Hydropathy profile analysis of the amino acid sequence of the Na\(^{+/}\) proline transporter of *Escherichia coli* (PutP) suggests that the protein consists of 12 transmembrane domains (TMs) which are connected by hydrophilic loops (Nakao, T., Yamato, I., and Anraku, Y. (1987) *Mol. Gen. Genet.* 208, 70–75). We have tested this prediction by applying a gene fusion approach in combination with a Cys accessibility analysis and site-specific proteolysis. Characterization of a series of PutP-alkaline phosphatase (PhoA) and PutP-β-galactosidase (LacZ) hybrid proteins yields a reciprocal activity pattern of the reporter proteins that is in agreement with the topology of TMs III to XII of the 12-helix model. Placement of the PutP-PhoA and PutP-LacZ junction sites closer to the N terminus does not yield conclusive results. As a prerequisite for further topology studies, a functional PutP molecule devoid of all five native Cys residues (Cys-free PutP) is generated. Subsequently, amino acids in Cys-free PutP are replaced individually with Cys, and the accessibility of the sulphydryl groups is analyzed. Surprisingly, Cys residues placed close to the N terminus of PutP (Ile-3 → Cys, Thr-5 → Cys) or into putative TM II (Ser-71 → Cys, Glu-75 → Cys) are highly accessible to membrane permeant and impermeant thiol reagents in intact cells. In contrast, Cys at the C terminus (Ser-502 → Cys) reacts only with the membrane permeant but not with the impermeant reagent in intact cells. These results contradict the 12-helix motif and indicate a periplasmic location of the N terminus whereas the C terminus faces the cytoplasm. In addition, a transporter with Cys in place of Leu-37 (putative TM II (Ser-71)), Asp-262 (cL7), and Ile-3 (putative TM III (Ser-71)) is proposed according to which the protein consists of 13 TMs with the N terminus on the outside and the C terminus facing the cytoplasm. The 13-helix structure is discussed as a common topological motif for all members of the Na\(^{+/}\)/solute cotransporter family.

The Na\(^{+/}\)/proline transporter of *Escherichia coli* (PutP) is an integral membrane protein that catalyzes the coupled translocation of Na\(^{+}\) ions and proline (1–3). PutP belongs to a family of homologous Na\(^{+}\)-dependent membrane transport proteins (Na\(^{+}\)/solute cotransporter family, SCF)\(^{1}\) that comprises more than 35 members of bacterial, yeast, insect, nematode, and mammalian origin (4, 5). Based on the hydropathy profile of the amino acid sequence of PutP, a secondary structure model has been proposed according to which the transporter consists of a short N-terminal tail, 12 transmembrane domains (TMs) in α-helical conformation that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a hydrophilic C-terminal tail (6) (Fig. 1). Experimental evidence for the cytoplasmic location of the C terminus comes from immunological studies (7). However, recent N-glycosylation scanning mutagenesis of the human Na\(^{+}\)/glucose transporter (SGLT1), another member of the SCF, suggests a 14-helix motif for this transporter (8). Because PutP lacks a C-terminal extension, 13 TMs are predicted for this and other prokaryotic members of the SCF (5).

In this study, we have tested the different topological predictions for PutP. In addition, our attention is focused on the topological location of the recently identified functional important amino acid residues, Asp-55 and Ser-57, which are proposed to be involved in ligand binding (9, 10). To obtain experimental evidence on the topology of PutP, we have generated and characterized a series of putP-PhoA and putP-LacZ fusions. In addition, information on the arrangement of the PutP polypeptide has been gained from a Cys accessibility analysis and site-specific proteolysis. Periplasmic alkaline phosphatase (PhoA) and cytosolic β-galactosidase (LacZ) have already been used successfully as reporter proteins to determine the topological arrangement of a variety of membrane proteins in the bacterial cytoplasmic membrane (11–13). Also, site-directed labeling and specific proteolysis of membrane proteins are established methods for topology analysis (14–16). The results obtained in the course of this study demonstrate that the N terminus of PutP is located on the periplasmic side of the membrane whereas the C terminus faces the cytoplasm. Furthermore, amino acids of former periplasmic loop (pL) 2 are proposed to form an additional TM (now TM II) whereas the boundaries of former TM II (now TM III) are shifted by eight amino acids toward the C terminus of PutP. The resulting 13-helix motif is discussed as a common structural feature of members of the SCF.

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1. The abbreviations used are: SCF, sodium solute cotransporter family; SM, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; BM, 4-(N-maleimidylpropionyl)-biocytin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Kp, potassium inorganic phosphate; clp, putative cytoplasmic loop; pL, putative periplasmic loop; PAGE, polyacrylamide gel electrophoresis; TM, putative transmembrane domain; Ni-NTA, nickel nitrilotriacetic acid.

* This work was supported by the Deutsche Forschungsgemeinschaft (SFB171/C19). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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DNA sequencing of double-stranded DNA using the dideoxynucleotide chain-termination method after alkaline denaturation (22).

**Proline Transport in Cells—**Active transport was measured in *E. coli* WG170 producing PutP or its variants with 5 μM [1-14C]proline in the presence of 20 mM L-tartrate and 50 mM NaCl at 25 °C using the rapid filtration method as described (10).

**Labeling of Put(PFH) in Cells—** *E. coli* WG170 harboring plasmid pTrc99a/put(PFH) encoding Cys-free or single Cys Put(PFH) was grown aerobically in LB medium (23) containing 100 μg/ml ampicillin at 37 °C, and expression was initiated by addition of 0.3 mM isopropyl-β-D-galactopyranoside (IPTG) at the middle of the exponential growth phase. Further cultivation was 65 min (or 45 min if necessary). The cells were harvested by centrifugation, washed with 100 mM KP, pH 7.5, containing 0.5 mM phenylmethylsulfonyl fluoride (buffer A) and resuspended in the same buffer to give a final protein concentration of 14 mg/ml. For blocking of periplasmic thiol groups, an aliquot of the cell suspension (1.5 ml) was preincubated with 400 μM (or 1 mM if indicated) of the membrane impermeant thiol reagent 4-aminocetamide-4-maleimidyl-stibine-2,2'-disulfonate (SM) at room temperature. After 30 min, cells were washed three times with buffer A and resuspended in the same buffer as described above. Aliquots (1.5 ml) of blocked and untreated cells were reacted with 400 μM 3-(N-maleimidylpropionyl)biocytin (BM) at room temperature for 30 min. Subsequently, labeled cells were washed three times with buffer A and disrupted by sonication followed by low speed centrifugation at 30,000 × g for 30 min at 4 °C to remove above unbroken cells. Membranes were collected by centrifugation at 230,000 × g for 45 min at 4 °C with 100 mM KP, pH 8.0, and resuspended in the same buffer. For solubilization of labeled Cys-free or single Cys Put(PFH), the membrane suspension was supplemented with 2 mM β-mercaptoethanol and 10% glycerol; β-D-deoxymaltoside was added dropwise 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 30,000 × g for 20 min. The supernatant was supplemented with 10 mM imidazole and 300 mM NaCl and loaded onto a Ni²⁺-NTA spin column pre-equilibrated with 100 mM KP, pH 8.0, 300 mM NaCl, 10 mM imidazole, 100 mM glycerol (w/v), 0.04% β-D-deoxymaltoside (w/v) (buffer E). Unbound protein was removed by washing the Ni²⁺-NTA spin column with 60 ml of buffer E containing 10 mM imidazole solution in buffer E. Aliquots of the purified protein were subjected to SDS polyacrylamide gel electrophoresis (PAGE) (10% acrylamide) (43). The amount of protein was judged after Coomasie Blue staining. Reaction of single Cys Put(PFH) with biotin maleimide was determined by Western blot analysis using streptavidin-peroxidase.

**Site-specific Proteolysis—**Put(PFH) was purified and reconstituted into proteoliposomes in an inside-out orientation as described (42). Proteoliposomes containing Put(PFH) were diluted in 10 mM Tris/HCl, pH 8.0, to yield a final protein concentration of 0.25 mg/ml. The endoprotease AspN was added at a Put(PFH)/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 or 17 h of incubation. Subsequently, the protein was solubilized in 1% SDS, subjected to SDS-PAGE (10% acrylamide) according to Schägger and Jagow (24), and stained with silver (25). N-terminal sequencing was performed as described (26).

**Generation of putP-phoA and putP-lacZ Gene Fusions—**Initially, a unique *Nhel* site was introduced at the 3’ end of a cassette version of the *putP* gene, which was generated by site-directed mutagenesis using plasmid pTrc7-Sput(pCassette) containing only a single thiol group. For overexpression, put(PFH) and its variants were cloned into plasmid pTrc99a using restriction endonucleases NcoI and HindIII. The resulting constructs were verified by DNA sequencing of double-stranded DNA using the dideoxynucleotide chain-termination method after alkaline denaturation (22).
were generated using PCR with antisense primers that were complementary to the 3' termini of the desired putP fragments. In addition, the primers created an NheI site at the 3' termini of the different putP fragments. The sense primer was complementary to an appropriate sequence upstream of the newly generated NheI site. The amplified fragments were digested with NheI and an appropriate enzyme upstream of the NheI site and then cloned into plasmids pT7-5/putP-SS02/phoA and pT7-5/putP-SS02/ lacZ digested with the same two restriction enzymes. For expression, the gene fusions were cloned into plasmid pBAD24. Desired gene fusions were identified by restriction analysis and finally sequencing of plasmid DNA (22).

Assay of Alkaline Phosphatase and β-Galactosidase Activities—E. coli CC181 harboring the desired putP-phoA or putP-lacZ fusion in plasmid pT7-5 (or pBAD24) was cultivated in LB medium at 37 °C. In the exponential growth phase, the cells were induced with 0.5 mM IPTG (or 0.2% arabinose (w/v) in case of pBAD24) for 2 h. Alkaline phosphatase and β-galactosidase activities were assayed by measuring the rate of hydrolysis of p-nitrophenyl phosphate and o-nitrophenyl-β-D-galactoside, respectively, by permeabilized cells (13, 28). Assays were performed in triplicate.

Immunological Analysis—E. coli CC181 harboring plasmid pBAD24 with the desired putP-phoA or putP-lacZ fusion was cultivated and induced as described above. Cells from a 1-ml aliquot of the cultures were harvested by centrifugation and resuspended in SDS lysis buffer (60 mM Tris/HCl, pH 6.8, 2 mM β-mercaptoethanol, 10% glycerol (v/v), 2% SDS (w/v), 0.005% bromphenol blue (w/v)). Ten micrograms of total cell protein from each culture were subjected to SDS-PAGE using 7.5% (PutP-LacZ) or 11% (PutP-PhoA) polyacrylamide gels. Proteins were then transferred to a nitrocellulose membrane (0.45 μm pore size), and hybrid proteins were probed with mouse antiserum raised against alkaline phosphatase or β-galactosidase followed by incubation with horseradish-peroxidase inked sheep-anti-mouse-IgG antibody. Protein Determination—Protein determination was performed using a modification of the method of Lowry (29) with bovine serum albumin as standard.

RESULTS

putP-phoA and putP-lacZ Fusion Analysis—For generation of gene fusions, a unique NheI site was introduced at the 3' end of full-length putP followed by the phoA or lacZ gene. Based on these constructs, further putP-phoA and putP-lacZ fusions were generated by shifting the NheI site (junction site) from the 3' end of putP toward the 5' end by oligonucleotide-directed, site-specific mutagenesis. The gene fusions were expressed in E. coli CC181 from the lac promoter in plasmid pT7-5, and the activity of the reporter proteins was determined in permeabilized cells (Table I). Hybrid proteins with junction sites after Glu-446, Pro-393, Leu-302, Glu-217, and Gly-157 of PutP showed high PhoA (>100 units) and low LacZ (<5 units) activities, indicating a location of these sites at or close to the periplasm. A reverse activity pattern (PhoA < 20, LacZ > 120 units) was observed when the junction site was placed after Ser-502, Thr-426, His-365, Met-259, Gln-190, Lys-121, Gly-116, Glu-102, and Arg-96 of the transporter. The latter results indicate that the C-terminal amino acids of the corresponding PutP fragments are located at or close to the cytosolic side of the membrane. Further analysis of PutP-PhoA hybrid proteins revealed a 5- to 10-fold increase in alkaline phosphatase activity upon shifting the junction site from Arg-96 to Trp-90 in PutP. Hybrid proteins with PutP-PhoA junction sites in the loop between putative TMs I and II showed intermediate alkaline phosphatase activities. The corresponding PutP-LacZ hybrids exhibited relatively high β-galactosidase activities. Because the latter results were not conclusive, the topology of the N-terminal part of PutP could not be further defined by the gene fusion method.

Attempts were made to estimate the amount of hybrid protein in cells of E. coli CC181 by Western blot analysis using antibodies directed against PhoA or LacZ. However, expression of the gene fusions from the lac promoter did not yield reproducible results because of the instability particularly of the inactive hybrid proteins. Therefore, putP-phoA and putP-lacZ gene fusions were cloned into plasmid pBAD24 containing the arabinose P_RAD promoter that, after induction with 0.2% arab-
influence, gave higher levels of gene expression than the lac system. Thus, using the pBAD vector higher reporter protein activities were found in *E. coli* CC181 cells than with the lac system (Table I). However, the overall alkaline phosphatase/β-galactosidase activity patterns of both expression system were similar. Furthermore, the pBAD system allowed the detection of PhoA and LacZ with specific antibodies raised against these proteins. Representative blots are shown in Figs. 2 and 3. As expected from the C-terminal truncation of PutP, hybrid proteins of decreasing size (starting from S502PhoA or S502LacZ) were detected on the blot. Most of the hybrid proteins showed some degree of instability, yielding degradation products that corresponded to the PhoA or LacZ moiety of the proteins. A similar instability of hybrid proteins was observed in other studies (30–32).

**Generation and Properties of Cys-free PutP**—As a prerequisite for site-directed labeling of PutP, all five native Cys residues of the transporter were replaced simultaneously with Ala (Cys-12, -141, -281) or Ser (Cys-344, -349). Cells of *E. coli* WG170, producing the resulting transporter, catalyzed Na+-coupled proline uptake with 50% of the *V*max and up to 100% of the steady-state level of proline accumulation of cells containing wild-type PutP. Further kinetic analysis of Cys-free PutP revealed an apparent *Km* for proline of 5 ± 0.2 μM wild-type. In contrast to the effect on wild-type PutP, *N*-ethylmaleimide did not inhibit transport catalyzed by the Cys-free protein. Furthermore, immunological analysis did not reveal significant differences between the amounts of wild-type and Cys-free PutP in the membrane. These results indicate that none of the Cys residues was essential for activity and/or insertion of the protein into the membrane.

**Influence of Amino Acid Substitutions on the Activity of Cys-free PutP**—To provide a chemical reactive group at a defined position in the primary structure of the transporter, different amino acids were replaced individually with Cys in Cys-free PutP. Analysis of Na+-coupled proline uptake revealed that the substitution of Ile-3, Thr-5, Ala-12, Ser-28, Leu-37, Phe-45, Ser-54, Ser-71, Glu-75, Ile-80, or Lys-91 by Cys has no or only little effect on the initial rates of proline transport (>70% of the Cys-free PutP value) and the steady-state levels of proline accumulation (>75% of the Cys-free PutP value) (Fig. 4). PutP-G39C,3 PutP-R40C, and PutP-M62C exhibited intermediate transport activities with transport parameters corresponding to 30–70% of the Cys-free PutP values. Proline uptake by PutP with Cys in place of Ser-50 or Ala-53 caused a dramatic decrease of the initial rate of proline uptake (<10% of the Cys-free PutP value) (Fig. 4). However, all PutP molecules showed a significant activity indicating that none of the substituted residues is essential for active transport.

![Fig. 2. Immunological detection of PutP-PhoA hybrid proteins in *E. coli* CC181.](Image 2)

**Fig. 2.** Immunological detection of PutP-PhoA hybrid proteins in *E. coli* CC181. Cells were cultivated as described under “Experimental Procedures.” Ten micrograms of total cells protein from each culture were separated by SDS-PAGE using 11% polyacrylamide gels. Proteins were then transferred to a nitrocellulose membrane (0.45 μm pore size), and hybrid proteins were probed with mouse antiserum raised against alkaline phosphatase followed by incubation with horseradish-peroxidase-linked sheep-anti-mouse-IgG antibody.

![Fig. 3. Immunological detection of PutP-LacZ hybrid proteins in *E. coli* CC181.](Image 3)

**Fig. 3.** Immunological detection of PutP-LacZ hybrid proteins in *E. coli* CC181. The experimental procedure was as described in Fig. 2 except for 7.5% polyacrylamide gels used for SDS-PAGE and anti-β-galactosidase antibodies used to probe β-galactosidase.

![Fig. 4. Influence of the replacement of different amino acids in the N-terminal part of Cys-free PutP on Na+-coupled proline uptake.](Image 4)

**Fig. 4.** Influence of the replacement of different amino acids in the N-terminal part of Cys-free PutP on Na+-coupled proline uptake. Cells of *E. coli* WG170 were grown and treated as described under "Experimental Procedures." Transport of L-[U-14C]proline (5 μM final concentration) was assayed in the presence of 50 mM NaCl and 20 mM sodium lactate (Na+ salt) as the electron donor at 25 °C under aerobic conditions using a rapid filtration method (10). Initial rates of proline uptake (black columns) and steady-state levels of proline accumulation (gray columns) are expressed as percentage of the corresponding Cys-free PutP value. Cys-free PutP catalyzed proline uptake with an initial rate of 6.5 nmol/min × mg of cell protein to a steady-state level of proline accumulation of 15 nmol/mg of cell protein.

**Location of the N-terminus of PutP**—Information on the location of the N terminus of PutP in intact cells was gained by monitoring the accessibility of the sulphydryl group in single Cys PutP-I3C and PutP-T5C to membrane permeant (BM) and impermeant (SM) sulphydryl reagents. Since the C terminus of PutP was shown to be located on the cytoplasmic side of the membrane by immunological studies (7) and gene fusion analysis (this study) single Cys PutP-S502C was used for comparison. Cells of *E. coli* WG170 producing the desired PutP variant were preincubated with SM to block periplasmic Cys residues. Blocked and untreated cells were then incubated with BM and labeled PutP was isolated as described under "Experimental Procedures." Reaction with BM was assessed by Western blotting using streptavidin-horseradish peroxidase. Applying this procedure, Cys at the position of Ile-3, Thr-5, or Ser-502 was...
shown to react with BM in untreated *E. coli* WG170 cells (Fig. 5). Furthermore, reaction of BM with PutP-S502C was blocked by preincubation with the membrane impermeant SM only in disrupted cells but not in intact cells. These results confirm a cytoplasmic location of the C terminus. In contrast to these experiments, the thiol groups at positions 3 and 5 of PutP were blocked almost completely already in intact cells by pretreatment with the membrane impermeant SM (Fig. 5). These results indicate a peri-plasmic location of the N terminus and suggest an uneven number of TMs.

**Accessibility of Cys Residues in pL2 and TM II of PutP**—To further investigate the topology of the N-terminal part of PutP, the accessibility of single Cys residues in pL2 and the adjoining putative TMs I and II to sulfhydryl reagents was analyzed. The studies revealed that Cys at the position of Ala-12 in TM I; Ser-28, Gly-39, Arg-40, Phe-45, Ser-50, Ala-53, and Ser-54 in pL2; Met-62, and Ile-80 in TM II; or Lys-91 in putative cL3 showed only a low reactivity toward BM or did not react at all (data not shown). Incubation of PutP containing a single Cys at the position of Leu-37 in pL2, or Ser-71 or Glu-75 in TM II, with 200 \( \mu \)M BM resulted in significant labeling of the transporter in intact cells. In addition, reaction of PutP-S71C and PutP-E75C with BM was blocked by preincubation of the cells with membrane impermeant SM. However, the latter compound had relatively little effect on the BM labeling of PutP-L37C in intact cells although SM inhibited reaction of the Cys with BM after cells disruption similar to that observed for PutP-S502C (Fig. 5). The results are consistent with the idea that residues of pL2 form an additional TM (now TM II) thereby placing Leu-37 onto the cytosolic side of the membrane. Furthermore, the accessibility of the thiol groups in PutP-S71C and PutP-E75C to sulfhydryl reagents was analyzed. The studies revealed that Cys at the position of Ala-12 in TM I; Ser-28, Gly-39, Arg-40, Phe-45, Ser-50, Ala-53, and Ser-54 in pL2; Met-62, and Ile-80 in TM II; or Lys-91 in putative cL3 showed only a low reactivity toward BM or did not react at all (data not shown).

**Proteolysis**—The modification of the topological arrangement of PutP proposed by the Cys accessibility analysis was further tested by site-specific proteolysis using the endoprotease AspN. This enzyme is known to cleave polypeptides before Asp residues (33, 34). For AspN treatment, PutP-wild type was purified and reconstituted into proteoliposomes in an inside-out orientation as described (42). In agreement with the 12-helix motif, AspN treatment of the proteoliposomes yielded peptide fragments within 30 min of incubation that contained Asp-112 (in cL3), Asp-262 (in cL7), or Asp-356 (in cL9) at the N terminus (Figs. 1 and 6 and Table II). In addition, PutP was cleaved before Asp-33 in pL2 while hydrolysis of peptide bonds at potential AspN sites in other periplasmic loops was not observed within 30 min. Only after an extended period of incubation of the proteoliposomes with AspN (6–17 h at 37 °C) and equilibration of the protease with the proteoliposome interior PutP was cleaved before Asp-228 in pL6 (Figs. 1 and 6, and Table II). In addition, an unspecific cleavage occurred before Leu-302 in pL8. These findings are consistent with the results of the Cys accessibility analysis and support the idea that an additional TM is formed by amino acids of former pL2, thereby moving amino acids around Asp-33 and Leu-37, from the periplasmic to the cytosolic side of the membrane.

**DISCUSSION**

The 12-helix secondary structure model of PutP (6) is based mainly on hydropathy profile analysis of the amino acid sequence. We have obtained experimental evidence on the topological arrangement of the secondary transporter by applying a gene fusion approach together with a Cys accessibility analysis.
The prerequisite for site-directed labeling indicates that Cys residues are shown to be completely replaceable in (LacY) or melibiose permease (MelB) of E. coli (36, 37). Furthermore, individual substitution of a variety of amino acids in the N-terminal part of Cys-free PutP by Cys had no or only little effect on transport activity, indicating also a structural integrity of the corresponding PutP molecules. The substitution of Arg-40, Ser-50, Ala-53, and Met-62 by Cys results in reduced proline uptake rates (Fig. 4). In case of Ser-50, Ala-53, and Met-62, the effect on transport activity might be because of a location close to Asp-55 and Ser-57, which are proposed to be involved in ligand binding (9, 10). Arg-40 is conserved within the members of the SCF (4). A preliminary substitution analysis indicates that this basic residue affects Na⁺-dependent proline binding.4

In intact cells, Cys residues placed at or close to the N terminus (I3C, T5C) of PutP are readily accessible to membrane permeant (BM) and impermeant (SM) sulfhydryl reagents. Under these conditions, a Cys at the C terminus of the protein (S502C) reacts only with the membrane permeant BM but becomes accessible to the highly polar SM after cell disruption (Fig. 5). This accessibility pattern can only be explained by a location of the N and C terminus on the periplasmic and cytosolic side, respectively, of the membrane. This conclusion contradicts the 12-helix motif and implies the existence of an uneven number of TMs. Furthermore, the high accessibility of sulfhydryl groups placed at the position of Ser-71 or Glu-75 (former TM II) to BM and SM in intact cells suggests a location of these residues in a periplasmic loop. This modification requires a shift of the boundaries of former TM II by at least eight amino acids toward the C terminus of PutP. Contrary to the general high accessibility of sulfhydryl groups particularly at or close to the protein termini, Cys residues at most of the positions in former pL2 and adjacent TMs tested show little or no reaction with sulfhydryl reagents independent from the membrane orientation (Fig. 5). The low reactivity of the sulfhydryl group might be because of a hydrophobic environment (38) or a location that buries the sulfhydryl group within the protein. These results are consistent with the idea that residues of former pL2 are located in a transmembrane domain. This idea also explains the finding that antibodies raised against a synthetic peptide corresponding to the hydrophilic segment between TM I and II does not react with the transporter (3). Strong support for the formation of an additional TM in this region comes from the fact that the membrane impermeant SM blocks reaction of PutP-L37C with BM completely only after cell disruption but not in intact cells similar to that observed for PutP-S502C. These results suggest a shift of Leu-37 from the former pL2 into a cytosolic loop. The formation of an additional TM in this region of the protein is confirmed by the pattern of PutP fragments obtained after AspN proteolysis of transporter reconstituted into proteoliposomes in an inside-out orientation. Thus, the endoproteinase readily cleaves the polypeptide not only in the known cytoplasmic loops (cL3, cL7, and cL9 according to the 12-helix motif) as expected but also before Asp-33 in the former pL2. In conclusion, the data suggest a placement of amino acids Arg-27 to Arg-40 from former pL2 into a cytoplasmic loop (now cL2), whereas amino acids

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4 M. Quick and H. Jung, unpublished data.
Ser-41 to Pro-65 form an additional TM (TM II).

The latter modification shifts amino acid residues proposed to be involved in ligand binding (Asp-55 and Ser-57) (9, 10) from former pL2 into the new TM II. This topological arrangement is in agreement with the fact that residues suggested to be involved, i.e. in binding of the coupling ion in transporters such as LacY and MelB, are located in TMs (31, 39). On the other hand, Glu-75 which is not important for PutP function (10), is shifted from the energetically unfavorable position in former hydrophobic TM II into pL3 (former pL2).

Although it is clearly not the aim of this study to investigate the mechanism of insertion of PutP into the cytoplasmic membrane, it is tempting to speculate how the transporter without a leader (signal) sequence translocates its N terminus across the membrane. Analysis of hybrid proteins generated from E. coli leader peptidase and the phage P3 coat protein reveals that short tails, which do not contain positively charged residues, are efficiently translocated independent of the Sec machinery (40). Depending on size and presence of acidic amino acid residues in the N-terminal tail, the translocation might be energized by a proton motive force (41). In the case of PutP, the short N-terminal tail and the adjoining first TM do not contain either positively or negatively charged residues. Instead, this part of the protein is highly hydrophobic. Therefore, it is possible that the energy required to translocate the short N-terminal tail of PutP across the membrane is gained from the transition of hydrophobic TM I from the aqueous phase into the apolar phase of the bilayer.

The new secondary structure model of PutP is in agreement with the recently proposed topological arrangement of TMs I to XIII of SGLT1, which is based on an N-glycosylation study (8). The results support the idea of a common topological motif for members of the SCF, according to which the bacterial transporters for proline (PutP) and pantothenate (PanF) and the mammalian Na\textsuperscript{+}/galactoside transporters (SGF) (4), are composed of 12 TMs as revealed by a melB-phoA fusion analysis (31). Thus, the 13-helix motif might be a characteristic feature of members of the SCF.

Acknowledgments—We thank Dr. K. Altendorf (University of Osnabrück) for generous support of the project, Dr. K. Jung (University of Osnabrück) for many critical discussions, and E.-M. Uhlemann for excellent technical assistance in the protein chemical part of the study. In addition, we thank Dr. C. Manoil (University of Washington) for providing E. coli CC181.

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