 mM KP_i, pH 7.5/2 mM β-mercaptoethanol/5 mM $MgSO_4$. After thawing the samples at room temperature, the proteoliposomes were extruded through a 400 nm filter to obtain unilamellar vesicles of relatively homogeneous size (*16*). Transport was started by 200-fold dilution of aliquots of the proteoliposome suspension into the desired buffer. The following buffers were used: Na^+-free 100 mM Tris/2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 7.5/2 mM β-mercaptoethanol/5 mM $MgSO_4$ containing 50 mM NaCl (creation of $\Delta\tilde{\mu}_{Na^+}$) or 50 mM LiCl (creation of $\Delta\tilde{\mu}_{Li^+}$) or no further additions (creation of $\Delta\psi$); Na^+-free 100 mM Tris/Mes, pH 6.0/2 mM β-mercaptoethanol/5 mM $MgSO_4$ (creation of $\Delta\tilde{\mu}_{H^+}$) or 100 mM KP_i, pH 7.5/2 mM β-mercaptoethanol/5 mM $MgSO_4$ (facilitated diffusion, control). All buffers contained 10 mM [^{14}C]proline (26 Ci/mol) and 0.2 μM valinomycin. Transport assays were terminated at a given time by quenching of the reaction with 3 mL of ice-cold 100 mM KP_i, pH 6.6/100 mM LiCl and immediate filtration using Millipore filters (type GSTF 02500, 0.2 mm pore size).

Analytical Methods. Silver staining was performed after protein separation by SDS−PAGE (*19*) using 10% acrylamide gels as described (*20*). Specific detection of PutP(FH) in cells and extracts was achieved by Western blot analysis using mouse anti-FLAG antibodies followed by incubation with sheep anti-mouse antibodies, horseradish peroxidase linked (*7*). Protein determination was performed by a modified Lowry method (*21*) with bovine serum albumin as standard.

RESULTS

Properties of PutP(FH). To facilitate immunological detection and protein purification, the Flag epitope and a 6His tag were attached to the C-terminus of the Na^+/proline transporter, yielding PutP(FH). Analysis of Na^+-coupled proline uptake revealed that cells producing PutP(FH) transported proline at an initial rate and to steady-state levels of proline accumulation that were identical to those producing wild-type PutP, while cells transformed with a plasmid devoid of *putP* were not able to transport the amino acid (data not shown). Furthermore, a kinetic analysis of PutP(FH) revealed apparent K_m values for proline and Na^+ of 2 ± 0.3 μM and 31 ± 4 μM, respectively, as it had been determined for the wild-type (*2*). These results demonstrated that the attachment of the Flag epitope and 6His tag to the C-terminus did not influence the function of PutP in a significant manner.

Protein Solubilization. Different uncharged and zwitterionic detergents were tested for the ability to solubilize PutP(FH) from *E. coli* membranes (protein concentration 5 mg/mL, detergent concentration 1.5%, w/v). More than 80% of the transporter was solubilized by β-D-decylmaltoside or β-D-dodecylmaltoside. After solubilization with zwittergents 3-14 and 3-12, lauryldimethylamine oxide, and β-D-octylglucoside, about 10, 20, 30, and 60%, respectively, of PutP(FH) was found in the soluble fraction. Addition of 0.6 M NaCl improved the ability of zwittergents 3-12 and 3-14 to solubilize the transporter [about 90% of PutP(FH) was in the soluble fraction]. However, addition of NaCl stimulated aggregate formation. In the presence of β-D-dodecylmaltoside, PutP(FH) showed the lowest tendency to form aggregates. Therefore, the latter detergent was selected for solubilization and purification of the transporter.

Purification of PutP(FH). The protocol used for the purification of PutP(FH) comprised two steps: purification by nickel chelate affinity chromatography and ion exchange chromatography. In a typical experiment, inverted membrane vesicles (5 mg/mL) were solubilized with 1.5% β-D-dodecylmaltoside as described under Experimental Procedures. Binding of PutP(FH) to the Ni^{2+}−NTA resin was most effective when the solubilized protein was incubated batchwise with preequilibrated resin for 45 min. Furthermore, selective binding of PutP(FH) was greatly enhanced by addition of imidazole (10 mM) and NaCl (300 mM). The protein−resin complex was filled into a column, and most of the contaminants were removed by washing the resin with 30 mM imidazole in equilibration buffer. The protein was eluted by increasing the imidazole concentration to 200 mM. The peak fractions contained PutP(FH) at a concentration of 3 mg/mL. The obtained protein was about 95% pure as judged from a silver-stained SDS gel (Figure 1).

To remove residual contaminating proteins, ion exchange chromatography was used as a further purification step. The desalted protein was loaded onto a DEAE-Sepharose CL-6B column, and PutP(FH) was eluted at a NaCl concentration of about 55 mM. The resulting protein was subjected to

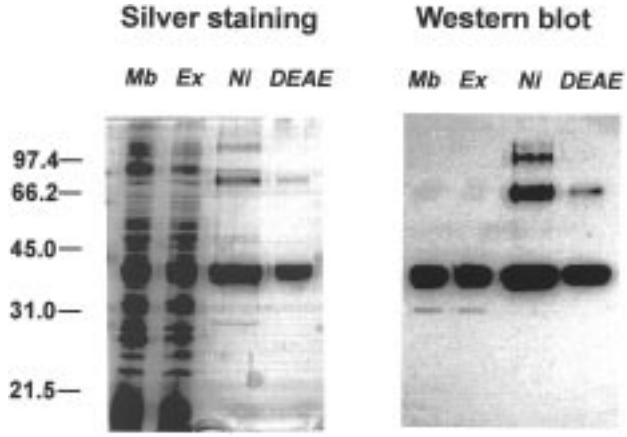

FIGURE 1: SDS−PAGE analysis of samples from different steps of the solubilization/purification procedure of Na^+/proline transporter. Proteins were resolved using 10% polyacrylamide gels and detected by silver staining and Western blot analysis with anti-Flag-M2 antibody (*7*). Mb, inverted membrane vesicles (10 μg); Ex, β-D-dodecylmaltoside extract (10 μg); Ni, solubilized Na^+/proline transporter eluted from a Ni−NTA column with 200 mM imidazole (3 μg); DEAE, solubilized protein eluted from a DEAE-Sepharose column at 55 mM NaCl (2 μg).

SDS−PAGE, and after silver staining, only one band with an apparent molecular mass of about 40 kDa was detectable (Figure 1). This protein band was identified as PutP(FH) by Western blot analysis (Figure 1). Overall, starting from 90 mg of membrane protein, about 6 mg of pure protein was obtained.

Reconstitution. For reconstitution of purified PutP(FH), preformed liposomes made of *E. coli* phospholipids were titrated with the detergents β-D-octylglucoside, β-D-dodecylmaltoside, or Triton X100, and solubilization was followed by turbidity measurements. The detergent concentration was adjusted to the onset or total solubilization of lipids. After mixing of the detergent-destabilized liposomes with the purified transporter, detergent was removed by addition of Bio-Beads SM-2 in three steps at room temperature. As shown in Figure 2, the highest proline uptake activities were obtained when Triton X100 was used for lipid solubilization followed by β-D-dodecylmaltoside and β-D-octylglucoside. Furthermore, with all three detergents, the concentration corresponding to the onset of lipid solubilization yielded higher activities compared to total lipid solubilization. This was particularly evident in the case of β-D-dodecylmaltoside and β-D-octylglucoside.

Orientation of PutP(FH) in Proteoliposomes. In intact cells, the C-terminus of PutP was shown to be located on the cytoplasmic side of the membrane by immunological studies (*5*), *putP-phoA* and *putP-lacZ* fusion analysis (R. Rübenhagen, K. Leifker, N. Tholema, S. Tebbe, and H. Jung, manuscript in preparation), and site-directed labeling (Figure 3, panel D). Therefore, the C-terminal region was selected for determination of the orientation of reconstituted PutP(FH) in proteoliposomes. The method was based on a functional transporter that contained only a single Cys residue placed at the C-terminus followed by the Flag epitope and 6His tag [single Cys PutP-S502C(FH)]. Replacement of Ser502 by Cys did not affect transport (data not shown). The accessibility of the Cys residue at position 502 to thiol reagents of different polarity and, therefore, solubility in the compartments of the system was analyzed for two reconstitution

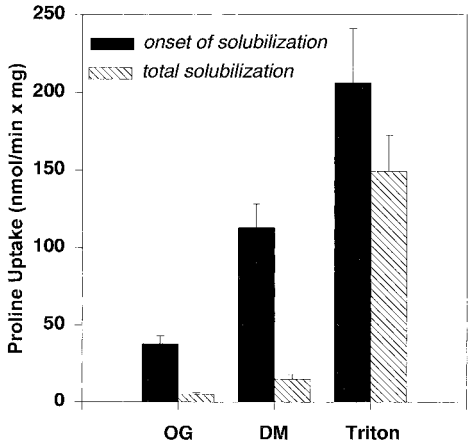

FIGURE 2: Influence of detergents used for liposome destabilization on Na^+/proline transporter activity in proteoliposomes. The transporter was purified and reconstituted using preformed liposomes destabilized with the different detergents at concentrations corresponding to the onset or total solubilization of lipids. After removal of the detergent, initial rates of $\Delta\tilde{\mu}_{Na^+}$-driven proline uptake were estimated from transport curves as shown in Figure 4. Each experiment was repeated 3 times, and the calculated standard deviations are shown. OG, β-D-octylglucoside; DM, β-D-dodecylmaltoside; Triton, Triton X100.

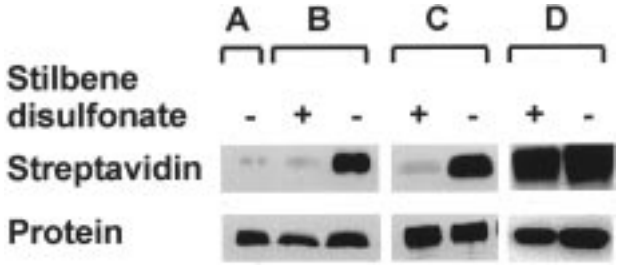

FIGURE 3: Orientation of Na^+/proline transporter in proteoliposomes. Proteoliposomes containing Cys-free (A) or single-Cys PutP-S502C(FH) reconstituted with Triton X100 (B) or β-D-octylglucoside (C) were treated with 200 μM biotin maleimide, subjected to three cycles of freeze/thaw/sonication, and incubated for 30 min at room temperature. When indicated, proteoliposomes were preincubated with 200 μM stilbene disulfonate for 10 min. Subsequently, the protein was solubilized in 1% SDS and subjected to SDS−PAGE. Reaction with biotin maleimide and the amount of protein were estimated by Western blot analysis using streptavidin−horseradish peroxidase (panel Streptavidin) and anti-Flag-M2 antibody (panel Protein), respectively. The reaction of the thiol reagents with single-Cys PutP-S502C(FH) in intact cells of *E. coli* WG170 is shown for comparison (D).

conditions differing in the nature of the detergent used for liposome destabilization (β-D-octylglucoside and Triton X100 at the onset of lipid solubilization). Purified and reconstituted single Cys PutP-S502C(FH) reacted in both cases with biotin maleimide as shown in Figure 3 (panels B and C). Subjection of the proteoliposomes and biotin maleimide to three cycles of freeze/thaw/sonication did not influence the extent of labeling. In contrast, the negative control, Cys-free PutP(FH), showed no reaction with biotin maleimide (Figure 3, panel A). Furthermore, preincubation of reconstituted PutP-S502C(FH) with the membrane-impermeable probe stilbene disulfonate blocked the reaction of the protein with biotin maleimide completely (Figure 3, panels B and C). The presence of equal amounts of protein was demonstrated by Western blot analysis (Figure 3, lower part) and protein determination. These experiments were carried out 3 times for every reconstitution condition. In

all trials, the C-terminal Cys residue of the reconstituted transporter molecules was highly accessible to the membrane-impermeant probe stilbene disulfonate, indicating that single-Cys PutP-S502C(FH) was inserted into proteoliposomes in an inside-out orientation. Use of Triton X100 or β-D-octylglucoside for liposome destabilization did not yield significant differences in the orientation of single-Cys PutP-S502C(FH) (Figure 3, panels B and C).

The inside-out orientation of the transporter reconstituted with Triton X100 was further supported by site-specific proteolysis using the endoproteinase AspN. The enzyme cleaved reconstituted PutP(FH) to about 90%. SDS−PAGE analysis of AspN fragments yielded a stable peptide band with an apparent molecular mass of 14 kDa. N-Terminal sequencing of the fragment revealed a cleavage site before Asp112 which is located on a cytoplasmic loop as it was shown by *putP-phoA* and *putP-lacZ* fusion analysis and site-directed labeling (R. Rübenhagen, K. Leifker, N. Tholema, S. Tebbe, and H. Jung, manuscript in preparation). Judging from size, the fragment contained amino acids Asp112 to Ala261 of PutP (calculated molecular mass 16.4 kDa). Thus, the AspN cleavage sites before Asp214 and Asp227 in the periplasmic loop between transmembrane domains V and VI did not appear to be accessible to proteolytic cleavage by AspN.

Properties of PutP(FH) in Proteoliposomes. Since liposomes destabilized with Triton X100 at the onset of lipid solubilization lead to the highest transport activities, the functional properties of the corresponding proteoliposomes were further investigated. The cation-coupled symport activity of reconstituted PutP(FH) was investigated by measuring [^{14}C]proline accumulation in proteoliposomes loaded with 100 mM KP_i, pH 7.5. A membrane potential ($\Delta\psi$, interior negative) and desired ion gradients were imposed across the proteoliposome membrane by creating an outward-directed K^+ diffusion gradient in the presence of valinomycin and changing the ionic composition and/or pH of the dilution buffer. It was shown that the imposition of an electrochemical Na^+ or Li^+ gradient ($\Delta\tilde{\mu}_{Na^+}$ or $\Delta\tilde{\mu}_{Li^+}$) caused accumulation of [^{14}C]proline in the proteoliposomes whereas in the absence of a driving force (dilution into 100 mM KP_i, pH 7.5) the amino acid only equilibrated with the liposome compartment (Figure 4). Accumulation of [^{14}C]-proline was also observed by application of $\Delta\psi$ in the presence of equal molar amounts of Na^+ ions in proteoliposomes and dilution buffer. In contrast, a pH gradient was neither able to drive uphill transport of proline nor stimulated $\Delta\psi$-driven proline transport (Figure 4). In addition, analysis of $\Delta\tilde{\mu}_{Na^+}$-driven transport at pH 6.0, 6.5, 7.0, or 7.5 did not reveal any significant differences in transport activity (data not shown). Further kinetic analysis of $\Delta\tilde{\mu}_{Na^+}$-driven transport yielded an apparent K_m for proline of 2 ± 0.3 μM and a V_{max} of 1130 ± 80 nmol min^{-1} (mg of protein)$^{-1}$. Assuming that all the transporter molecules were reconstituted functionally, this reflected a turnover number of 1 s^{-1}. The apparent K_m for Na^+ was determined as 730 ± 90 μM compared to 31 ± 4 μM in intact cells (see above).

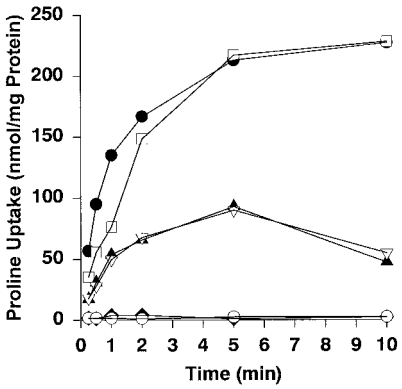

FIGURE 4: Ion dependence of proline uptake by proteoliposomes containing purified Na^+/proline transporter. Proteoliposomes in 100 mM KP_i, pH 7.5, were diluted 200-fold into 100 mM Tris/Mes, pH 7.5, containing 2 mM β-mercaptoethanol, 5 mM $MgSO_4$, 0.2 mM valinomycin, 10 μM [^{14}C]proline, and 50 mM NaCl ($\Delta\tilde{\mu}_{Na^+}$) (●) or LiCl ($\Delta\tilde{\mu}_{Li^+}$) (□), or no further additions ($\Delta\psi$) (▽). In addition, proteoliposomes were diluted into 100 mM Tris/Mes, pH 6.0, containing 0.2 μM valinomycin ($\Delta\tilde{\mu}_{H^+}$) (▲), or 100 mM KP_i, pH 6.0 (ΔpH) (◆), or 100 mM KP_i, pH 7.5 (control) (○). Transport was stopped by rapid filtration as described under Experimental Procedures.

DISCUSSION

This paper describes an efficient procedure for solubilization, large-scale purification, and oriented functional reconstitution of PutP of *E. coli*. While the protein can be efficiently solubilized by different nonionic and zwitterionic detergents, it is most stable in β-D-dodecylmaltoside. By utilizing Ni−NTA affinity and ion exchange chromatography, milligram quantities of the purified transporter are obtained. The strategy of PutP reconstitution is based on the studies of Rigaud et al. (*22*, *23*). Thus, purified PutP is inserted into preformed, detergent-destabilized liposomes. The nature and concentration of the detergent used for liposome destabilization are critical for the functional reconstitution of the transporter. The highest PutP activities are measured when Triton X100 is used for liposome destabilization. Furthermore, a detergent concentration leading to saturation of the preformed liposomes with detergent rather than complete solubilization gives the highest activities.

Site-directed labeling and site-specific proteolysis indicate a unidirectional inside-out orientation of the purified PutP in proteoliposomes. Clearly, the mechanism by which proteins associate with phospholipids is critical for the final orientation of the protein in the bilayer. A possible mechanism explaining the unidirectional orientation is that the protein is always inserted into the membrane with its mostly hydrophobic moiety first (*23*, *24*). Thus, using preformed liposomes, a large hydrophilic domain may prevent a scrambled orientation as it has been shown, e.g., for F_oF_1-ATPase, Ca^{2+}-ATPase (*23*), and the LacS protein (*25*). In the case of PutP, the insertion of a highly polar peptide composed of 17 amino acids at the C-terminus may lock the secondary transport protein in an inside-out orientation. Interestingly, use of β-D-octylglucoside or Triton X100 for liposome destabilization does not affect the orientation of the protein in proteoliposomes. Thus, differences in transport activities measured for the different reconstitution conditions cannot be attributed to an effect on transporter orientation. Rather, the low transport activities measured when β-D-octylglucoside is present during reconstitution might be explained by the high tendency of PutP to form aggregates under these conditions.

Reconstituted PutP exhibits transport characteristics similar to those observed with whole cells and membrane vesicles containing the transporter. Thus, accumulation of proline in proteoliposomes is achieved in the presence of $\Delta\tilde{\mu}_{Na^+}$, $\Delta\tilde{\mu}_{Li^+}$, or $\Delta\psi$ while a pH gradient neither causes proline uptake nor stimulates $\Delta\psi$-driven transport. Taking the inside-out orientation of PutP into account, the studies indicate that the direction of proline transport is reversed if the applied electrochemical Na^+ gradient is reversed as it has been shown, i.e., for the Na^+/glucose transporter (*26*).

Comparison of the apparent affinity of PutP for proline in proteoliposomes and intact cells (*27*) does not yield significant differences. However, the apparent affinity of reconstituted PutP(FH) for Na^+ is decreased by a factor of about 25. Judging from the unaltered apparent proline affinity and the high activity of the transporter, it is unlikely that the decreased Na^+ affinity is caused by misfolding of the protein. Taking the inside-out orientation of the transporter into account, the lower ion affinity can rather be explained by a functional asymmetry of the transporter. Thus, the cation affinity on the cytosolic side of PutP appears to be lower than on the outside. This idea is consistent with the finding that the apparent K_m for Na^+ uptake into intact cells or membrane vesicles is about 100 times lower than the K_d value for Na^+ representing ion binding to both sites of the membrane (*28*).

Furthermore, PutP reconstituted into preformed, detergent-destabilized liposomes catalyzes $\Delta\tilde{\mu}_{Na^+}$-driven proline uptake with a 20-fold higher V_{max} than PutP reconstituted by detergent dilution (*29*). Since there is no significant difference in protein purity, the higher activity has to be attributed to the improved reconstitution conditions described in this paper.

In summary, this study provides an efficient procedure for large-scale purification and oriented reconstitution of PutP into proteoliposomes in a highly active form. It opens the possibility for protein chemical and spectroscopic studies as well as attempts at crystallization. Finally, the kinetic analysis suggests a lower cation affinity on the cytosolic side of PutP relative to the outside.

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