Sites Important for Na\(^{+}\) and Substrate Binding in the Na\(^{+}\)/Proline Transporter of *Escherichia coli*, a Member of the Na\(^{+}\)/Solute Symporter Family*

Received for publication, November 16, 2001, and in revised form, December 20, 2001

Published, JBC Papers in Press, December 26, 2001, DOI 10.1074/jbc.M111008200

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To elucidate the functional importance of transmembrane domain II in the Na\(^{+}\)/proline transporter (PutP) of *Escherichia coli* we analyzed the effect of replacing Ser-54 through Gly-58. Substitution of Asp-55 or Met-56 dramatically reduces the apparent affinity for Na\(^{+}\) and Li\(^{+}\) in a cation-dependent manner. Conversely, Cys in place of Gly-58 significantly reduces only the apparent proline affinity while substitution of Ser-57 results in a dramatic reduction of the apparent proline and cation affinities. Interestingly, upon increasing the proline concentration the apparent Na\(^{+}\) affinity of Ser-57 replacement mutants converges toward the wild-type value, indicating a close cooperativity between cation and substrate site(s). This notion is supported by the fact that Na\(^{+}\)-stimulated site-specific fluorescence labeling of a single Cys at position 57 is completely reversed by the addition of proline. Similar results are obtained upon labeling of a Cys at position 54 or 58. Taken together, these results indicate that Asp-55 and Met-56 are located at or close to the ion-binding site while Ser-54, Ser-57, and Gly-58 may be close to the proline translocation pathway. In addition, the data point at an involvement of the latter residues in ligand-induced conformational dynamics that are crucial for cation-coupled transport.

The Na\(^{+}\)/solute symporter family (SSF, 1 TC 2.A.21) comprises more than 80 similar proteins of pro- and eukaryotic origin (1–3). These integral membrane proteins utilize the Na\(^{+}\) electrochemical gradient to drive the coupled uphill transport of a variety of substrates (sugars, amino acids, vitamins, osmolytes, ions, myo-inositol, urea, and water). Among the eukaryotic members of the SSF the Na\(^{+}\)/glucose transporter (SGLT1), the Na\(^{+}\)/iodide transporter, and the Na\(^{+}\)/multivi-

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB431/D4. 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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\(^{1}\) The abbreviations used are: SSF, Na\(^{+}\)/solute symporter family (TC 2.A.21); PutP(Cys), engineered transporter devoid of all five native Cys residues; TM, putative transmembrane domain; Mes, 4-morpholinoethanesulfonic acid.

\(^{2}\) The initial findings on the role of Asp-55 and Ser-57 in Na\(^{+}\)-coupled proline uptake we analyzed the functional importance of amino acids in the vicinity of both residues, namely Ser-54, Met-56, and Gly-58. In addition, former work on Asp-55 and Ser-57 was extended. To this end, we systematically studied the effect of individual amino acid substitution on (i) proline uptake activity, (ii) ion selectivity, and (iii) the influence of...
ligand binding on the accessibility of Cys individually placed at positions 54 to 58. The results indicate that the region comprising Ser-54 to Gly-58 is particularly crucial for binding and translocation of the coupling ion (Na+ or Li+) and the substrate. Furthermore, it is shown that this part of the protein undergoes functional relevant conformational alterations that are essential for cation-coupled transport.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(\frac{1}{14}\text{C} \) Proline (241 µCi/µmol) was obtained from ICN. Mouse anti-FLAG M2-antibody was from Integra Biosciences. Sheep anti-\(\text{IgG} \)-horseradish peroxidase conjugate was from Amersham Bioscience, Inc. Restriction endonucleases, \(\text{Taq} \)-DNA polymerase, T4-DNA ligase, and alkaline phosphatase were purchased from New England Biolabs. Synthetic oligonucleotides were from Eurogentec and Invitrogen. E. coli DH5α competent cells were obtained from Invitrogen. Fluorescein 5-maleimide was from Molecular Probes. The SDS-PAGE low-range molecular mass standard was purchased from Bio-Rad. N-Dodecyl-\(\beta\)-D-maltoside was from Anatrace and Ni\(^{2+}\)-NTA spin columns were from Qiagen. All other chemicals were of analytical grade and obtained from commercial sources.

**Bacterial Strains and Plasmids**—E. coli DH5α (F \(\delta\delta\delta\delta\text{ lacZAM15 ΔlacZYA-argF169 deoR recA1 endA1 hsdR17(rK- mK-) phoA galK86 relA1 galK86 relA1 hsdS20(rK- mK-) F’traD36 proAB supE44 thyA45 recA1 lacI977Δ (lacZΔM15 proAB) rpsL20 mpl1408::Tn10}) was used as host for all cloning procedures. E. coli W3110 (F \(\text{F’} \text{ lacZM15 galK86 relA1 lacI977Δ (lacZΔM15 proAB) rpsL20 mpl1408::Tn10}) served as carrier for the plasmids described.

**Site-directed Mutagenesis**—Amino acid substitutions were created using a two-step polymerase chain reaction (PCR) protocol with plasmids \(\text{pT7-5/putP} \) or \(\text{pT7-5/putP/Cys} \) as templates. Mutagenic primers encoding the desired amino acid substitution, and suitable sense and antisense primers binding up- and downstream, respectively, of the site of substitution were used similarly as described (16, 18). PCR fragments were digested with restriction endonucleases ApaI and XbaI and ligated to similarly treated plasmids \(\text{pT7-5/putP} \) and \(\text{pT7-5/putP/Cys} \) incubated with alkaline phosphatase to avoid religation of the vector. For overexpression of PutP, the mutated putP genes were cut with NcoI and HindIII and ligated to similarly treated vector \(\text{pT7/putA} \) incubated with alkaline phosphatase. The resulting constructs were verified by sequencing double-stranded plasmid DNA using dyeoxydeoxyxynucleotide chain termination (23) after alkaline denaturation (24).

**Transport Assay**—Active transport was measured in E. coli W170 (PutP A+) harboring derivatives of plasmids \(\text{pT7-5/putP} \) or \(\text{pT7-5/putP/Cys} \) encoding PutP with given amino acid replacements. The cells were grown aerobically in Luria-Bertani medium (25) containing 100 µg/ml ampicillin at 37 °C. Overnight cultures were diluted 25-fold and were allowed to grow to an optical density at 420 nm (\(\text{OD}_{420} \)) of 1.0, followed by induction with 0.5 mM isopropyl-1-thio-\(\beta\)-D-galactopyranoside for 2 h. Cells were harvested by centrifugation at 13,200 \(\times g \) for 10 min and washed up to 6 times with 100 mM Tris/Mes, 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 \(\text{L-}^{14}\text{C} \) proline (if not otherwise indicated) was assayed under standard test conditions in the presence of 20 mM N-acetate (Na+ salt) and 50 mM NaCl or, for determination of the ion dependence on proline uptake (apparent affinities for Na+ and Li+), in the presence of 0.005 to 250 mM NaCl or LiCl at 25 °C using the rapid filtration method as described (16). Initial rates of transport were calculated from the initial linear portion of the time course data and were plotted according to Eadie-Hofstee. Standard deviations were determined from at least three independent experiments.

**Cysteine Accessibility Analysis**—E. coli W170 harboring derivatives of plasmid \(\text{pT7/putA} \) encoding PutA\(\text{Delta} \)Cys encoding PutP\(\text{Delta} \)Cys with given amino acid replacements was grown aerobically (500 ml of culture) to an \(\text{OD}_{600} \) of 1.5 before induction with 0.5 mM isopropyl-1-thio-\(\beta\)-D-galactopyranoside and further growth for 2 h. Cells were harvested, washed in 100 mM Tris/Mes, pH 7.0, and sonicated for 4 × 30 s using a Branson Sonifier. Cell debris was removed by centrifugation at 13,200 \(\times g \) for 10 min at 4 °C and the supernatant was subjected to ultracentrifugation at 230,000 \(\times g \) for 45 min at 4 °C. The resulting membrane pellet was washed in 100 mM Tris/Mes, pH 7.0, resuspended in the same buffer, and stored in liquid nitrogen until use.

After thawing, 150-µl aliquots of the membrane suspension (protein concentration adjusted to 10 mg/ml) were preincubated in the presence of different concentrations of ligand at 25 °C for 10 min. Subsequently, 200 µl fluorescein 5-maleimide was added and incubation was continued for further 10 min. The reactions were stopped by addition of 5 mM β-mercaptoethanol. PutP was solubilized from the membrane and purified by Ni\(^{2+}\)-NTA affinity chromatography as recently described (8). Equal amounts of protein were subjected to 10% SDS-PAGE. Fluorescein 5-maleimide fluorescence of PutP was visualized using the Multi-ImageQuant\textsuperscript{TM} system (Molecular Dynamics, Sunnyvale, CA) with an excitation wavelength of 380 nm and quantified by means of the QuantityOne\textsuperscript{TM} software. After staining the gel with Coomassie Blue the density of the stained bands served to correct the fluorescence values for differences in the amount of protein using the ImageQuant\textsuperscript{TM} software. The corrected fluorescence value of each PutP derivative obtained upon labeling in the absence of ligand was set to 100. Standard deviations were determined from at least three independent experiments.

**Immunological Analysis**—Relative amounts of PutP wild-type and PutP with given amino acid replacements in membranes of E. coli W170 were estimated by Western blot analysis. Immunoblotting was performed with mouse anti-FLAG IgG against the Flag epitope at the C terminus of each PutP variant followed by incubation with horseradish peroxidase-linked sheep anti-(mouse IgG) antibody by the enhanced chemiluminescence method as described (18).

**Protein Determination**—The protein concentration of membrane suspensions was determined by the method of Peterson (26). Solubilized protein was quantified according to Bradford (27). Bovine serum albumin was used as standard.

**RESULTS**

**Generation of Mutants**

Asp-55 and Ser-57 in TM II of PutP were demonstrated to be important for ligand binding and transport (16, 18). To elucidate the function of amino acids located in the vicinity of these crucial residues, Ser-54, Met-56, and Gly-58 were individually replaced with Cys by oligonucleotide-directed, site-specific mutagenesis. In addition, Ser-54 was substituted by Ala and Thr. PutP molecules with verified amino acid replacements were subjected to detailed kinetic analyses and site-directed thio-labeling studies. Other mutants used in this study (PutP-D55C, -D55E, -D55N, -S57A, -S57C, -S57G, and -S57T) were generated and initially characterized previously (16, 18).

**Time Course of Proline Uptake by PutP Containing Substitutions for Ser-54, Met-56, or Gly-58**

To determine the effect of the newly generated amino acid substitutions on PutP activity, active transport of proline was assayed under standard test conditions (50 mM Na+, 10 µM proline) by using E. coli W170 which lacks PutP and PutA (proline dehydrogenase), and therefore, cannot metabolize proline. Cells transformed with plasmids encoding PutP-S54A, -S54C, or -S54T exhibited initial rates and steady-state levels of proline accumulation comparable to those observed for PutP wild-type (Fig. 1). Severe effects on Na+ -coupled proline uptake were observed upon replacement of Met-56 and Gly-58. In particular the initial rate of proline uptake was reduced below 10% compared with PutP-wild-type (PutP-M56C, 6.7 ± 1.4%, -G58C, 1.9 ± 0.8%), while the steady-state level of proline accumulation was less affected.

**Immunological Analysis**

Relative concentrations of PutP molecules bearing given replacements in membranes of E. coli W170 were approximated by Western blot analysis using an anti-FLAG-antibody as de-
Fig. 1. Influence of the replacement of different amino acids in TM II of PutP on proline uptake. Cells of E. coli WG170 were grown and treated as described under “Experimental Procedures.” Transport of L-[U-14C]proline (10 μM final concentration) was assayed in the presence of 50 mM NaCl and 20 mM β-lactate (Na+ salt) as the electron donor at 25 °C under aerobic conditions using a rapid filtration method (16). Initial rates of proline uptake (black columns) and steady state levels of proline accumulation (striped columns) are expressed as percentage of the corresponding PutP-wild-type value. PutP-wild-type catalyzed proline uptake with an initial rate of 26 ± 7 nmol/min × mg of cell protein to a steady-state level of proline accumulation of 28.6 ± 3 nmol/mg of cell protein. Standard deviations were determined from at least three independent experiments.

Kinetic Analyses

To further characterize the effect of the individual replacement of amino acids Ser-54 through Gly-58 on PutP function, the apparent proline affinity (Km(pro)) was determined under Na+ saturation (50 mM Na+). Furthermore, to obtain information on a possible participation of the described region in cation binding and/or coupling of cation and substrate transport, proline uptake was analyzed with varying Na+ or Li+ concentrations. The proline concentration in the latter experiments was 10 μM (5-fold above the apparent Km (pro) of PutP-wild-type) if not otherwise indicated. The cation concentration causing half-maximum stimulation of proline uptake (K0.5Na+ or K0.5Li+) was used as a measure of the apparent cation affinity, while VmaxNa+ and VmaxLi+ correspond to the maximum rates of proline uptake under saturating Na+ or Li+ concentrations. The obtained kinetic data led to a classification of the mutants into three groups and the results are presented accordingly (Table I).

Mutants with an Altered Ion Dependence (Group I Mutants)—Group I comprises PutP bearing substitutions for Asp-55 or Met-56. The mutants are characterized by their low apparent affinity for the coupling ion, so, similar as previously reported for PutP-D55E (16), also substitution of Met-56 by Cys caused severe effects on K0.5Na+ (20-fold increase) and VmaxNa+ (reduction to 14% of the wild-type value) (Table I). Furthermore, replacement of Asp-55 with Glu or Met-56 with Cys had a much more pronounced effect on K0.5Li+ than on K0.5Na+ (K0.5Li+ > 100 mM) yielding a dramatic change in the K0.5Li+/K0.5Na+ ratio. While this ratio for PutP wild-type was about 5, PutP-D55E and -M56C exhibited a ratio of K0.5Li+/K0.5Na+ of above 100, thereby suggesting a more distinct discrimination between the two coupling cations. Contrary to the apparent Li+ affinity, the maximum rate of Li+-coupled proline uptake (VmaxLi+) of both mutants was in the same order of magnitude as the corresponding Na+ value. Compared with the apparent ion affinities, the apparent proline affinity was only moderately altered upon substitution of Asp-55 by Glu or Met-56 by Cys (about 8-fold increase of Km (pro)) (Table I). Finally, as shown for Na+ (16), Li+ also proved unable to drive proline transport by PutP-D55C and -D55N.

Mutants with Altered Affinities for Coupling Ion and Proline (Group II Mutants)—Group II encompasses PutP containing substitutions for Ser-57 and Gly-58. Group II mutants differ from group I mutants by their highly reduced apparent affinities for proline in addition to defects in ion binding (Table I). Most severe effects were observed upon replacement of Ser-57.

Substitution of this residue by Ala, Cys, Gly, or Thr increased K0.5Na+ by up to 2 orders of magnitude (18). In addition, this study revealed severe defects in ion binding. Depending on the nature of the amino acid side chain at position 57, K0.5Na+ was increased by factors of 12 (PutP-S57T), 42 (-S57C), 45 (-S57G), and 79 (-S57A). PutP-S57A, -S57C, and -S57T exhibited a VmaxNa+ ≤ 4% of the wild-type activity. A higher but still reduced Vmax was observed for PutP-S57G (40% compared with PutP-wild-type). Since the proline concentration used for the latter studies (10 μM) was far below the apparent proline affinity of PutP containing replacements for Ser-57, the Na+ dependence of proline uptake of PutP-S57A and -S57C was also analyzed at higher proline concentrations. Saturating proline concentrations could not be used since the ion stimulatory fraction of the transport signal was too low to allow a precise calculation of kinetic parameters. However, in the presence of 100 μM proline K0.5Na+ for PutP-S57A and -S57C was about 8-fold reduced compared with K0.5Na+ determined at 10 μM proline. Furthermore, the VmaxNa+ obtained at 100 μM proline was ~5-fold increased compared with that determined in the presence of 10 μM proline. The 10-fold increase of the proline concentration had no significant effect on K0.5Na+ and VmaxNa+ for PutP-wild-type (data not shown).

Substitution of Ser-57 with Cys, Gly, or Thr affected VmaxNa+ and K0.5Na+ to a similar extend as observed for the corresponding Na+ parameters (Table I). However, Li+ did not stimulate proline uptake by PutP-S57A (Li+ concentrations of up to 250 mM were used) which was independent on the proline concentration (10 or 100 μM).

Substitution of Gly-58 by Cys resulted in a highly reduced apparent affinity for proline (40-fold increase of K0.5pro), similar as described above for PutP containing substitutions for Ser-57. The apparent Na+ affinity of PutP-G58C was less dramatically affected (5-fold increase of K0.5Na+) while the apparent Li+ affinity was more severely altered (19-fold increase of K0.5Li+). The changes in the apparent ligand affinities of the mutant were accompanied by reduced maximum rates of proline uptake (30-fold reduction of VmaxNa+; 8-fold reduction of VmaxLi+).
Proline uptake kinetics of PutP bearing replacements of given amino acid residues

To determine $K_m^{\text{ser}}$, initial rates of $^L$[U-14C]proline uptake by E. coli WG170 producing either PutP-wild-type or PutP with given replacements were measured in the presence of 50 mM NaCl and 20 mM $\beta$-lactate (Na$^+$ salt) at proline concentrations from 0.2 to 100 $\mu$M. For determination of the ion specific parameters ($K_{0.5}^{\text{Na}}$, $K_{0.5}^{\text{Li}}$, $V_{\text{max}}^{\text{Na}}$, $V_{\text{max}}^{\text{Li}}$) transport of 10 $\mu$M (or 100 $\mu$M if indicated) $^L$[U-14C]proline was measured in the presence of 0.005 to 200 mM NaCl or LiCl at 25°C. The resulting data were plotted according to Eadie-Hofstee. Standard deviations were determined from at least three independent experiments.

Table I

| Mutant group | PutP | $K_m^{\text{ser}}$ | $K_m^{\text{Li}}$ | $K_{0.5}^{\text{Li}}$ | Ratio, $K_{0.5}^{\text{Li}}/K_{0.5}^{\text{Na}}$ | $V_{\text{max}}^{\text{Na}}$ | $V_{\text{max}}^{\text{Li}}$ |
|-------------|------|-----------------|-----------------|-----------------|-----------------------------|-----------------|-----------------|
| Wild-type   |      | 2.1 ± 0.2       | 38 ± 7          | 184 ± 61        | 4.8                         | 8.5 ± 0.5       | 12.3 ± 2.5      |
| I           | D55E | 8.9 ± 2.0       | 1,025 ± 475     | >100,000        | >100                        | 0.74 ± 0.2      | 0.41 ± 0.05     |
|             | M56C | 18.5 ± 3.3      | 753 ± 229       | 106,500 ± 20,800| 145                        | 1.2 ± 0.4       | 4.2 ± 0.8       |
| II          | S57A | 830 ± (418 ± 118)$^c$ | 3,000 ± 700     | 9,000 ± 1,200   | 5.6                         | 0.34 ± 0.01     | 0.3 ± 0.02      |
|             | S57C | 400 ± (200 ± 59) | 1,600 ± 332     | 1,200 ± 1,200   | (1.5 ± 0.05)                | 1.5 ± 0.1       | 2.91 ± 0.4      |
|             | S57G | 35 ± (20 ± 5)   | 1,700 ± 153     | 22,500 ± 3,600  | 13.2                        | 3.36 ± 0.3      | 2.91 ± 0.4      |
|             | S57T | 137 ± 450 ± 67  | 5,500 ± 654     | 12.2            | 0.12 ± 0.03                 | 0.90 ± 0.02     |
|             | G59C | 837 ± 154 ± 68  | 3,429 ± 34      | 22.5            | 0.22 ± 0.07                 | 1.63 ± 0.2      |
| III         | S54A | 1.5 ± 0         | 66 ± 13         | 297 ± 30        | 4.5                         | 21.5 ± 2.1      | 15 ± 4          |
|             | S54C | 3.7 ± 1         | 221 ± 33        | 1,500 ± 155     | 6.8                         | 10 ± 1.6        | 21 ± 2.0        |
|             | S54T | 1.5 ± 0         | 62 ± 22         | 736 ± 71        | 11.9                        | 11.6 ± 1.9      | 8.4 ± 1.1       |

$^a$ $K_m^{\text{ser}}$ values of PutP-D55E and PutP-S57A, -S57C, -S57G, and -S57T were previously determined (16, 18). The standard deviation of $K_m^{\text{ser}}$ of the Ser-57 substitutions was reported not to exceed 25% (18).

$^b$ $V_{\text{max}}^{\text{Li}}$ did not or only slightly stimulate transport thereby precluding accurate determination of $K_{0.5}^{\text{Li}}$.

$^c$ Values in parentheses are from transport measurements with 100 $\mu$M $^L$[U-14C]proline.

Mutants with Minor Effects on Kinetic Parameters (Group III Mutants)—Group III contains PutP with substitutions for Ser-54. Substitution of Ser-54 by Ala, Cys, or Thr had the least effect on all kinetic parameters tested. The apparent affinity of the transporter for proline ($K_m^{\text{ser}}$) was not significantly affected by the replacements (Table I). Furthermore, $K_m^{\text{Na}}$ and $K_m^{\text{Li}}$ were increased by factors of maximum 6 and 8 (PutP-S54C). $V_{\text{max}}^{\text{Na}}$ and $V_{\text{max}}^{\text{Li}}$ were in the same order of magnitude as the corresponding wild-type values.

Influence of Ligands on Cys Accessibility

To obtain further information on the role of this region in ligand binding and ligand-induced conformational alterations, the accessibility of Cys residues placed individually at positions 54 to 58 was analyzed in the absence and presence of different ligands (Na$^+$, Li$^+$, and proline). For this purpose Cys was individually introduced into a functional PutP derivative devoid of all five native Cys residues (PutPΔCys) (8). The effects of the substitutions on transport activity of Cys-free PutP were similar to that observed in the wild-type background (data not shown). All mutants exhibited Na$^+$-coupled proline uptake except for PutPΔCys)-D55C which was already reported to be completely inactive in an earlier study (16). Labeling experiments were performed with randomly oriented, de-energized membrane vesicles using the fluorescent dye fluorescein 5-maleimide as sulfhydryl reagent.

Contrary to the Cys-free transporter all five single-Cys PutP derivatives showed significant fluorescence labeling and the degree of labeling varied with the position of the Cys and the absence or presence of ligand (Fig. 3). In the absence of ligand (the Na$^+$ concentration of the buffer was below 5 $\mu$M) highest relative fluorescence was observed upon incubation of PutPΔCys)-S54C (group III mutant), -D55C, and -M56C (group I mutants) with the fluorescence probe while PutPΔCys)-S57C and -G58C (group II mutants) were only poorly labeled under these conditions (Fig. 3, lanes 1). Addition of Na$^+$ or Li$^+$ had little or no effect on the fluorescence labeling of Cys at positions 54, 55, and 56 (Fig. 3, lanes 2 and 4). However, Na$^+$ ions caused a substantial stimulation of the labeling reaction of PutPΔCys)-S57C and -G58C. In contrast to Na$^+$, Li$^+$ ions did not significantly affect labeling at position 57 and had only a small stimulatory effect on the reaction of Cys at position 58 with the fluorescent maleimide. In the absence of either cation no significant change of labeling was observed for any single Cys-PutP derivative upon the addition of proline (not shown). However, proline caused efficient protection of Cys at position 54 in the presence of Na$^+$ or Li$^+$ (Fig. 3, lanes 3 and 5). Furthermore, in the presence of Na$^+$, proline inhibited labeling of PutPΔCys)-S57C and G58C thereby reversing the stimulatory Na$^+$ effect. Labeling of PutPΔCys)-M56C was slightly reduced by proline in the presence of Na$^+$. Finally, reaction of PutPΔCys)-D55C with the fluorescent probe remained unaffected under all conditions tested.

Discussion

The present report investigates the role of amino acids Ser-54 to Gly-58 in TM II of PutP in ligand binding and the coupling of cation and substrate transport. The studies are based on earlier observations demonstrating that Asp-55 and Ser-57 are crucial for coupled transport (16, 18). The kinetic analysis of the effect of the individual substitution of amino acids Ser-54 through Gly-58 reveals three groups of mutations with the following properties (Fig. 4). Group I comprises PutP-D55E and -M56C, both of which have an altered ion dependence. For these constructs Li$^+$-dependent transport is much more affected than Na$^+$-coupled transport. The apparent affinity of the transporter for proline is only slightly changed. Group II encompasses PutP molecules with replacements at position Ser-57 or Gly-58 exhibiting a highly reduced affinity for proline and for the coupling cation. Group III (PutP with substitution of Ser-54) shows transport kinetics similar to PutP-wild-type. In more detail, PutP containing neutral replacements for Asp-55 is not only unable to catalyze Na$^+$-driven proline uptake as previously reported (16) but also cannot utilize the energy stored in the electrochemical Li$^+$ gradient to drive substrate transport. However, PutP with Glu in place of Asp-55 (as well as PutP-M56C) catalyzes Na$^+$- and Li$^+$-coupled proline uptake with reduced maximum rates and reduced apparent ion...
affinities. A striking feature of the latter mutants is the finding that the apparent \( Li^+ \)/\( H_1 \) affinity of the transporter is more than 100-fold lower than the apparent affinity for \( Na^+ \)/\( H_1 \). In PutP-wild-type both parameters differ only by a factor of 4.8. This change of ion discrimination may be due to the fact that the larger \( Na^+ \)/\( H_1 \) ion can undergo more interactions with the protein than the smaller \( Li^+ \)/\( H_1 \) ion, and thus alteration of the binding site is therefore less dramatic for \( Na^+ \)/\( H_1 \) than for \( Li^+ \)/\( H_1 \). In addition to previous findings, this study identifies also Met-56 as a residue which is crucial for high-affinity ion binding. Since the side chain of Met is nonpolar and cannot interact with monovalent cations, e.g. via electrostatic interactions or hydrogen bond formation, a direct participation of Met-56 in ion binding is highly unlikely. Nonetheless, the observed strong effects on the apparent affinities for \( Na^+ \) than for \( Li^+ \). In addition to previous findings, this study identifies also Met-56 as a residue which is crucial for high-affinity ion binding. Since the side chain of Met is nonpolar and cannot interact with monovalent cations, e.g. via electrostatic interactions or hydrogen bond formation, a direct participation of Met-56 in ion binding is highly unlikely. Nonetheless, the observed strong effects on the apparent affinities for \( Na^+ \) and \( Li^+ \) may reflect an altered geometry of the ion-binding site caused by the relatively modest alteration of the side chain (replacement of Met-56 by Cys). The latter idea is in perfect agreement with the proposed role of Asp-55 in ion binding (16).

However, if Asp-55 and Met-56 are indeed located at or close to the ion-binding site one might expect a competition of ion and sulphydryl reagents for Cys placed at either one of these positions. But this is clearly not the case even though both positions are accessible to sulphydryl reagents. These results can be interpreted in either of two ways: ion binding takes place at a site different from this part of TM II or ion binding is prevented by the alteration of the cation site caused by the mutation itself. The kinetic data of the mutants strongly support the latter idea. In conclusion, we suggest a location of Asp-55 and Met-56 at (or in the vicinity of) the ion-binding site(s) in which Asp-55 directly interacts with the coupling cation(s).

To date there is no direct evidence for an involvement of amino acid residues in ion binding in other members of the SSF (28). Recently, evidence has been presented suggesting a participation of Ala-166 of the human \( Na^+ \)/glucose transporter (SGLT1) in coupling \( Na^+ \) to sugar transport (29). In the well studied melibiose permease (MelB) of \( E. coli \), amino acids also located in the N-terminal domain of the protein (Asp-55, Asp-58, and Asp-124) are implicated in \( Na^+ \) binding (30–32). However, PutP clearly differs from the latter sugar transporter by the fact that in PutP the carboxylate at only one position (Asp-55) is essential for substrate transport while other acidic residues in the N-terminal domain prove to be dispensable for function (16).

In addition, members of group II mutants are characterized by substantially reduced apparent affinities for \( Na^+ \) and \( Li^+ \) suggesting that the amino acids at the corresponding
Substitution of Ser-54 (group III mutants) has little or no effect on transport kinetics. These results suggest that this residue does not play a primary role in ligand binding by PutP. Nonetheless, the reaction of Cys in place of Ser-54 with fluorescein maleimide is inhibited in the presence of both, Na⁺ (or Li⁺) and proline, while the cation alone has no influence on the labeling reaction. This result points out the possibility that the accessibility of Ser-54, which is located on the same α-helical phase of TM II as Ser-57, from the aqueous environment has changed due to conformational changes caused by high-affinity binding of proline to Ser-57 (Fig. 4). In fact, a proline-induced conformational change has previously been demonstrated by side-directed spin labeling of Cys individually placed at position 37 in the cytoplasmic loop preceding TM II or at position 45 on the same putative helix (TM II) as Ser-54 (9).

Taken together, the results emphasize the crucial role of N-terminal regions (in particular TM II) in high-affinity ligand binding and coupling of ion and solute transport for PutP, a member of the SGLT family. Results obtained with SGLT1 identify that the C-terminal domain forms the major part of the substrate (sugar) pathway (10–12, 33). Since a truncated version of SGLT1 consisting of the last five C-terminal membrane domains catalyzes Na⁺-independent facilitated diffusion of sugar with a highly reduced sugar affinity, these results directly support the findings of the present report. In conclusion, the consensus idea might be that multiple regions in individual members of the SSF contribute to efficient substrate binding. For PutP the idea is also supported by the fact that deletion mapping of putP mutants with an altered substrate specificity cluster in three distinct regions of the putP gene (34). Furthermore, the proposed close proximity of ion and organic solute binding sites in the tertiary structure of this class of membrane proteins, thereby ensuring strong cooperativity, might be the key for the molecular mechanism of cation/solubrane co-transport.

Acknowledgment—We thank S. Landmeier for generating PutP-M56C and -G58C.

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J. Biol. Chem. 2002, 277:8790-8796.
doi: 10.1074/jbc.M111008200 originally published online December 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M111008200

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