Role of Ser-340 and Thr-341 in Transmembrane Domain IX of the Na\(^+\)/Proline Transporter PutP of *Escherichia coli* in Ligand Binding and Transport* **

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The Na\(^+\)/solute symporter family comprises more than 400 members of pro- and eukaryotic origin. Using the Na\(^+\)/proline transporter PutP of *Escherichia coli* as a model, the role of two conserved residues, Ser-340 and Thr-341, is investigated to obtain insights into the mechanism of transport catalyzed by members of this family. Substitution of these amino acids alters the transport kinetics of cells and proteoliposomes containing the PutP variants significantly. In particular, the apparent affinities for Na\(^+\) and Li\(^+\) are reduced by 2 orders of magnitude or more. Also proline binding is affected, albeit to a lesser extent than ion binding. Thereby, the presence of a hydroxyl group at position 341 is essential for high affinity ligand binding. Furthermore, Cys placed at position 340 or 341 reacts with sulphydryl reagents of different polarity, indicating accessibility from the water phase. In addition, Cys cross-linking suggests proximity of the residues to other amino acids previously shown to be crucial for ligand binding. For these reasons it is suggested that Ser-340 and Thr-341 are located in a ligand translocation pathway. Furthermore, it is proposed that the side chain of Thr-341 directly participates in Na\(^+\) binding.

Members of the Na\(^+\)/solute symporter (SSS) family (TC 2A.21, SLC5) are found in Archaea, bacteria, and eukaryotes (1–4). They have the capability to couple electrochemical Na\(^+\) gradients with the transport of solutes like glucose, nucleosides, proline, pantothenate, or iodide. Some transporters of the SSS family are implicated in human disease (e.g. the Na\(^+\)/glucose transporter SGLT1) or play an important role in medical therapy (e.g. the human Na\(^+\)/iodide symporter NIS) (6, 7). Furthermore, bacterial transporters like the Na\(^+\)/proline transporter PutP of *Staphylococcus aureus* contribute to virulence (8).

A crystal structure is not available yet for any member of the SSS family. Beside intensive trials to crystallize SSS proteins, it is obvious that information on structure-function relationships is required in addition to structure to understand the mechanism of respective transport processes at the molecular level. For this purpose we utilize PutP of *Escherichia coli* as a model since it can easily be genetically manipulated and produced in milligram amounts (9, 10).

PutP transports Na\(^+\) and proline with a 1:1 stoichiometry following an ordered binding mechanism at low proline concentrations, whereas at high concentrations binding can be unordered (11, 12). Current experimental data indicate that the transporter is composed of 13 transmembrane domains (TMs), a motif suggested being common to all SSS proteins (10, 13–16). Furthermore, TM II of PutP was recently proposed to form part of the substrate translocation pathway. The TM contains residues Asp-55, Ser-57, and Gly-58, implicated in Na\(^+\) and/or proline binding (17–19). In addition, it participates in the formation of a water-filled cleft that is open to the cytoplasm in the absence of ligands and closed by proline binding in the presence of Na\(^+\) (20).

Inspired by the three-dimensional structure of the leucine transporter LeuT\(_{\text{Ac}}\) (Na\(^+\)/neurotransmitter symporter (NSS) family), which revealed an involvement of two adjacent polar residues (Thr-354, Ser-355) in Na\(^+\) binding (21), PutP was screened for a similar arrangement. Here, first evidence is presented indicating that TM IX of PutP is located close to TM II and contains residues Ser-340, Thr-341 that are particularly important for high affinity Na\(^+\) and proline binding. It is suggested that Ser-340 and Thr-341 are located in a ligand translocation pathway; thereby, Thr-341 probably directly participates in Na\(^+\) binding.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *E. coli* JM109 (endA1 recA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) (F’tra363pro AB’ lacI Q ΔM15) (22) and *E. coli* DH5α (F’Δ80d lacZ ΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsd R17 (rK-,mK+) phoA supE44 λ-thi-1 gyrA96 relA1) were used as carriers for the plasmids generated in this study. *E. coli* WM170 (F’ trp lacZ

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\(^\text{5}\) The abbreviations used are: SSS family, Na\(^+\)/solute symporter family (TC 2A.21); BMH, 1,6-bis-(maleimido) hexane; CuPh, copper (1,10-phenanthroline); Lm, lithium motive force; NEM, N-ethyl maleimide; Ni\(^{2+}\)-NTA, Ni\(^{2+}\)-nitritotriacetic acid; p-PDM, N,N’-p-phenylenedimaleimide; PutP(LCys), engineered transporter devoid of all native cysteine residues; smf, sodium motive force; TM, putative transmembrane domain; NIS, Na\(^+\)/iodide symporter; Mes, 2-(N-morpholino)ethanesulfonic acid.
Role of Ser-340 and Thr-341 of PutP

rpsL thi Δ(putPA)101 proP219 (23), harboring given plasmids, was used for overexpression of the putP gene and transport assays. The following plasmids, derivatives of pT7-5 (24) containing the lac promoter/operator for expression of the putP gene, were used for all gene manipulations; pT7-5/putP and pT7-5/putPΔCys, each of which harboring a cassette version of the putP gene encoding PutP-wild-type and an engineered transporter devoid of all five native Cys residues [PutP(ΔCys)], respectively, and a C-terminal-attached amino acid sequence resembling the FLAG epitope and a His6 tag (9). Vector pTrc99a (25) was used for overexpression.

Site-directed Mutagenesis—Desired nucleotide substitutions in putP were generated by PCR with Taq-DNA polymerase using plasmid pT7-5/putP as a template and synthetic mutagenic oligonucleotides. Resulting PCR fragments were digested with PstI and ApIII and ligated with similarly treated plasmid pT7-5/putP or pT7-5/putPΔCys additionally incubated with alkaline phosphatase. The resulting plasmid DNA was verified by sequencing using an ABI 377 HT device. Double-Cys mutants were generated by restriction fragment replacement by sequencing using an ABI 377 HT device. Double-Cys mutants were generated by restriction fragment replacement with PstI and Spel. For overexpression, the mutated putP gene was cut out with restriction endonucleases Ncol and HindIII and ligated to similarly treated vector pTrc99a additionally incubated with alkaline phosphatase.

Proline Transport in Intact Cells—Active transport was measured in E. coli WG170 (PutPΔ-Δ) harboring derivatives of plasmids pT7-5/putP or putPΔCys encoding PutP with given amino acid replacements. The cells were grown aerobi-
cally in Luria-Bertani (LB) medium (26) containing 100 μg/ml ampicillin at 37 °C. Overnight cultures were diluted 25-fold and were allowed to grow up to an optical density at 420 nm (A420) of 1.0 followed by induction with 0.5 mM isopropyl β-D-1-thio-d-galactopyranoside for 2 h. Cells were harvested by centrifugation at 13,200 × g for 10 min and washed up to 6 times with 100 mM Tris/Mes buffer, pH 6.0, at 4 °C to reduce the Na + contamination below 5 μM. For transport assays cells were resuspended in the same buffer and adjusted to a total protein concentration of 0.35 mg/ml. Transport of 10 μM L-[1-14C]proline (26 Ci/mol) (if not otherwise indicated) was assayed under standard test conditions in the presence of 20 mM D-lactate (Na + salt) and 50 mM NaCl. Transport assays were terminated at various time points (0, 0.17, 0.5, 1, 2, 5, 10, 30 min) using the rapid filtration method as described (19). Initial rates of transport were calculated from the initial linear portion of the time course, and steady-state levels of proline accumulation were taken from time points after leveling off of the uptake curve. S.D. were determined from at least three independent experiments.

Immunological Analysis—Relative amounts of PutP with given amino acid replacements in membranes of E. coli WG170 were estimated by Western blot analysis with horseradish per-oxidase-linked mouse anti-FLAG IgG directed against the FLAG epitope at the C terminus of each PutP variant as described before (18).

Cys Accessibility Analyses—The experiments were performed with right-side-out membrane vesicles prepared from E. coli WG170 transformed with pTrc99a/putP(ΔCys) harboring given mutations following the protocol of Kaback (27). Before Cys labeling, the right-side-out vesicle suspension (10 mg total membrane protein/ml) was reduced with 1 mM dithi-othreitol or 1 mM Tris(2-carboxyethyl)phosphine hydrochloride. The vesicles were washed and resuspended in 100 mM Tris/Mes buffer, pH 6.0. Two hundred-μl aliquots of the suspension (10 mg of total membrane protein/ml) were incubated with 500 μM [14C]NEM (8 μCi/μmol) at 25 °C for 10 min [PutP(ΔCys)-T341C] or 15 min [PutP(ΔCys)-S340C]. The reaction was stopped by the addition of 2 μl dithiothreitol. When indicated, 500 μM methanethiolosulfonate ethylammonium (MTSEA), methanethiolosulfonate ethylsulfonate (MTSES), or methyl methanethiosulfonate (MMTS) were added before [14C]NEM. Similarly, the effect of PutP ligands on Cys accessibility was analyzed by incubation with 50 mM Na +, Li +, and/or 10 mM proline Na +/proline before [14C]NEM labeling. After labeling, PutP was solubilized by the addition of 1.5% β-dode-
cycl maltoside under stirring at 4 °C for 30 min. Then PutP was purified via Ni2+-NTA affinity chromatography as described before (17). After protein determination, equal amounts of protein were subjected to a 10% SDS-PAGE. Gels were stained with Coomassie Blue and scanned for estimation of relative amounts of protein. Radioactivity was detected with a PhosphorImager SI of GE Healthcare. The software ImageQuant was used for quantitative analysis of the images.

In Situ Site-directed Cross-linking—For cross-linking experiments, 0.4 liters of E. coli WG170 transformed with pT7-5/putP(ΔCys) encoding PutP with single or double Cys replacement and an introduced double thrombin cleaving site were grown and harvested as described above. Inverted mem-
brane vesicles were prepared as described (9) with 1 mM Tris(2-carboxyethyl)phosphine hydrochloride as the reducing compound.

Before cross-linking, the membrane vesicles were washed twice and resuspended in 50 mM KP, buffer, pH 7.0. One hundred μg total membrane protein (1 mg total membrane protein/ml) were incubated with 0.5 mM Cu(1,10-phenanthro-
line)3 (CuPh) or with 2 mM concentrations of one of the homobifunctional cross-linker 1,6-bis-(maleimido) hexane (BMH) or N,N'-p-phenylenedimaleimide (p-PDM). Stock solutions of 30 mM CuPh (in ethanol) and 200 mM concentrations of the maleimide reagents (in Me2SO) were prepared. Reactions were performed at 4 °C for 60 (CuPh) or 120 min (maleimide reagents) and at 25 °C for 30 or 60 min to estimate the depend-
ence of cross-linking efficiency on thermal backbone motions. After termination with 10 mM EDTA (CuPh) or 10 mM dithio-
 thermostat (BMH and p-PDM), the inverted membrane vesicles were subjected to ultracentrifugation at 250,000 × g for 30 min at 4 °C. The membranes were resuspended in thrombin cleavage buffer (Invertogen) containing 1% β-dodecyl-maltoside and, after a 2-fold dilution in thrombin cleavage buffer, digested overnight at 4 °C with 1.3 units of thrombin protease. The digested proteins were subjected to non-reducing SDS-PAGE and immunoblot analysis as described above.

Purification and Reconstitution of PutP—PutP was purified by Ni2+-NTA affinity chromatography and reconstituted into proteoliposomes prepared from E. coli polar lipid extracts (Avanti Polar Lipids, Inc., Alabaster, Alabama) at a lipid to protein ratio of 100 to 1 (w/w) as described before (9).
Transport Assay with Proteoliposomes—L-[14C]Proline uptake was measured with proteoliposomes containing PutP(FH) (~2 mg of protein/ml) preloaded with 100 mM KP, pH 7.5, 2 mM β-mercaptoethanol, 5 mM MgSO₄. The proteoliposomes were extruded through a 400 nm filter before use. Transport was started by a 400-fold dilution of aliquots of the proteoliposome suspension into the desired buffer. The following buffers were used: Na⁺-free 100 mM Tris/Mes, pH 7.5, 2 mM β-mercaptoethanol, 5 mM MgSO₄ containing 50 mM NaCl (creation of Δμ₉Na⁺) or 50 mM LiCl (creation of ΔμH⁺) or no further additions (creation of Δψ); Na⁺-free 100 mM Tris, Mes, pH 6.0, 2 mM β-mercaptoethanol, 5 mM MgSO₄ (creation of ΔμH⁺) or 100 mM KP, pH 7.5, 2 mM β-mercaptoethanol, 5 mM MgSO₄ (facilitated diffusion, control). All buffers contained 10 μM L-[14C]proline (26 Ci/mol) and 0.2 μM valinomycin. For the measurement of proline counterflow (cfl), proteoliposomes preloaded with 10 mM L-proline were diluted 400-fold into 100 mM KP, pH 7.5, 2 mM β-mercaptoethanol containing L-[14C]proline at a final concentration of 25 μM (15 Ci/mol). Transport assays were terminated at various time points (0, 0.17, 0.5, 1, 2, 5, 10 min) by quenching of the reaction with 3 ml of ice-cold 100 mM KP, pH 6.6, 100 mM LiCl and immediate filtration using Millipore filters type GSTF 02500, 0.2-μm pore size as described (9). Initial rates of transport were determined from the initial linear part of the transport curve. S.D. were determined from at least three independent experiments.

Determination of Na⁺—Na⁺ concentrations in buffers used for transport assays and Cys accessibility analyses were determined with a Varian AA240 atomic absorption spectrometer.

Protein Determination—Determination of protein was performed according to a modified Lowry method (28) for total membrane protein, according to Bradford (29) for detergent-solubilized protein, and by the Amido Black method (30) for protein in proteoliposomes.

RESULTS

Conserved Amino Acids of TM IX—Searching for amino acid residues of potential structural and/or functional significance (e.g. residues involved in ligand binding), a multiple sequence alignment of members of the SSS family was performed. The analysis revealed that Ser-340 and Thr-341 of E. coli PutP are conserved within prokaryotic and eukaryotic family members irrespective of their substrate specificity (e.g. transporters for proline, pantothenate, sugars, or iodide). In fact, the transporters analyzed contained Ser at the position corresponding to Ser-340 and Ser or Thr at the position corresponding to Thr-341 of E. coli PutP (supplemental Fig. 1). The high degree of conservation suggests that both residues fulfill similar functions in all related transporters. In addition, a similar amino acid arrangement (Thr-354, Ser-355) was found to be involved in Na⁺ binding in the non-homologous leucine transporter LeuT_Aa (21). Therefore, we set out to elucidate the role of Ser-340 and Thr-341 in PutP of E. coli.

Effect of the Substitution of Ser-340 and Thr-341 on Proline Uptake and putP Expression In E. coli WG170—Ser-340 and Thr-341 were individually replaced by Ala, Cys, Thr (Ser-340) or Cys, Ser, Val (Thr-341). In addition, double mutants S340A/T341C and S340A/T341V were generated.

In a first step, time courses of L-proline uptake into E. coli WG170 were recorded under standard test conditions (70 mM Na⁺, 10 μM L-[14C]proline) (Fig. 1A). Based on the observed activities, the mutants were divided into three groups: I) mutants with moderately reduced initial rates (S340A 20%, S340T 40%, T341S 60% of the wild-type type) but steady-state values of L-proline accumulation close to wild-type (80–100% of the wild-type value); II) mutants with highly reduced initial rates (S340C 9%, T341C 3%, T341V 3.5% of the wild-type rate) and reduced steady-state values (S340C 60%, T341C 35%, T341V 20% of the wild-type type); III) mutants with only marginal levels of activity (<1% of the wild type) (S340A/T341C, S340A/T341V). Western blot analysis of membranes prepared from cell suspensions used for the transport assays indicated that the observed reduced proline uptake rates were not because of reduced amounts of transporter molecules in the membrane (Fig. 1B). To test whether the inhibitory effects of the Cys substitution at position 340 or 341 were due to intra- or intermolecular disulfide bridge formation (e.g. with one of the five native Cys residues), the Cys substitutions were introduced into PutP(ΔCys). Recording of time courses of transport yielded
similar inhibitory effects as observed in the wild-type background (data not shown). The results suggested that the altered amino acid side chains per se and not subsequent reactions caused the detected transport defects.

Taken together the results indicated that the nature of the amino acid side chains at positions 340 and 341 of PutP of E. coli was crucial for the proline uptake rate. Highest rates were observed when hydroxyl groups were retained at both positions (e.g. S340T, T341S). Overall, the steady-state value of proline accumulation was much less affected than the uptake rate.

Influence of the Substitutions on the Ion Selectivity of PutP—To test for possible changes of the ion selectivity, the energetic requirements of the transport process were investigated under more defined conditions using purified and reconstituted transport protein. It should be noted that the protocol used for reconstitution yields proteoliposomes containing PutP in an inside-out orientation (9).

First, determination of the initial rates of Na\(^+\)-coupled (sodium motive force (smf)-driven) transport in proteoliposomes confirmed in principal the defects described above for intact cells, although individual values varied to some extent (Fig. 2). Mutants of group I (S340A, S340T, T341S) showed again the highest activities. However, differing from intact cells, neither the S340A nor the T341S substitution caused significant inhibition of transport. This difference may be attributed to the previously described functional asymmetry of the transporter (9, 31). The initial rates of the mutants T341C and T341V of group II were highly reduced (about 2.5% of the wild type) as in intact cells. PutP-S340C (9% residual activity in intact cells) showed only marginal activity (<1% of the wild type) in proteoliposomes (Fig. 2). Reducing conditions were maintained during all steps of the preparation to prevent a potential participation of Cys-340 in formation of a disulfide bridge with a native Cys. Nevertheless, oxidation of Cys-340 during PutP preparation could have added to the deleterious effect of the substitution in proteoliposomes. Finally, activities of mutants of group III (S340A/T341C, S340/T341V) were reduced to marginal values (<2% of the wild type) similar as observed with intact cells (not shown).

Secondly, analysis of various energetic conditions revealed that in all cases transport was strictly dependent on an inwardly directed electrochemical Na\(^+\) (smf) or Li\(^+\) gradient (lithium motive force (lmf)) (Fig. 2). Δψ-Driven transport of the mutants was only marginal and most likely due to Na\(^+\) impurities of buffer components (leading to Na\(^+\) concentrations in the assay of about 5–10 μM). Establishment of a H\(^+\) gradient in addition to Δψ (proton motive force-driven transport) did not further stimulate transport. These measurements indicated that the replacements at positions 340 or 341 did not cause a dramatic change in ion selectivity (e.g. from Na\(^+\)- to H\(^+\)-coupled transport). However, comparison of smf- and lmf-coupled transport of PutP-T341S (smf >90% and lmf 30% of wild-type activity) hints at subtle alterations of the ion preferences of the transporter (Fig. 2).

Besides ion-coupled transport, the impact of the substitutions on proline counterflow was analyzed. This transport mode neither required a membrane potential nor an ion gradient. Instead, accumulation of externally applied L-[\(^1\)\(^4\)C]proline in proteoliposomes was driven by downhill movement of unlabeled l-proline from preloaded proteoliposomes. Substitution of Ser-340 (by Cys or Thr) or Thr-341 (by Cys or Val) led to significantly reduced counterflow activities (remaining activities below 13% of the wild-type value) (Fig. 2). Exceptions were PutP-S340A and PutP-T341S, which catalyzed counterflow almost like the wild type. The latter results confirmed the functional significance of Ser-340 and Thr-341 and suggested that these residues are important for proline binding or transport.
Kinetic Analyses—More detailed kinetic analyses revealed substantial defects in proline AND Na+ binding. Thereby, binding parameters of group II mutants were more severely affected than the ones of group I mutants. The maximum rate of proline uptake was in general less dramatically affected (reduced to maximum 50% of wild-type V_{max}) than binding parameters (Table 1; Fig. 3). Transport by group III mutants (S340A/T341V; S340A/T341C) was not significantly stimulated by Na+ although PutP-T341C and -T341V was no longer significantly affected than the ones of group I mutants. The maximum rate of transport by group III mutants PutP-T341C or -T341V was finally stimulated by Na+ (and in case of T341V, also by Li+). The same elevation of the proline concentration increased the apparent Na+ and Li+ affinities of PutP-wild type and -S340C about 10-fold (other mutants not tested). These data suggested cooperative interactions between ion and proline binding sites. Importantly, ion binding remained impaired also in the presence of saturating proline concentrations, suggesting that the observed defects in ion binding were not secondary to proline binding defects.

Because the ion dependence of PutP-T341C and -T341V in intact cells remained unclear, the apparent Na+ affinities were analyzed in the more defined proteoliposome system. Here, proline uptake into proteoliposomes containing either purified PutP-T341C or -T341V was finally stimulated by Na+ concentrations of 50 mM or higher, and the K_{0.5(Na+)} was estimated to exceed 250 mM. A precise determination of K_{0.5(Na+)} was not possible since NaCl concentrations in the molar range inhibited PutP-dependent transport in proteoliposomes (32). In contrast to the Cys and Val substitution, PutP with Ser in place of Thr-341 showed transport kinetics close to wild type. These results confirmed the particular significance of a hydroxyl group at position 341 for high affinity Na+ binding.

Contrary to position 341, evidence for a specific function the hydroxyl group at position 340 was not obtained with the proteoliposome system. Rather, size and polarity of the residue mattered for transport as already observed for the uptake kinetics of intact cells. Therefore, the kinetic data obtained with proteoliposomes confirmed the results with intact cells (compare above).

Taken together, the kinetic analyses indicated that Ser-340 and Thr-341 are essential for the high affinities of PutP for coupling ion and substrate. Furthermore, the data suggested a particular crucial role of the hydroxyl group at position 341 in binding of Na+ or Li+.

Accessibility to Sulfhydryl Reagents—To determine whether positions 340 and 341 are in contact with the water phase, the accessibility of Cys placed at either one of these positions in

**TABLE 1**
Proline uptake kinetics of PutP bearing replacements of given amino acid residues

| PutP       | K_{m(pro)} | V_{max} | K_{0.5(Na+)} | K_{0.5(Li+)} |
|------------|------------|---------|--------------|--------------|
| Wild type  | 2.1 ± 0.2  | 44.5 ± 3.9 | 0.038 ± 0.007 (0.2 ± 0.09) | 0.295 ± 0.057 |
| S340A      | 31.3 ± 3.7 | 39.5 ± 7.1 | 5.3 ± 1.0 (5.2 ± 0.49) | 32.3 ± 7.9   |
| S340C      | 46.3 ± 7.8 | 53.7 ± 2.6 | 40.1 ± 5.7  | 32.1 ± 2.8   |
| S340T      | 0.7 ± 0.2  | 19.7 ± 5.8 | 3.3 ± 0.3 (11.9 ± 1.7) | >100         |
| T341C      | 60.8 ± 7.8 | 21.6 ± 0.8 | NS (>250)   | NS           |
| T341S      | 3.0 ± 1.0  | 40.2 ± 11.2 | 0.051 ± 0.001 | 2.0 ± 0.29   |
| T341V      | 117.1 ± 4.6| 27.5 ± 2.0 | NS (>250)   | NS           |

*Values in parentheses were obtained with proteoliposomes containing the purified PutP variants.

*NS, no stimulation of proline uptake into intact cells by increasing concentrations of Na+ or Li+.
Role of Ser-340 and Thr-341 of PutP

PutP(ΔCys) to sulfhydryl reagents of various sizes and polarities was analyzed. Labeling experiments were performed with right-side-out membrane vesicles containing the corresponding PutP variant.

In initial studies it was shown that PutP(ΔCys)-S340C and PutP(ΔCys)-T341C readily react with [14C]NEM. Reaction of Cys-340 resulted in about 60% labeling of the protein within 10 min. Reaction of the maleimide with Cys-341 was faster, yielding about 100% protein labeling within the same time. These data suggested a somewhat more buried position of Cys-340. In contrast, the negative control PutP(ΔCys) did not react with [14C]NEM under the conditions of the experiment even after extended periods of time (Fig. 4, A and B). Because reaction of the maleimide required deprotonation of the sulfhydryl group, the results indicated a polar environment at positions 340 and 341. The latter conclusion was strongly supported by the observation that charged reagents methanethiosulfonate ethylammonium (MTSEA) and methanethiosulfonate ethylsulfonate (MTSES) as well as neutral methyl methanethiosulfonate (MMTS) efficiently inhibited [14C]NEM labeling of Cys at both positions (Fig. 4B). Taken together, the data suggested that Ser-340 and Thr-341 are located in a water-filled cavity in the membrane that is at least transiently open to the periplasm. Finally, the influence of PutP ligands on Cys modification by [14C]NEM was investigated. Repeated experiments did not reveal a significant effect of Na⁺, Li⁺, proline, or a combination thereof on the reaction (data not shown).

Proximity between TM II and TM IX—Because the dramatic changes of the apparent Na⁺ and proline affinities suggested an involvement of TM IX in ligand binding, a possible proximity between residues of TM IX and the functionally important TM II was tested by in situ Cys cross-linking. For this purpose the following pairs of Cys residues were individually introduced in TMs II and IX of PutP(ΔCys): S41C/C344, D55C/S340C and D55C/T341C, S57C/S340C and S57C/T341C, M62C/A327C. In addition, thrombin cleavage sites were introduced into periplasmic loop 7 to facilitate determination of cross-linking products (compare the supplemental material for details on the establishment of protease cleavage sites and activities of all PutP variants used for cross-linking).

Cross-linking of Cys in TMs II and IX was performed with BMH (3.47–15.64 Å), p-PDM (9.2–12.29 Å), and CuPh (zero-length) (33) (Fig. 5). Thrombin digestion of PutP variants in the absence of cross-linker resulted in C-terminal fragments of the expected apparent size of 30 kDa, whereas cross-linked and cleaved PutP migrated at the size of intact PutP (44 kDa) (Fig. 5).

All three cross-linkers (CuPh, BMH, p-PDM) caused intramolecular cross-linking between Cys in TMs II and IX, although the efficiency of cross-linking varied with the cross-linker and position analyzed (Fig. 5). Cys residues at positions 57 and 341 were most efficiently cross-linked (>90% intramolecular cross-linking) not only with the zero-length cross-linker CuPh but also with BMH and p-PDM, indicating a temporal close proximity of both positions. The fact that the two bifunctional maleimides were also well accommodated hints at a structurally flexible part of the protein. Positions 57 and 340 were efficiently cross-linked (>90%) by BMH and p-PDM and to a significant lesser extent by CuPh (15–50% depending on temperature), suggesting a predominant distance between these positions in the range of 9.2 and 12.3 Å. Also, the Cys pairs S41C/C344, D55C/S340C, and D55C/T341C were cross-linked by all three cross-linkers albeit the cross-linking efficiencies were lower (20–40% intramolecular cross-linking) than that of S57C/T341C. Finally, M62C/A327C showed significant cross-linking by CuPh (30% intramolecular cross-linking) only at 25 °C, whereas reaction at 4 °C was ineffective, proposing that a conformational alteration preceded disulfide bridge formation. In contrast to CuPh, M62C/A327C could be cross-linked with BMH or p-PDM (40 to 45% intramolecular cross-linking) already at 4 °C, suggesting that under this condition the distance between both positions lied in the range of 9.2 and 12.3 Å (distance spanned by rigid p-PDM).

To test for possible ligand-induced changes of distances between TMs II and IX, the effect of Na⁺ and proline on Cys cross-linking was analyzed (supplemental Fig. 4). The results did not reveal any significant effect of the ligands on cross-linking.
DISCUSSION

In a search for residues involved in ion and/or substrate binding in the SSS family member PutP, the role of two polar amino acids, Ser-340 and Thr-341, in Na⁺/proline transport is analyzed. Both amino acids are located in the middle of TM IX and are conserved in pro- and eukaryotic members of the family independent of their substrate specificity (e.g. proline, pantotenate, glucose, iodide). This investigation demonstrates that the two residues are of particular functional significance. This is documented by the fact that already relative conservative replacements (S340A, S340C, or S340T; T341C, T341S, or T341V) cause a significant inhibition of proline uptake into intact cells as well as proteoliposomes containing the reconstituted PutP variants. In addition, simultaneous substitution of both residues (group III mutants) inhibits transport almost completely. Kinetic analyses reveal that inhibition of transport of group I (S340A, S340T, T341S) and group II mutants (S340C, T341C, T341V) are primarily due to drastic changes of the apparent affinities of PutP for its ligands. By contrast, maximum rates of proline uptake are only marginally affected. The results suggest that events associated with ligand binding rather than with subsequent translocation steps are hampered by the alterations.

Most severe defects are observed for binding of the coupling ion. Substitution of Ser-340 decreases the apparent Na⁺ and Li⁺ affinities of PutP by 2–3 orders of magnitude (Fig. 3). These dramatic changes ascribe Ser-340 a specific role in Na⁺ (and Li⁺) binding. However, the size of the side chain at this position had a stronger impact on activity than the presence of a hydroxyl group. Therefore, it is proposed that the residue is located in the direct surroundings of the ion binding site of PutP. Alternatively, it may participate in ion binding via its main chain carbonyl group.

Substitution of the neighboring Thr-341 results in a somewhat different phenotype, indicating a specific role the hydroxyl group of the side chain in ion binding. Although the most conservative replacement with Ser has only little effect on ion binding, Na⁺ does not significantly stimulate proline uptake into intact cells containing PutP-T341C or -T341V. Using the more defined proteoliposome system, Na⁺ stimulation of transport containing either one of these mutants is observed only at elevated ion concentrations, suggesting a highly reduced (minimum 2 orders of magnitude) ion affinity. Why is this stimulation not seen with intact cells? Clearly, it is easier to establish defined energetic conditions with proteoliposomes than with intact cells in which not only PutP but a number of other membrane proteins (e.g. Na⁺/H⁺ antiporter) interfere with electrochemical ion gradients. Furthermore, high concentrations of Na⁺ have an inhibitory effect on various proteins and cellular processes (34). In fact, proline uptake into PutP wild-type cells is inhibited already at Na⁺ concentrations of 250 mM and higher. This is exactly the concentration range by which proline uptake into PutP-T341C or -T341V proteoliposomes becomes stimulated. Therefore, because of the complex scenario in intact cells, stimulatory and inhibitory effects of Na⁺ on proline transport may partially compensate each other. In any event, the observed distinctive effects of the Thr-341 substitutions on the apparent ion affinity implicate the residue in binding of the coupling ion.

Besides the effects of the amino acid replacements on ion binding, proline binding and transport is also affected. The sub-

Taken together, these results demonstrated an at least temporal proximity of TMs II and IX. The distances between selected residues of these domains were not well defined probably due to the structural flexibility of this protein region.
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substitutions inhibit also counterflow activity, which does not require an electrochemical ion gradient. In addition, the proline affinity is affected, albeit to a lesser extent than the ion affinities (Fig. 3). These results provide additional evidence for the particular functional significance of Ser-340 and Thr-341 and indicate that both residues are important for ion and proline binding. Similar observations have already been made upon replacement of other residues (Asp-55, Ser-57, Gly-58 of TM II) supposed to be involved in ligand binding (17–19). Obviously, there is an interdependence of ion and proline binding as shown by this and other investigations (9, 12, 31, 35). A simple explanation for the observed phenomena is a close proximity or even an overlapping of ion and proline binding sites. Such an arrangement would ensure tight coupling of ion and substrate transport. But what would be the primary function of Ser-340 and Thr-341? The following results argue for a primary role of the residues in ion binding: (i) the amino acid replacements have a stronger impact on ion than on proline binding; ii) apparent ion affinities remain reduced even in presence of proline, since amino acids in NIS (Ser-353 and Thr-354) are demonstrated to be particularly crucial for Na\(^{+}\) binding and/or translocation (38).

FIGURE 6. Model showing the participation amino acids of TM II and IX in ligand binding and transport. The model is based on the current analysis as well as on previous investigations (17–19). It represents a working hypothesis according to which TMs II and IX participate in the formation of a ligand translocation pathway; Asp-55, Ser-57, and Thr-341 directly participate in Na\(^{+}\) or proline binding. Further structural information is necessary to prove or disprove the model.

If Ser-340 and Thr-341 participate in ligand binding, are the residues located close to other amino acids implicated in Na\(^{+}\) binding (Asp-55) and proline binding (Ser-57, Gly-58)? Here, it is shown that pairs of Cys residues placed at these positions 55/340, 55/341, 57/340, and 57/341 form disulfide bridges. Albeit the cross-linking efficiency varies between the pairs, the results support the idea of a functional interaction of TMs II and IX. However, the necessary amino acid replacements in (or close to) the proposed ligand binding sites of PutP impair transport activity almost completely. Therefore, pairs of Cys residues are also introduced at either end of TMs II and IX. The resulting functional PutP variants are also cross-linked, indicating an at least temporal proximity of TMs II and IX. Furthermore, the observation that cross-linkers of varying lengths cross-link positions in both TMs suggests a conformational flexibility of these parts of the transporter. The latter idea is further supported by the temperature dependence of cross-linking of M62C/A327C. The proposed proximity between TMs II and IX is in general agreement with earlier analyses reporting a clustering of mutations causing an altered substrate specificity within the N- and C-terminal domains of PutP (36).

Despite comprehensive trials, a significant effect of Na\(^{+}\) and proline binding on cross-linking efficiencies (e.g. via altering intramolecular distances) was not observed, although a participation of TM II in ligand-induced conformational alterations was previously reported (20). Also, in view of the in-part dramatic effects of the Cys substitutions on apparent ligand affinities, it is assumed that the PutP variants are even in the presence of ligands still dynamic enough to allow efficient cross-linking of Cys pairs within the given labeling period. In addition, Cys cross-linking is known to underestimate distances (37), thereby potentially obscuring ligand-induced changes.

Can the model presented in Fig. 6 be generalized for all members of the SSS family? The conclusions drawn here are in good agreement with previous analyses of NIS, which like PutP is a member of the SSS family. In fact, the corresponding amino acids in NIS (Ser-353 and Thr-354) are demonstrated to be particularly crucial for Na\(^{+}\) binding and/or translocation (38). The general importance of Asp-55 and Ser-57 is less clear as these residues are not as conserved within the SSS family as Ser-340 and Thr-341. So, it is possible that Na\(^{+}\) binding to the
N-terminal domains differs significantly between members of the SSS family. These differences may be attributed to the different Na\textsuperscript{+}:substrate stoichiometries (e.g. 1:1 in PutP; 2:1 in NIS, SGLT1). In addition, because ion and substrate binding sites are supposed to closely interact with each other, the properties of the substrates (e.g. charged proline versus polar sugar) may also have influenced the precise mechanism of Na\textsuperscript{+} binding. In human SGLT1, the N-terminal half of the protein is thought to contain two Na\textsuperscript{+} binding sites, whereas the C-terminal part is made responsible for sugar binding and translocation (39–42). Thereby, sugar binding is supposed to occur in a hydrophilic cavity formed by TMs 10–13 (5, 7). Residues directly participating in Na\textsuperscript{+} binding are not yet identified.

Finally, it must be considered that the complete sets of residues involved in ion and/or substrate binding are neither known for PutP, SGLT1, or other members of the SSS family. Furthermore, although the results presented here support an involvement of Ser-340 and Thr-341 of PutP in ligand binding, it must be stated that the observed binding defects can also be explained by indirect effects, e.g. distortion of the structural arrangement of neighboring residues participating in the formation of a binding cavity. Along this line, Ser-340 and Thr-341 could simply be required for stabilizing intramolecular contacts between N- and C-terminal domains, which in turn may be necessary for high affinity ligand binding. Clearly, more information on transporter structure is required to fully understand the phenomena described here.

In summary, the results demonstrate that Ser-340 and Thr-341 of PutP are particularly important for high affinity Na\textsuperscript{+} and proline binding. Being placed in the middle of TM IX, the residues are accessible from the water phase and located close to residues of TM II, which are also implicated in ligand binding. For these reasons it is suggested that Ser-340 and Thr-341 are located in a ligand translocation pathway. Furthermore, it is proposed that Thr-341 directly participates in Na\textsuperscript{+} binding.

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