The Na⁺/proline transporter PutP of *Escherichia coli* is a member of a large family of Na⁺/substrate symporters. Previous work on PutP suggests an involvement of the region ranging from Asp-55 to Gly-58 in binding of Na⁺ and/or proline (Pirch, T., Quick, M., Nietschke, M., Langkamp, M., Jung, H. (2002) *J. Biol. Chem.* 277, 8790–8796). In this study, a complete Cys scanning mutagenesis of transmembrane domain II (TM II) of PutP was performed to further elucidate the role of the TM in the transport process. Strong defects of PutP function were observed upon substitution of Ala-48, Ala-53, Trp-59, and Gly-63 by Cys in addition to the previously characterized residues Asp-55, Ser-57, and Gly-58. However, except for Asp-55 none of these residues proved essential for function. The activity of eight mutants was sensitive to N-ethylmaleimide inhibition with the sensitive positions clustering predominantly on a hydrophilic face in the cytoplasmic half of TM II. The same face was also highly accessible to the bulky sulfhydryl reagent fluorescein 5-maleimide in randomly oriented membrane vesicles, suggesting an unrestricted accessibility of the corresponding amino acid positions via an aqueous pathway. Na⁺ stimulated the reactivity of Cys toward fluorescein 5-maleimide at two positions while proline inhibited reaction of the sulfhydryl group at nine positions. Taken together, the results demonstrate that TM II of PutP is of particular functional importance. It is proposed that hydrophilic residues in the cytoplasmic half of TM II participate in the formation of an aqueous cavity in the membrane that allows Na⁺ and/or proline binding to residues located in the middle of the TM (e.g. Asp-55 and Ser-57). In addition, the data indicate that TM II participates in Na⁺ and proline-induced conformational alterations.

Members of the Na⁺/substrate symporter family (SSSF, 1 TC 2.A.21) couple electrochemical Na⁺ gradients to the uphill transport of a variety of substrates including amino acids, sugars, vitamins, ions, and others (1–4). Members of the SSSF share a common topological motif of 13 α-helical domains whereby N- or C-terminal extensions may occur (3, 5–7). We use the Na⁺/proline transporter PutP of *Escherichia coli* as a model system to study the mechanism of sodium motive force-driven substrate uptake. The putP gene has been cloned and sequenced and the gene product has been solubilized, purified, and shown to be solely responsible for sodium-coupled uptake of proline in a reconstituted system (8–10). Obviously, missing information on the three-dimensional structure is limiting access to the molecular mechanism of Na⁺-coupled substrate transport. Because NMR and crystallization approaches have proven unsuccessful for members of the SSSF until now, genetic and biochemical methods are used to elucidate structure-function relationships. Analyses of PutP have identified amino acid residues crucial for PutP function (11). Asp-187 in the cytoplasmic loop between transmembrane domains (TM) V and VI is one of these residues and has been proposed to participate in the coupling of Na⁺ and proline transport (12).

Particular attention has recently been paid to the role of amino acids in putative TM II in PutP function. Asp-55 in the TM has been identified as the only negatively charged amino acid of the N-terminal domain of PutP essential for function and has been proposed to participate in binding of the coupling ion (13). Furthermore, kinetic and Cys accessibility analyses implicate Ser-57 and Gly-58 in Na⁺ and proline binding and suggest partly overlapping binding sites of both ligands (14, 15). In addition, Arg-40 located at the cytoplasmic end of TM II is crucial for coupling of Na⁺ and proline transport (16).

To extend knowledge on the role of TM II for PutP function, a complete Cys scanning mutagenesis of the TM (amino acid positions 41 to 65) has been performed in this study. Analysis of the consequences of the amino acid substitutions for transport function identified amino acids that are crucial for transport in addition to the previously analyzed residues at positions 55, 57, and 58. Furthermore, Cys accessibility analyses suggest a participation of TM II in the formation of a hydrophilic cleft or half-channel in the membrane and implicate the TM in ligand-induced conformational alterations.

**EXPERIMENTAL PROCEDURES**

**Materials—**[^1] L-[U-¹⁴C]Proline (240 Ci/mol) and sheep anti-(mouse IgG)-horseradish peroxidase conjugate were purchased from Amer sham Biosciences. Mouse anti-FLAG M2-IgG was from Integra Bio sciences. Restriction endonucleases, Taq-DNA polymerase, T4-DNA ligase, and alkaline phosphatase were purchased from New England Biolabs or Invitrogen. Isopropyl-β-D-thiogalactoside was obtained from BIOMOL. Synthetic oligonucleotides (primer) were from Eurogentec, MWG, or Invitrogen. *E. coli* DH5α competent cells were also from Invitrogen. Fluorescein 5-maleimide was from Molecular Probes, N-ethylmaleimide (NEM) was obtained from Sigma. SDS-PAGE low range molecular mass standards were purchased from Bio-Rad. n-Undecyl deoxy-D-maltoside was from Anatrace and Ni²⁺-NTA spin columns were from Qiagen. All other chemicals were of analytical grade and obtained from commercial sources.

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Bacterial Strains and Plasmids—E. coli JM109 (endA1 recA1 gyrA96 thi hsdR17 supE44 relA1 lacIqZM15 tnaC) (17) and E. coli DH5α (F- φ80d lacZ ΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 Δ(mcrBC-mcrA) φ80lac Pro+ F′ traD36 proAB lacZΔM15 (tcr-1)) (18) were used as carriers for the plasmids generated in this study. E. coli WG170 (F- trp lacZ rpsL, thi Δ(lacPA1019) pO12209) (13) harboring the given plasmids were used for overexpression of the putP gene and transport assays. 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: pT7-5/putP (wild-type and an engineered transporter devoid of all five native Cys residues (PutP(Cys5))).

Transport Assays—Transport activities were measured in E. coli WG170 transformed with plasmid pT7-5/putP(Cys5) harboring the given mutations. Rates of proline transport were calculated from the initial linear portion of the time course of Na⁺/proline symport. A decrease in the initial rate of proline uptake was defined as inactive transport.

Results

1. Cys Scanning of TM II of PutP

(a) Immunological Analysis—Relative amounts of PutP with given amino acid replacements in membranes of E. coli WG170 were estimated by Western blot analysis as described (14). For membrane preparation, remaining cells of the transport assay were disrupted and membranes were prepared as described above. Twenty μg of total membrane protein was loaded onto a 10% SDS-PAGE and subsequently blotted onto a nitrocellulose membrane. 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. Detection was carried out according to the enhanced chemiluminescence method.

(b) Protein Determination—Determination of protein was performed according to Bradford (23) for purified protein and a modified Lowry method (24) for total membrane protein with bovine serum albumin as standard.

2. Transport Activities of Single Cys PutP Derivatives—A multiple alignment of members of the SSSF was performed to identify conserved amino acids in the N-terminal domain of PutP. The alignment showed that predominantly polar amino acids in the region of TM II of PutP are conserved within both prokaryotic and eukaryotic members of the family (Arg-40, Ser-50, Gly-63) or within the PutP subfamily (Ala-53, Ser-50, Asp-55, Ser-57, Gly-58, Trp-59, Pro-65, Gly-66) (Fig. 1). Previous work on the role of amino acids Ser-54 to Gly-58 has demonstrated that Asp-55 and Ser-57 are of particular importance for PutP function (13-16). To further clarify the role of the N-terminal region of PutP in Na⁺/proline symport, a complete Cys scanning mutagenesis of TM II was performed.

In a first step, each amino acid of TM II (Ser-41 to Pro-65) was individually replaced with Cys in a functional PutP molecule devoid of all five native Cys residues (PutP(Cys5)). Active transport was measured under standard test conditions (50 mM NaCl, 10 μM proline) by using E. coli WG170, which lacks PutP and PutA and therefore cannot metabolize proline. Most severe effects on transport were observed upon replacement of amino acids in the C-terminal (periplasmic) half of TM II (Ala-53 to Pro-65) (Fig. 2). In contrast, little or no effect on PutP function was detected upon substitution of amino acids in the N-terminal (cytoplasmic) half of TM II (Ser-41 to Gly-52) (24). The levels of maximum accumulation of proline were generally less affected than the initial rates of transport. Besides the known residues, Arg-40, Asp-55, Met-56, Ser-57, and Gly-58, substitution of Ala-48 and Gly-63 had the most dramatic effect on transport (initial rates and steady-state levels of proline accumulation below 5% and 10%, respectively of PutP(Cys5)) (22). Significant but less dramatic effects were detected upon replacement of Ala-53, Trp-59, Leu-64, and Pro-65 (initial rates between 10 and 30% of PutP(Cys5), steady-state levels of proline accumulation were not affected).

(c) Cys Accessibility Analyses—To investigate accessibility of individual introduced Cys residues to the sulfhydryl-specific dye fluorescein 5-maleimide, 500 μl of E. coli WG170 transformed with pT7-5/putP(Cys5) harboring given mutations were grown aerobically to an A600 of 1.5 before induction with 0.5 mM isopropyl-β-D-thio-galactoside and further growth for 2 h. Cells were harvested, washed in 100 mM Tris/Mes buffer, pH 7.0, and sonified 4× 30 s using a Branson sonifier. The cell debris was removed by centrifugation at 13,200 × g and 4 °C for 10 min, and the supernatant was subjected to ultra centrifugation at 230,000 × g for 45 min. The resulting membrane pellet was washed and resuspended in 100 mM Tris/Mes buffer, pH 7.9. After protein determination, membrane pellets were stored in liquid nitrogen until further use. For accessibility analyses, 150-μl aliquots of the membrane suspension containing 10 mg/ml total protein were incubated with or without Na⁺ and/or proline at given concentrations at 25 °C for 10 min. Subsequently, 200 μM fluorescein 5-maleimide was added and incubation was continued at 25 °C for an additional 10 min. Reactions were stopped by addition of 5 mM β-mercaptoethanol. After labeling, PutP had undergone substitution of 1.5% β-dodecylmalto side at stirring at 4 °C for 30 min. Then, samples were purified via Ni²⁺-NTA spin columns and eluted with 200 mM imidazole as described before (15). After protein determination, equal amounts of protein were subjected to a 10% SDS-PAGE. Fluorescent bands of PutP were visualized using the MultiFinder device (Bio-Rad) and the software Quantity One or MultiAnalyser with the following settings: excitation wavelength 360 nm, UV illumination trans, high resolution mode. After analysis of fluorescent PutP bands, the gel was stained with Coomassie Blue to detect total amounts of protein.
Effect of NEM Labeling on the Activity of Single Cys PutP Derivatives—To further evaluate the role of amino acids in TM II, the influence of the sulfhydryl reagent NEM on the activity of the single Cys PutP derivatives was investigated in cells of *E. coli* WG170 (Fig. 4). The effect of NEM treatment of PutP(ΔCys) (about 10% inhibition of the initial rate of proline uptake) was probably a side effect because of labeling of other proteins that could, e.g., be important for the energetic status of the cells. Analysis of the effect of NEM on the initial rate of proline uptake of the single Cys PutP derivatives revealed an inhibitory effect of 50% and more at eight (PutP(ΔCys)-L42C, -T47C, -A51C, -S54C, -M56C, -S57C, -G58C, and -M62C) of 24 positions analyzed (Fig. 4). Most of the sites of NEM inhibition cluster on one phase of TM II (Thr-47, Ala-51, Ser-54, Gly-58, Met-62) (Fig. 5). However, the periodicity of inhibition was interrupted in the middle of TM II comprising the functionally important residues Ser-54 to Gly-58. Here, individual NEM labeling of every residue led to inhibition of transport. PutP-D55C could not be analyzed in the activity assay because the protein is completely inactive from the beginning.

Accessibility of Cys to Fluorescein 5-Maleimide—The activity assay used to analyze NEM labeling did not discriminate between inaccessible residues and residues that were modified without significant effect on PutP activity. To obtain a comprehensive picture of the accessibility of Cys at the various positions of TM II, a fluorescence approach was used. For this purpose randomly oriented membrane vesicles containing the corresponding single Cys PutP derivatives were labeled with fluorescein 5-maleimide. The maleimide is known to react with sulphydryl groups in polar but not in apolar environments (25, 26). After vesicle labeling, the PutP derivatives were solubilized from the membranes, purified by Ni²⁺-NTA affinity chromatography, and subjected to SDS-PAGE. Fluorescence of PutP labeled with fluorescein 5-maleimide was detected by UV excitation.

PutP(ΔCys) was used as a negative control and was not modified by the fluorescent compound (Fig. 5). Despite the fact that amino acids Ser-41 to Pro-64 are supposed to form a membrane spanning domain (TM II), Cys placed at several positions within this region was modified by fluorescein 5-maleimide (Fig. 5). In general, positions in the N-terminal (cytoplasmic) half of TM II showed a higher accessibility than positions in the C-terminal (periplasmic) half of the TM (Fig. 6A). Cys in place of Ser-41, Leu-42, Thr-47, Ala-48, Ala-51, Ser-54, Asp-55, and Gly-63 was particularly well accessible to fluorescein 5-maleimide (fluorescence densities >10,000 arbitrary units) (Fig. 5C). This region of the protein must therefore be well exposed to an aqueous environment. Except for Leu-42 and Gly-63 at the ends of TM II, the residues are all located on one face of the putative α-helix and might participate in the formation of a water-filled cavity or half-channel in the membrane (Fig. 6B). This idea is in good agreement with the proposed role of Asp-55 and Ser-57 in Na⁺ and/or proline binding. An intermediate accessibility was observed for Cys in place of Pro-44, Phe-45, Val-46, Leu-49, Ser-50, Met-56, Met-62, and Leu-64 (Fig. 5). PutP(ΔCys) derivatives containing a single Cys at the positions of Gly-43, Gly-52, Ala-53, Ser-57, Gly-58, Trp-59, Leu-60, and Pro-65 showed only a low reactivity toward fluorescein 5-maleimide or did not react at all (Fig. 5). The data indicate that these residues are either buried within the protein or exposed to the lipid environment. Although most of the inaccessible residues cluster on the phase of TM II that is located opposite to the well accessible positions at 41, 47, 48, 54, 51, and 55, there is no clear periodicity of fluorescein 5-maleimide accessibility (Fig. 6). For example, Leu-42 and Gly-63 are located on a face of low accessibility but were significantly modified by the sulphydryl reagent. On the other hand, Cys in place of Gly-58 or Pro-65 are located on a face of high accessibility showed no or only little accessibility to fluorescein 5-maleimide. This inconsistency may be explained by structural perturbations caused by the replacement of the native amino acids at these positions with Cys, an idea supported by the fact...
that Gly and Pro residues frequently fulfill specific roles in protein structure (27, 28). Alternatively, the results can be taken as an indication that the polypeptide chain comprising amino acids 41 to 65 of PutP has some irregular structure and does not form a straight α-helix.

**Influence of Ligand on Cys Accessibility** — To further explore the role of TM II in PutP function, the influence of ligand (Na⁺, proline) on the accessibility of Cys individually introduced at every position in TM II of PutP(Cys) was tested. For this purpose, randomly oriented membrane vesicles containing the single Cys PutP derivatives were labeled posterior to incubation with or without ligand(s). Subsequently, PutP was isolated and fluorescence was detected as described above.

The analysis revealed four groups of single Cys PutP derivatives, with the following characteristics. (a) Cys residues individually introduced at the positions of Phe-45, Val-46, Thr-47, Leu-49, Ser-50, Ala-51, Ser-54, Gly-63, or Leu-64 were labeled by fluorescein 5-maleimide and the labeling reaction was inhibited by proline in the presence of Na⁺. Na⁺ alone did not affect labeling (Fig. 7 II). (b) Cys in place of Ser-41, Leu-42, Ala-48, Ser-50, and Asp-55 was highly accessible to fluorescein 5-maleimide and the labeling reaction was neither significantly influenced by Na⁺ nor by proline (data not shown). (c) Cys at the positions of Gly-43, Pro-44, Gly-52, Ala-53, Met-56, Leu-60, Leu-61, Met-62, and Pro-65 showed only a low reactivity toward fluorescein 5-maleimide and the labeling reaction was neither significantly influenced by Na⁺ nor by proline (data not shown). (d) As demonstrated in a previous study (15), Na⁺ stimulates the reactivity of Cys placed at positions 57 or 58, an effect that is prevented by the simultaneous addition of Na⁺ and proline.

Analysis of the dependence of the reactivity of Cys at position 42945

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**Fig. 2. Influence of amino acid replacements in TM II on proline uptake.** Cells of *E. coli* WG170 were grown and treated as described under “Experimental Procedures.” Transport of t-[U-14C]proline (10 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 using a rapid filtration method (14). A, initial rates of proline uptake are expressed as percentage of the corresponding value of PutP(ΔCys). PutP(ΔCys) catalyzed proline uptake with an initial rate of 17.6 ± 4 nmol/min mg of cell protein. B, steady-state levels of proline accumulation were determined after leveling out of the uptake curve usually between 5 and 30 min of transport and are given in nanomoles of proline/mg of protein.

**Fig. 3. Immunological detection of PutP containing replacements of amino acids in TM II.** Cells were cultivated as described under “Experimental Procedures.” Twenty micrograms of total membrane protein from each culture were separated by 10% SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane (0.45 μm pore size) and probed with mouse anti-FLAG M2 monoclonal antibody followed by incubation with horseradish peroxidase-linked sheep anti-mouse IgG antibody. The detection was performed according to the enhanced chemiluminescence method.
In any case, the results indicate that residues of TM II are of particular functional importance. The observed substrate protection can be explained by direct stimulatory effect (Fig. 4). In the case of PutP-G58C a significant stimulatory effect is already observed at a Na⁺ concentration of 5 mM that is in the order of the Na⁺ binding constant of PutP.

Most of the amino acid positions protected by proline (group A substitutions) cluster on the phase of TM II at which Ser-57 and Gly-58 reside (15) (Fig. 8). However, also residues at the opposite phase of the putative α-helix (e.g. Phe-45 and Gly-63) were protected from modification by fluorescein 5-maleimide. The observed substrate protection can be explained by direct steric hindering and/or by ligand-induced conformational alterations, which either bury the corresponding residue within the protein or move it from an aqueous into an apolar environment. In any case, the results indicate that residues of TM II are of particular functional importance.

**DISCUSSION**

Identification of the sites of Na⁺ and substrate binding and translocation is a central issue of ongoing studies on members of the SSSF. Analysis of human SGLT1 shows that a fragment comprising the last five TMs of the transporter is sufficient to catalyze Na⁺-independent facilitated diffusion of the sugar (29, 30). Little is known about the role of the N-terminal half of proteins of the SSSF. For PutP of *E. coli* it has previously been demonstrated that TM II of the protein harbors amino acid residues (Asp-55, Ser-57, Gly-58) of special functional importance (13–15). The residues have been proposed to directly participate in binding of Na⁺ and proline thereby implicating TM II in the formation of a substrate translocation pathway. To further investigate the role of TM II, a complete Cys scanning mutagenesis of TM II is performed in this study. This involves a determination of effects of the replacements on proline uptake as well as NEM inhibition studies. In addition, Cys accessibility analyses have been carried out in the presence and absence of Na⁺ and proline to learn more about membrane topology, possible ligand binding sites, and functional dynamics of PutP.

Cys scanning of TM II reveals strong defects of PutP function upon alteration of 7 of 24 amino acids tested. Ala-48, Ala-53, Trp-59, and Gly-63 prove particularly crucial for function in addition to the previously analyzed residues at positions 55, 57, and 58. Gly-63 is conserved within the SSSF, whereas Ala-53 and Trp-59 are conserved within the subfamily of putative Na⁺/proline transporters (Fig. 1). Although Ala-48 is not conserved within the SSSF, a small amino acid (mostly Gly or Ala, rarely Thr) is found at this position in other members of the family. Substitution of Ala-53 and Trp-59 may cause local structural perturbations affecting the adjacent functionally important region around Asp-55 and Ser-57 thereby explaining the reduced transport activities of the corresponding mutants. Ala-48 is located on the same face as Asp-55 and may be part of a substrate translocation pathway. The latter suggestion is supported by the fact that both residues are highly accessible to sulphydryl reagents (Fig. 5) (see also discussion below). Gly-63 may be important for the structure of TM II and/or its conformational flexibility. A similar role is described, *e.g.* for specific Gly residues in LacY of *E. coli* (31–33). In fact, TM II of PutP is involved in ligand-induced conformational alterations as shown in this and previous studies (15, 34). Taken together, the results of the activity measurements confirm and extent the particular functional significance of TM II in the transport process.

The activity of eight mutants proves sensitive to NEM inhibition (Fig. 4). Remarkably, positions only in the cytoplasmic half and in the middle of TM II are affected by the NEM treatment (Fig. 6A). In addition, five NEM-sensitive positions are clustered on the face of TM II that contains a high number of polar amino acid residue (Ser-41, Thr-47, Ser-54, Asp-55) (Fig. 6B). The same face is also highly accessible to the bulky sulphydryl reagent fluorescein 5-maleimide in randomly oriented membrane vesicles, suggesting an unrestricted accessibility of the corresponding amino acid positions via an aqueous pathway. In addition, it is noteworthy that positions predominantly located in the cytoplasmic half of TM II show the highest reactivity. Taken together, the data suggest that the polar face of the putative α-helix is exposed to an aqueous cavity that is
open to the cytoplasmic side of the membrane. This cavity most likely extends from the cytoplasmic end of TM II to the middle of the domain (residues 41 to 55) (Fig. 9). As a consequence the studies suggest that PutP accommodates predominantly to an “inward facing” conformation in the absence of Na\(^+\), proline,

and a membrane potential. Na\(^+\) and/or proline binding may occur to residues at the inner end of the cavity, an idea that is consistent with the previously described functional importance of Asp-55 and Ser-57 of TM II (13–15). The existence of a hydrophilic cleft within the membrane has recently also been described, for example, for the ABC multidrug transporter LmrA of Lactococcus lactis and the glycerol 3-phosphate trans-

**FIG. 5.** Accessibility of Cys individually introduced at every position in TM II to fluorescein 5-maleimide. Randomly oriented membrane vesicles containing the indicated PutP derivative were prepared as described under “Experimental Procedures.” One-hundred-fifty μl of a suspension of membranes of *E. coli* WG170 containing given PutP derivatives (10 mg of total membrane protein/ml) were incubated with 200 μM fluorescein 5-maleimide for 10 min. The labeling reactions were stopped by addition of 2 mM β-mercaptoethanol. After solubilization with 1.5% n-dodecyl-β-maltoside and purification via Ni\(^{2+}\)-NTA affinity chromatography, 5 μg of protein were loaded onto a 10% SDS-PAGE and separated. Fluorescent bands were detected using a MultiImager (Bio-Rad) with a scan time of 60 s for each gel (A). Afterward, the same gel was stained with Coomassie Blue (B). Fluorescence intensities detected by MultiImager were corrected for background fluorescence and quantitatively analyzed using software Quantity One™ and plotted (C).

**FIG. 6.** Schematic presentation of the Cys accessibility of TM II. The figure summarizes the Cys accessibility data shown in Figs. 4 and 5. Residues labeled with NEM are sensitive to inhibition by N-ethylmaleimide. Residues in black ellipses are highly accessible to fluorescein 5-maleimide (fluorescence intensity > 10,000 arbitrary units); residues in gray ellipses show an intermediate accessibility (fluorescence intensity between 5,000 and 10,000 arbitrary units); residues in white ellipses are not or only slightly accessible (fluorescence intensity < 5,000 arbitrary units). A, side view of TM II. B, helical wheel projection of TM II.

**FIG. 7.** Ligand-induced accessibility changes of residues in TM II. Membrane vesicles containing the indicated PutP derivatives were prepared as described in the legend of Fig. 5. Aliquots (150 μl) of a suspension of membranes of *E. coli* WG170 containing given PutP(ΔCys) derivatives (10 mg of total membrane protein/ml) were preincubated in the presence or absence of ligand(s) at 25 °C for 10 min. Subsequently, labeling of the samples with 200 μM fluorescein 5-maleimide was performed for 10 min. Labeled PutP was isolated and analyzed as described in the legend of Fig. 5. A, fluorescent proteins bands. B, Coomassie Blue-stained protein. I, residues depicting Na\(^+\)- and proline-induced accessibility changes. Ligands were added as follows: 1) no addition; 2) plus 50 mM KCl; 3) plus 0.5 mM NaCl; 4) plus 5 mM NaCl; 5) plus 50 mM NaCl; 6) plus 50 mM NaCl and 10 mM proline. II, residues showing proline-induced accessibility changes. Ligands were added as follows: 1) no addition; 2) plus 50 mM NaCl; 3) plus 0.5 mM NaCl and 0.1 mM proline; 4) plus 50 mM NaCl and 1 mM proline; 5) plus 50 mM NaCl and 10 mM proline.
Cys Scanning of TM II of PutP

behavior is likely because of the conformational flexibility of the domain that may facilitate trapping of individual positions by sulfhydryl reagents.

As demonstrated in a previous study (15), Na$^+$ stimulates the reactivity of Cys placed at positions 57 or 58, an effect which is prevented by the simultaneous addition of Na$^+$ and proline. This phenomenon has been explained by a Na$^+$-induced conformational alteration that makes these positions accessible to the aqueous phase and leads to proline binding at or close to these residues. Analysis of the dependence of the reactivity of Cys at position 57 on the Na$^+$ concentration demonstrates that high Na$^+$ concentrations (50 mM) are required to see a significant stimulatory effect (Fig. 7I). This finding is in agreement with the highly decreased apparent Na$^+$ affinity of PutP-S57C (15). In the case of PutP-G58C a significant stimulatory effect is already observed at a Na$^+$ concentration of 5 mM, which is in the order of the Na$^+$ binding constant of PutP. Extension of the analysis of the effect of Na$^+$ on the reactivity of Cys individually placed at all the other positions in TM II did not reveal any significant effect (Fig. 7II, lanes 1 and 2).

In the presence of Na$^+$, proline inhibits the reactivity of Cys at 9 of 15 positions that previously exhibited a high or intermediate accessibility to fluorescein 5-maleimide in addition to the inhibitory effect on Cys accessibility at positions 57 and 58 (Fig. 7II, lanes 3–5). The protective effect of proline may be explained by direct steric hindering as discussed for positions 57 and 58 (15). However, while substitution of either of these two residues dramatically reduces the apparent proline affinity, replacement of many of the other protected residues had little effect on transport kinetics. In addition, the latter residues are distributed along almost the entire TM and are in part found on different faces of the putative α-helix (Fig. 8B). Therefore it is highly unlikely that protection of these positions is achieved by direct steric hindering by the substrate. Rather, the findings may be explained by a proline-induced conformational alteration that buries the residues within the protein. Independent support for the latter idea comes from spin labeling experiments that revealed Na$^+$- and proline-dependent changes in the mobility of the nitroxide side chain attached to positions 45 in TM II and 37 in the preceding loop (34). Furthermore, the proline protective effect may partially be attributable to a reduced conformational flexibility of the Na$^+$-proline-PutP complex and a tighter packing of the protein. The latter explanation is in general supported by FTIR and H/D exchange experiments with the Na$^+$/melibiose transporter MelB of E. coli and the Na$^+$/galactose transporter vSGLT of Vibrio para-hemolyticus, which revealed a reduced accessibility of water to the protein backbone and an increase of the compactness of transporter structure upon substrate binding (36, 37). In addition, it can be envisaged that the ternary complex undergoes a conformational alteration that switches PutP from an inward facing into an outward facing conformation thereby allowing transport of Na$^+$ and proline through the membrane. Although PutP can function in both directions (10), the latter idea remains highly speculative. Experimental data indicating an outward oriented aqueous cavity are not available yet. Furthermore, while proline protection is observed at a variety of positions in TM II and other parts of the protein, a stimulatory proline effect has not been found yet (this study).2 Because Na$^+$-coupled proline uptake is not detected in the absence of an electrical potential,3 inclusion of the effect of a membrane potential in the studies might lead to new insights into PutP function.

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2 T. Pirch and H. Jung, unpublished data.
3 A. Hackmann, M. Nietschek, and H. Jung, unpublished data.
In conclusion, the experimental data presented in this study demonstrate that TM II is of particular importance for PutP function. It is proposed that predominantly hydrophilic residues in the cytoplasmic half of TM II participate in the formation of an aqueous cavity in the membrane that allows Na\textsuperscript{+} and/or proline binding to residues located in the middle of the membrane (e.g., Asp-55 and Ser-57 of TM II). In addition, the data indicate that TM II participates in Na\textsuperscript{+}- and proline-induced conformational alterations.

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