Core Transmembrane Domain 6 Plays a Pivotal Role in the Transport Cycle of the Sodium/Proline Symporter PutP*

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Crystal structures of transporters with a LeuT-type structural fold assign core transmembrane domain 6 (TM6') a central role in substrate binding and translocation. Here, the function of TM6' in the sodium/proline symporter PutP, a member of the solute/sodium symporter family, was investigated. A complete scan of TM6' identified eight amino acids as particularly important for PutP function. Of these residues, Tyr-248, His-253, and Arg-257 impact sodium binding, whereas Arg-257 and Ala-260 may participate in interactions leading to closure of the inner gate. Furthermore, the previous suggestion of an involvement of Trp-244, Tyr-248, and Pro-252 in proline binding is further supported. In addition, substitution of Gly-245, Gly-247, and Gly-250 affects the amount of PutP in the membrane. A Cys accessibility analysis suggests an involvement of the inner half of TM6' in the formation of a hydrophilic pathway that is open to the inside in the absence of ligands and closed in the presence of sodium and proline. In conclusion, the results demonstrate that TM6' plays a central role in substrate binding and release on the inner side of the membrane also in PutP and extend the knowledge on functionally relevant amino acids in transporters with a LeuT-type structural fold.

The solute/sodium symporter (SSS) family (TC 2A.21, SLC5) comprises integral membrane transport proteins that use an electrochemical sodium gradient to drive the uptake of various organic and inorganic solutes into cells (1–3). Well characterized members of this family include transporters for glucose (SGLTs) (4), iodide (Na+/I− symporter) (5), vitamins (sodium-dependent multivitamin transporters) (6), and proline (PutP) (7). The galactose/glucose transporter of Vibrio parahaemolyticus (vSGLT) is so far the only SSS transporter whose crystal structure is resolved by crystallography (8, 9). Based on the vSGLT structure, SSS proteins are thought to share the same structural fold with the bacterial sodium-dependent acid amino transporter LeuT (neurotransmitter/sodium symporter family, TC 2.A.22) (8, 10–12). The fold is characterized by a core of 10 transmembrane domains (TMs) that are arranged in two five-helix inverted repeats (11, 12). Like other transporters, the SSS proteins are thought to catalyze transport via alternating access of the substrate binding site to either side of the membrane (13). The mechanism is supported by numerous biochemical analyses and X-ray crystallographic data of transporters from different families (e.g. see Refs. 14–16).

PutP contains 13 TMs (17) of which TMs 2–11 correspond to TMs 1–10 of the LeuT core. Based on the structure of vSGLT (Protein Data Bank code 3DH4) (8), a homology model of PutP was generated (18) and further advanced using experimentally determined intramolecular distances (10). Comprehensive cysteine accessibility analyses suggest an inward open conformation as the most stable conformation of PutP under our experimental conditions (19). Thereby, the cytoplasmic halves of TMs 1' and 8' are proposed to participate in the formation of an inwardly directed hydrophilic cavity (19, 20), whereas external loop 4 is thought to be part of the outer gate (10). Substitution analyses suggest that binding of sodium involves amino acids of TMs 1' and 8' (18, 21) similar to that observed for the Na2 site in LeuT (12) (Fig. 1, A and B). Furthermore, amino acids of TMs 1', 3', 6', and 8' are thought to participate in proline binding to a central site (18, 22). Recent computational and binding analyses hint at the existence of two proline binding sites: a more externally localized proline binding site (Sc) formed by amino acids of TMs 1', 2', 6', and 10' and a more centrally localized site (Ss) constituted by amino acids of TMs 1', 6', and 8' (23) (Fig. 1, A and B). The Ss site fits to the location of the substrate in the crystal structure of vSGLT (Protein Data Bank code 3DH4) (8), and the Ss site corresponds to the location of the substrate in the crystal structure of LeuT (Protein Data Bank code 2A65) (12).

Crystal structures of LeuT and structurally related transporters suggest that TM6' plays a pivotal role in substrate binding and coordination of conformational alterations in the transport cycle (for example, see Refs. 12, 14, 15, and 24–27). In this functional analysis, we set out to advance our knowledge on the role of TM6' in the transport cycle of PutP of Escherichia coli. For this purpose, a complete substitution analysis of TM6' was performed. By this means, amino acids important for PutP function were identified. Furthermore, Cys accessibility analyses demonstrated a participation of TM6' in the formation of a pathway open to the inside. The pathway is shown to close upon binding of sodium and proline.
Results

Functionally Important Amino Acids of TM6’—TM6’ in PutP is composed of an external mostly α-helical segment (TM6’ex, positions 235–248), a loop region (TM6’loop, positions 249–253), and an internal α-helical segment (TM6’in, positions 254–259 and position 260 in the adjacent loop) (Fig. 2A). The TM contains a number of conserved amino acids among which Arg-257 is found in all and Tyr-248 is found in most of the aligned pro- and eukaryotic SSS family members; Gly-247, Gly-250, Pro-252, and His-253 are found in all prokaryotic SSS proteins; and Trp-244, Gly-247, and Gln-251 are found in orthologs with proline specificity (Fig. 2B). Aiming to test whether these and other amino acids of TM6’ are of structural and/or functional significance for PutP, a complete Cys-scan-mutagenesis of the TM was performed in a functional PutP molecule devoid of all five native Cys residues (PutP(ΔC)), and the consequences of the substitutions for the amount of PutP in the membrane and sodium-coupled proline transport were analyzed. We found highly reduced amounts of the transporter in the membrane when Gly-245 or Gly-247 was substituted by Cys, whereas PutP(ΔC)-G250C could not be detected by Western blotting. All other substitutions had little or no impact on the amount of PutP in the membrane (Fig. 2C). Next, we compared transport activities of all PutP(ΔC) variants under standard test conditions (50 mM NaCl, 100 μM L-[14C]proline). To exclude protein quantity effects, all initial rates measured were normalized to the amount of transporter in the membrane. Highly reduced transport activities (normalized initial rates ≈35% of PutP(ΔC)) were observed when Trp-244, Gly-247, Tyr248, Pro-252, Arg-257, or Ala-260 was replaced by Cys (Fig. 2C). In agreement with the lack of PutP(ΔC)-G250C in Western blotting analysis, proline uptake was not observed with this PutP variant. On the contrary, in the case of G245C, the normalized transport activity was significantly increased compared with PutP(ΔC) (Fig. 2C). When tested in the wild-type (PutP(WT)) background, also substitutions Q251C and H253C led to significantly reduced initial rates of proline uptake (33 ± 9 and 41 ± 9% of PutP(WT)). For all other substitutions, the effects on PutP(ΔC) and PutP(WT) were similar (data not shown).

A more detailed kinetic analysis according to Michaelis and Menten revealed significantly reduced maximum rates of transport ($V_{max}$) for PutP(WT)-Q251C, -P252C, -H253C, -R257C, and -A260C (11–58% of PutP(WT)) (Table 1). In addition, the proline concentration at half-maximum $V_{max}$ ($K_{m(pro)}$) was increased about 2-fold for PutP(WT)-P252C (23), and the sodium concentration causing half-maximum stimulation of proline uptake ($K_{0.5(Na^{+})}$) was 24–2-fold higher for PutP(WT)-H253C and -R257C, respectively, than the wild-type value (Table 1). Differing from the Cys substitution, Gln or Lys in place of Arg-257 yielded kinetic parameters similar to PutP(WT), suggesting that a polar side chain or a positive charge at position 257 is required for optimum activity. Additional substitutions for Ala-260 (Ser and Val) confirmed the significance of this position for the transport cycle. A260S reduced $V_{max}$ to 58% of PutP(WT), whereas $K_{m(pro)}$ and $K_{0.5(Na^{+})}$ were only marginally affected (Table 1). The A260V replacement resulted in a transport-inactive PutP variant that similarly to the A260C variant (transport activity <2% of PutP(WT)) prevented determination of reliable Michaelis-Menten parameters (Fig. 2D). Furthermore, proline binding analyses (equilibrium dialysis) of PutP(WT)-R257C and -A260C revealed $K_{d(pro)}$ values of 1.9 ± 0.5 and 3.7 ± 0.6 μM, respectively, that are similar to the $K_{d(pro)}$ of the central binding site $S_c$ (about 2 μM). Also, in the differential radial capillary action of ligand assay (DRAALA), proline binding was not significantly affected by the latter substitutions (Fig. 2E). Therefore, we concluded that both Arg-257 and Ala-260 in TM6’in are unimportant for proline binding. On the contrary, previous transport and proline binding analyses demonstrated that substitutions for Trp-244 (W244C or -F; 102–103-fold increase of $K_{m(pro)}$ (18), Tyr-248 (Y248C, -F, or -T; up to 10-fold increase of $K_{m(pro)}$ (18, 23), and Pro-252 (Cys) affect binding of proline to PutP (Ref. 23 and this investigation). The severely increased $K_{m(pro)}$ of W244C (18) is consistent with the reduced binding signal observed here with the DRAALA (Fig. 2E). In addition, the substitutions for Tyr-248 increased $K_{0.5(Na^{+})}$ about 102-fold (18). Taken together, the results indicate that eight amino acids of TM6’ (Trp-244, Gly-247, Tyr-248, Gln-251, Pro-252, His-253, Arg-257, and Ala-260) are particularly crucial for ligand recognition.
Role of TM6' in PutP

The Cytoplasmic Half of TM6' Participates in the Formation of a Hydrophilic Cavity—To probe the positioning of TM6' in PutP, the accessibility of Cys individually introduced at every position of the TM in the PutP(H9004C) background to fluorescein 5-maleimide (FM) was investigated. FM labeling was performed with randomly oriented membrane vesicles, allowing the identification of positions accessible from the aqueous phase (Fig. 3A). After labeling, the protein was purified and subjected to SDS-PAGE. Fluorescence was detected upon UV excitation and normalized to the amount of protein. The highest observed relative fluorescence was detected at position 253 and was set to 1 arbitrary unit. The level of unspecific labeling of PutP(H9004C) corresponded to 0.14 arbitrary unit. FM reacted essentially with all single Cys residues in the inner half of TM6' starting with Q251C in TM6' loop and extending into TM6' in (relative fluorescence, 0.39–1 arbitrary unit) with a local minimum of 0.31 at position 255 (Fig. 3B). The pattern of relative fluorescence intensities observed for this part of TM6' was in agreement with a partially unwound α-helix. In contrast, the relative accessibility of positions in TM6' ex was with 0.17–0.34

binding and/or other steps in the transport cycle of PutP (Fig. 2A).

FIGURE 2. Structure and functional significance of TM6' of PutP. A, structural model of TM6' of PutP with functionally and/or structurally important amino acids highlighted. Proline (surface presentation) is located as described previously (18). B, sequence alignment of amino acids of TM6' of SSS family members. The alignment was performed with Clustal Omega (47) and adjusted if reasonable. Arrows indicate (partially) conserved amino acids shown here to be of functional significance. C, impact of amino acid substitutions in TM6', i, relative amounts of PutP with given amino acid replacements in E. coli membranes as determined by Western blotting analysis. ii, initial rates of transport (black) and maximum accumulation of proline inside the cells (gray) were determined by measuring uptake of 10 μM L-[14C]proline (10 Ci mol⁻¹) into E. coli WG170 in 0.1 M Tris/MES buffer, pH 6.0, in the presence of 50 mM NaCl and 20 mM D-lactate (sodium salt) at 25 °C under aerobic conditions using a rapid filtration method as described (29). Transport activities are shown as the percentage of PutP(ΔC) and were normalized to the amount of PutP based on Western blotting analysis. Standard deviations were calculated from triplicate determinations. nc, negative control with E. coli WG170 transformed with pTrc99a without putP. Ec, E. coli; Hp, Helicobacter pylori; Sa, Staphylococcus aureus; Bs, Bacillus subtilis; Pp, Pseudomonas putida; hNIS, human Na⁺/I⁻ symporter; hSGLT1, human SGLT1; hSMVT, human sodium-dependent multivitamin transporter.
TABLE 1
Uptake kinetics of PutP bearing replacements of given amino acids
Transport of 10 μM L-[14C]proline into E. coli WG170 p77-5/putP (37, 38, 41) was assayed under aerobic conditions using a rapid filtration method as described (29).

<table>
<thead>
<tr>
<th>Substitution</th>
<th>( K_{m(Pro)} )</th>
<th>( V_{max} )</th>
<th>( K_{15(NaCl)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PutP(WT)</td>
<td>2.2 ± 0.4</td>
<td>64.6 ± 1.7</td>
<td>35.6 ± 1.0</td>
</tr>
<tr>
<td>Q251C</td>
<td>1.5 ± 0.3</td>
<td>24.8 ± 0.9</td>
<td>32.3 ± 2.4</td>
</tr>
<tr>
<td>P252C</td>
<td>5.2 ± 0.5*</td>
<td>27.3 ± 1.1</td>
<td>(28.7 ± 7.0) × 10^a</td>
</tr>
<tr>
<td>H253C</td>
<td>1.9 ± 0.2</td>
<td>26.0 ± 0.4</td>
<td>843.3 ± 81.9</td>
</tr>
<tr>
<td>R257C</td>
<td>0.7 ± 0.1</td>
<td>7 ± 0.6</td>
<td>63.3 ± 3</td>
</tr>
<tr>
<td>R257K</td>
<td>1.3 ± 0.1</td>
<td>40 ± 0.3</td>
<td>38.6 ± 2</td>
</tr>
<tr>
<td>R257Q</td>
<td>1.7 ± 0.2</td>
<td>25 ± 0.2</td>
<td>36.4 ± 1</td>
</tr>
<tr>
<td>A260C</td>
<td>ND*</td>
<td>ND</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>A260S</td>
<td>1.50 ± 0.41</td>
<td>37.2 ± 1.9</td>
<td>19.6 ± 2.5</td>
</tr>
</tbody>
</table>

* Two values for \( K_{15(NaCl)} \) were determined due to a biphasic curve shape. In contrast to PutP(WT), where already 50 mM NaCl inhibits transport, a leveling off of the curve was not observed. Therefore, the second value is only an estimate.

ND, not determined. Transport activities were below 3% of PutP(WT), preventing reliable determination of kinetic parameters.

arbitrary unit, not or only slightly increased compared with PutP(DC), suggesting a location buried in the protein or in an apolar environment. A similar labeling pattern was previously obtained for TMs 1 and 8 (19, 20). The results suggest that TM6 joins TMs 1 and 8 in forming a pathway that is open to the inside in the absence of ligands. This conclusion is in agreement with previous homology models of PutP (10, 18) (Fig. 3C) and supports the idea that PutP is most stable in an inward open conformation under our experimental conditions.

**Ligand-induced Conformational Alterations**—To test potential effects of ligands on the conformation of PutP, the impact of sodium and proline on Cys modification by FM was investigated. For this purpose, labeling with FM was performed after pre-equilibrating the membranes with 50 mM NaCl or 50 mM NaCl, 10 mM L-proline (Fig. 4). Pre-equilibration with sodium alone did not affect or only slightly affected FM labeling of Cys at any position in TM6. Similarly, the addition of sodium together with proline had no or only very limited effects on Cys modification at positions 235 and 240–247 (data not shown). However, simultaneous addition of sodium and proline decreased the level of Cys labeling at almost all positions in TM6 (Fig. 4). Compared with labeling in the absence of ligands, the most severe inhibitory effects were observed for PutP(DC)-R257C (8-fold) and -A256C (5-fold). Also Cys at positions 252, 255, and 258 showed significantly decreased labeling (Fig. 4). As ligand protection was observed along the whole inner part of TM6, steric hindrance by the ligand alone cannot explain the results. We suggest that ligand binding induces conformational alterations that lead to closure of the inner pathway. Because an opening of an outer pathway could not be observed (Refs. 19 and 20 and this investigation), PutP seems to adopt an occluded state upon sodium and proline binding under our experimental conditions. The results under-

**Discussion**

Based on a complete scan of TM6, we have shown that Trp-244, Gly-247, Tyr-248 (TM6_ex), Gln-251, Pro-252, His-253 (TM6_loop), Arg-257, and Ala-260 (TM6_in) are particularly crucial for PutP function. Furthermore, a Cys accessibility analysis suggests a participation of the inner half of TM6 in the formation of a pathway that, under physiological conditions, most likely allows the release of ligands from the central binding site into the cytoplasm (amino acids at positions 252–258). Under our experimental conditions, the inner pathway appears to be open in the absence of ligands and closed upon addition of sodium and proline. Taken together with our previous results on TMs 1 and 8 (19, 20), we conclude that
the three TMs play a central role in ligand binding and translocation in PutP (Fig. 5).

Of the crucial amino acids of TM6', Trp-244, Tyr-248, and Pro-252 may participate in proline binding as already suggested previously (18, 23). The idea agrees well with the central role of TM6' in substrate binding described for other transporters with a LeuT-type structural fold (8, 12, 14, 24–26, 28). For example, in TM6' of vSGLT, Ala-259, Asn-260, Tyr-263, and Trp-264 (Trp-244, Gly-245, Tyr-248, and Phe-249 in PutP (Fig. 2B)) are facing the sugar substrate (Protein Data Bank code 3DH4 (8)). Asn-260 of vSGLT participates in substrate binding, and its substitution by Ala prevents transport, whereas Tyr-263 stacks with the pyranose ring of galactose and establishes the internal gate along with the flanking amino acids Tyr-262 and Trp-264 (8). In this scenario, Tyr-263 in vSGLT temporarily interacts with Asn-64 in TM1 upon gate opening and ligand release (9). Transferring this idea to PutP, we speculate that opening of the internal gate involves temporal interactions of Tyr-248 with the essential Asp-55 in TM1' (29). The idea is also in principal agreement with the crystal structure of LeuT in different conformations that demonstrate changing interactions of the corresponding amino acid Phe-253 with TMs 1 and 10 (15). In the two-substrate binding site model for PutP and vSGLT, Tyr-248 and Tyr-263, respectively, are proposed to separate the S\textsubscript{i} site from the more outwardly located S\textsubscript{o} site (23) (Figs. 1A and 5). In this scenario, Trp-244 and Tyr-248 in TM6'\textsubscript{10} (Ala-259 and Tyr-263 in vSGLT) form together with amino acids of TMs 1', 2', and 10' the S\textsubscript{o} site, whereas Gln-251, Pro-252, and His-253 in TM6'\textsubscript{10} (Asn-267, Gln-268, and Tyr-269 in vSGLT) are located at or close to the S\textsubscript{i} site (23). Also in the latter model, Asp-55 and Tyr-248 (Asn-64 and Tyr-263 in vSGLT) are thought to fulfill a gating function.

Furthermore, replacement of Tyr-248, His-253, or Arg-257 in PutP affects sodium binding (Ref. 18 and Table 1). These results are unexpected because sodium is predicted to bind elsewhere involving conserved amino acids of TMs 1 and 1' (Refs. 18 and 21 and Fig. 5), resembling the Na\textsubscript{2} site of LeuT (12). Clearly, the effect can be indirect and the result of structural changes over a distance caused by the substitutions in TM6'. Alternatively, amino acids of TM6' may contribute to sodium binding at a second yet unrecognized site in PutP. In fact, Thr-254 in TM6 of LeuT forms together with amino acids of TMs 1', 2', and 10' the S\textsubscript{o} site, whereas Gln-251, Pro-252, and His-253 in TM6'\textsubscript{10} (Asn-267, Gln-268, and Tyr-269 in vSGLT) are located at or close to the S\textsubscript{i} site (23).
to coupling between ion and substrate transport (32). However, the presence and position of the Na\textsubscript{1} site in LeuT-like transporters are not conserved (8, 14, 30, 33). For example, the sodium/galactose stoichiometry of vSGLT is 1:1 (34), and only one sodium binding site (equivalent to Na\textsubscript{2} of LeuT) has been identified so far (8). In view of these facts and a sodium/proline stoichiometry of 1:1 (35), the idea of a second sodium binding site remains highly speculative.

Furthermore, the functional defects observed upon replacement of Arg-257 and Ala-260 in TM6\textsubscript{in} may result from disruption of the interaction network involved in closing the inner gate. In fact, substitution of Tyr-268 at the inner end of TM6 of LeuT contributes to stabilization of the transporter in an inward open conformation (15, 36). In conclusion, our results extend the knowledge on functionally relevant amino acids in inward open conformation (15, 36). In conclusion, our results demonstrate that TM6 plays a central role in substrate binding and release on the inner side of the membrane also for PutP and extend the knowledge on functionally relevant amino acids in transporters with a LeuT-type structural fold.

### Experimental Procedures

**Bacterial Strains and Plasmids—**E. coli DH5\textalpha{} (F\textsuperscript{−} φ80d lacZ ΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsd R17(λλ prophag44 λλ thi-1 gyrA96 relA1)) was used as carrier for plasmids. E. coli WG170 (F\textsuperscript{−} trp lacZ rpsL thi Δ(putPA)101 proP219) (37) harboring the given plasmids was used for expression of the putP gene of E. coli and biochemical assays. The following plasmids, derivatives of pT7-5 (38), containing the lac promoter/operator for expression of the putP gene were used for all gene manipulations: pT7-5/putP and pT7-5/putP(ΔC), each harboring a cassette version of the putP gene encoding PutP wild type and an engineered transporter devoid of all five native Cys residues (PutP(ΔC)), respectively, and a C-terminally attached amino acid sequence resembling the FLAG epitope and a His\textsubscript{6} tag (39). Vector pTrc99a (40) was used for overexpression.

**Site-directed Mutagenesis—**Desired nucleotide substitutions in putP were generated by PCR with Phusion DNA polymerase using plasmid pT7-5/putP or pT7-5/putP(ΔC) as a template and synthetic mutagenic oligonucleotides. Resulting PCR fragments were cloned in plasmid pT7-5/putP or pT7-5/putP(ΔC). The plasmid DNA was verified by sequencing using an ABI 3730 capillary sequencer.

**Transport Assay—**Active transport of l-[U-\textsuperscript{14}C]proline was measured in E. coli WG170 harboring plasmid pT7-5/putP encoding PutP with given amino acid replacements as described (29). Initial rates of transport were calculated from the initial linear portion of the time course, and steady-state levels of l-[U-\textsuperscript{14}C]proline accumulation were taken from time points after leveling off of the uptake curve. Standard deviations were calculated from at least triplicate determinations.

**Immunological Analysis—**Relative amounts of PutP with given amino acid replacements in membranes of E. coli WG170 harboring plasmid pT7-5/putP were estimated by Western blotting analysis with HRP-linked mouse anti-FLAG IgG directed against the FLAG epitope at the C terminus of each PutP variant as described (41).

**Cys Accessibility to Sulfhydryl Reagents—**The accessibility of Cys to FM was determined with randomly oriented membrane vesicles prepared from E. coli WG170 following the protocol of Raba et al. (20). In short, vesicles were incubated with 200 μM FM at 25 °C for defined periods of time, and then labeling reactions were stopped by addition of 10 mM β-mercaptoethanol. For testing the influence of ligands on FM labeling, membranes were preincubated in the absence or presence of 50 mM NaCl or 50 mM NaCl, 10 mM l-proline for 10 min and subsequently labeled as described. After labeling, PutP was purified (39), and equal amounts of protein were subjected to SDS-PAGE. Fluorescent bands of PutP were detected, and fluorescence was normalized to the total amount of PutP.

**Proline Binding Assays—**Binding assays were performed as described (42). In brief, membrane vesicles of E. coli WG170 expressing putP with given mutations were prepared using a high pressure cell disruption system (Constant Systems). The resulting vesicles were resuspended in 100 mM potassium phosphate buffer, pH 7.5, 2 mM β-mercaptoethanol and preincubated with 50 mM NaCl, 20 μM carbonyl cyanide m-chlorophe-nylhydrazone, and 5 μM monensin at 25 °C for 30 min.

For the DRAcALA, the protocol of Roelofs et al. (43) was followed. For binding, 1 μM l-[U-\textsuperscript{14}C]proline (55 μCi/μmol) was added to the vesicle suspension (9 mg/ml total protein), and samples were incubated at 25 °C for 10 min. Five-microliter aliquots were subsequently pipetted onto dry nitrocellulose (GE Healthcare) in triplicates. Radioactivity was detected using a Storage Phosphor Screen and a Typhoon Trio Imager (Amer- sham Biosciences).

For quantitative binding assays, aliquots of the preincubated membrane vesicles containing 12.5 μg/ml total protein were incubated with 50 mM NaCl and l-[U-\textsuperscript{14}C]proline (55 μCi/μmol; final concentrations from 0.2 to 50 μM) using a Thermo Scientific Single-Use RED Plate with RED inserts (8000 dalton molecular weight cutoff). The plate was shaken for 4 h in an Eppendorf Thermomixer comfort for 22 h. Aliquots of each chamber were mixed with ECOLUMETM liquid scintillation mixture (MP Biomedicals), and radioactivity was measured using a PerkinElmer Life Sciences Tri-Carb® 2910TR liquid scintillation analyzer. The k\textsubscript{d} values were determined using the Enzyme Kinetics module of SigmaPlot 12.5.

**Determination of Sodium—**Sodium concentrations in buffers used for transport assays were determined with a Varian AA240 atomic absorption spectrometer.

**Protein Determination—**Determination of protein was performed according to a modified Lowry method (44) for total membrane protein and according to Bradford (45) for detergent-solubilized protein.

**Molecular Graphics and Analyses—**Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS, National Institutes of Health Grant P41-GM103311) (46).

Author Contributions—S. B. and H. J. conceived the study, supervised the work, analyzed all results, and wrote the manuscript. C. C. S. participated in mutant generation, analyses of transport kinetics, and Cys accessibility analyses. S. I. D. participated in the Cys accessibility analyses.
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