Transmembrane Domain II of the Human Bile Acid Transporter SLC10A2 Coordinates Sodium Translocation*

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Human apical sodium-dependent bile acid transporter (hASBT, SLC10A2) is responsible for intestinal reabsorption of bile acids and plays a key role in cholesterol homeostasis. We used a targeted and systematic approach to delineate the role of highly conserved transmembrane helix 2 on the expression and function of hASBT. Cysteine mutation significantly depressed highly conserved transmembrane helix 2 on the expression and function of hASBT. Cysteine mutation significantly depressed transport activity for >60% of mutants without affecting cell surface localization of the transporter. All mutants were inaccessible toward chemical modification by membrane-impermeant MTSET reagent, strongly suggesting that transmembrane 2 (TM2) plays an indirect role in bile acid substrate translocation. Both bile acid uptake and sodium dependence of TM2 mutants revealed a distinct α-helical periodicity. Kinetic studies with conservative and non-conservative mutants of sodium sensitive residues further underscored the importance of Gln75, Phe76, Met79, Gly83, Leu86, Phe90, and Asp91 in hASBT function. Computational analysis indicated that Asp91 may coordinate with sodium during the transport cycle. Combined, our data propose that a consortium of sodium-sensitive residues along with previously reported residues (Thr134, Leu138, and Thr149) from TM3 may form the sodium binding and translocation pathway. Notably, residues Gln75, Met79, Thr83, and Leu86 from TM2 are highly conserved in TM3 of a putative remote bacterial homologue (ASBTNM), suggesting a universal mechanism for the SLC10A transporter family.

hASBT (SLC10A2) plays an essential role in the reuptake of bile acids from the intestinal lumen (1, 2). Bile acids are synthesized in the liver from cholesterol, stored in the gall bladder, and excreted into intestinal lumen in the fed state (3), playing a critical role in the digestion and absorption of fat. Hypcholesterolemia associated with idiopathic bile acid malabsorption demonstrates that hASBT is a viable pharmacological target for cholesterol-lowering therapy (4, 5). In addition, hASBT can be used as a potential prodrug target for increasing oral bioavailability of poorly permeable drugs (6, 7).

Bile acid uptake via hASBT is electrogenic (8) using a sodium gradient as the driving force for the transport of bile acids, with a 2:1 Na+:bile acid coupling stoichiometry. Using N-glycosylation and an epitope insertion approach, previous studies from our laboratory have definitively confirmed a 7-transmembrane (TM) topology for this 348-amino acid transporter (see Fig. 1A) (9). Recently, a crystal structure of a putative bacterial homologue of hASBT with ~24% sequence identity and 10 TMs was reported (10), isolated from Neisseria meningitidis (ASBTNM); although its physiological role in Neisseria, its specificity toward bile acids, and its phylogenetic relationship to mammalian ASBT remain to be established, this structure may aid in the understanding of human SLC10A structure-function relationships.

Our laboratory has carried out a systematic analysis to unveil residues or regions that are important for the expression, function, and stability of hASBT. For example, mutations on ~70% TM1 residues resulted in impaired function (11), with residue Leu30 critical for both bile acid and sodium binding. The large hydrophilic region lining the cytosolic half of TM3 may be partially involved in substrate exit during bile acid translocation (12). Consistent with the orientation of TM4 in our three-dimensional model (13), experimental data ruled out the direct involvement of TM4 in sodium translocation; instead, the high solvent accessibility of the cytosolic half of the transmembrane domain indicates an important role of TM4 in substrate transport (14). Conversely, TM5 may not be directly involved in substrate translocation, yet the presence of a helix–helix interacting motif, GXXXG, suggests that it may play a role in structural stability of the transporter (15). Functional flexibility enabled by residues Pro234, Gly237, and Gly241 allow TM6 to form a “conformational switch” for substrate turnover (16). Both experimental data and computational modeling suggest a pivotal role for TM7 in substrate translocation (17, 18). Overall, these studies have provided a roadmap for interpreting hASBT structure and function.

The present study analyzes the role of TM2 in the expression, function, and/or stability of hASBT using the substituted cysteine...
teine accessibility method (SCAM). Each individual residues of TM2 was mutated to cysteine, and the effect of these mutations on hASBT surface expression, function, sodium sensitivity, and aqueous accessibility were assessed. Conservative and non-conservative mutations were generated on functionally impaired and sodium-sensitive residues to further elucidate their functional importance/requirement.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radiolabeled [3H]taurocholic acid (TCA; 1 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), bovine serum albumin and TCA were from Sigma. [2-((trimethylammonium)ethyl] methanethiosulfonate (MTSET) reagent was from Toronto Research Chemicals, Inc. (North York, ON, Canada), EDTA-free protease inhibitor mixture tablets were from Roche Applied Science, Ez-link™ Sulfo-NHS-SS-Biotin was from Pierce, and cell culture media and supplies were from Invitrogen. All other reagents and chemicals were of highest purity available commercially.

**Cell Culture and Transient Transfection**—The monkey kidney fibroblast cell line, COS-1 (ATCC CRL-1650), was maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 4.5 g/liter glucose, 0.1 mM nonessential amino acid at 37 °C in a humidified atmosphere with 5% CO2. Transfections were carried out as previously described (19). Briefly, cells were seeded at a density of 6 × 10^4 cells/ml on 12- or 24-well plates (Costar, Corning, NY). The next day, after reaching ~70–90% confluence, cells were transfected with TurboFect transfection agent (Thermo Scientific). 40–48 h after the transfection, cells were used for uptake and surface protein labeling studies.

**Site-directed Mutagenesis**—Mutagenesis primers were custom-synthesized and purchased from Sigma. To generate mutations on each individual residue of TM2, C270A or wild type (WT) plasmid of hASBT in pCMV5 vector was used as the template. Our previous studies demonstrated that the C270A mutant is functionally active and has resistance to thiol modification (20). The mutations were generated by QuikChange site-directed mutagenesis. Plasmids were purified using kits from Qiagen (Valencia, CA). The sequences of the mutants were verified by DNA sequencing at the University of Maryland Medical School sequencing facility.

**TCA Uptake Study**—Uptake studies were carried out as previously described (11). Briefly, COS-1 cells were transiently transfected with pCMV5 plasmid containing C270A mutant, TM2 mutants, or WT_ASBT DNA. 48 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and incubated in 200 μl of modified Hanks’ balanced salt solution containing 5.0 μM cold TCA spiked with 1 μCi/ml [3H]TCA at 37 °C. The uptake study was carried out for 12 min to ensure steady-state kinetics. Uptake was halted by washing the cells with ice-cold PBS (pH 7.4) containing 0.5 mM TCA. Cells were lysed by incubation in 350 μl of 1 N NaOH for 2 h followed by neutralizing with 50 μl of concentrated HCl. 250 μl of sample from each well were subjected to analysis by scintillation counting using and LS6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Uptake activity was normalized to total protein in each well, which was quantified using Bradford protein assay (Bio-Rad). The final uptake activity was expressed as pmol of [3H]TCA/min/mg of protein.

**Protein Membrane Expression**— transiently transfected COS-1 cells in 12-well plates were first washed with ice-cold PBS followed by incubating with 1.2 mg/ml Sulfo-NHS-SS-Biotin in PBS for 30 min at 4 °C with gentle rocking. The reaction was quenched with 1 M Tris buffer (pH 8.0) followed by washing with Tris-buffered saline (TBS). Cells were lysed in 200 μl of lysis buffer (50 mM Tris buffer (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1× sigma protease inhibitor mixture) for 30 min at 4 °C. Cell lysates were centrifuged at 10,000 × g for 2 min. 30 μl of the supernatant was used for total protein expression assessment. The remaining supernatants were mixed with 250 μl of neutravidin resin (Pierce) and rocked overnight at 4 °C. The supernatant was removed after centrifugation at 2500 × g for 2 min. The resins were mixed with 2× sample buffer after washing twice with TBS and lysis buffer and heated for 10 min at 95 °C. The suspension was centrifuged at 5000 × g for 5 min, and the supernatants were subjected to SDS-PAGE and Western blot analysis.

For SDS-PAGE and immunoblotting studies, the total protein or the surface-labeled proteins were separated on 12.5% SDS-polyacrylamide gel (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). Blots were probed with rabbit anti-ASBT primary antibody (Santa Cruz Biotechnology; 1:1000 dilutions). The absence of a 90-kDa band for calnexin (mouse anti-calnexin; Sigma; 1:1000 dilutions) and a 140-kDa band for cadherin (mouse anti-cadherin; Abcam; 1:1000 dilutions) was used as an indicator for surface and total expression of hASBT. Goat anti-rabbit IgG (H+L) Dylight 800 conjugated and goat anti-mouse IgG (H+L) Dylight 680 conjugated (Thermoscientific, 1:10,000 dilutions) were used as the secondary antibodies. An Odyssey imaging system (LI-COR, Lincoln, NE) was used to visualize hASBT and control bands. The anti-ASBT primary antibody detects both the glycosylated (~41 kDa) and unglycosylated (~38 kDa) ASBT proteins.

**Sodium Activation and Kinetics**—To assess Na+ sensitivity of individual mutants, [3H]TCA uptake was measured at equilibrative (12 mmol/L) and physiological (137 mmol/L) Na+ concentrations. The results are expressed as a ratio of uptake at 12 mmol/L over 137 mmol/L Na+ to determine sodium sensitivity of each mutant. A ratio less than 1 indicates greater necessity for physiological sodium concentration for proper function. TCA uptake kinetics or Na+ activation kinetics were measured at TCA concentrations ranging from 0 to 200 μM and Na+ concentrations ranging from 0 to 137 mmol/L.

**Solvent Accessibility Studies**—The possible involvement of the TM2 residues in substrate translocation was analyzed on the cysteine mutants by assessing thiol accessibility of these mutants to positively charged, membrane impermeable MTSET. COS-1 cells transiently transfected with TM2 mutants were preincubated with MTSET for 10 min at room temperature and washed twice with Hanks’ balanced salt solution followed by [3H]TCA uptake at 37 °C for 12 min. MTSET working solution was freshly prepared to ensure stability. Control wells were treated with buffer in the absence of MTSET reagent and run parallel with the MTSET-treated cells.
Involvement of hASBT TM2 in Sodium Translocation

Molecular Dynamic Simulation—The structural model of the hASBT dimer was developed using an elaborate homology modeling scheme followed by refinement using both implicit and explicit protein-membrane molecular dynamics (MD) simulations as has been described in detail elsewhere. The hASBT dimer model showed excellent correlation with experimental substituted cysteine accessibility method profiles. In this study we performed additional analysis on the hASBT dimer trajectory specifically to probe the interactions with sodium ions. Briefly, bilayer protein-membrane simulations on the hASBT dimer were performed using the biomolecular simulation program CHARMM (22) and NAMD (23). The CHARMM22 protein force field (24) with CMAP (dihedral correction map (25)) modified the TIP3P water model (26), and the CHARMM lipid force field was used for the in silico modeling. A mixed 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine/cholesterol lipid bilayer was used to model the membrane (27). The electrostatic interactions were treated via the particle mesh Ewald method with a real-space cutoff of 12 Å, a $\kappa$ value of 0.34 Å$^{-1}$, and a sixth-order spline (28). Nonbond interaction lists were updated heuristically out to 16 Å with a force switch smoothing function from 10 to 12 Å used for the Lennard-Jones interactions. SHAKE algorithm was used to constrain all covalent bonds involving hydrogen atoms (29). All the bilayer calculations were performed at 303.15 K. A salt (NaCl) concentration of 0.137 M was used. A Langevin coupling coefficient of 1 ps$^{-1}$ with a temperature bath of 303.15 K was applied to all atoms to achieve constant temperature. A piston oscillation period of 200 fs and a barostat damping time scale of 100 fs were used to maintain a piston pressure of 1 atm. A time step of 2 fs was used. After initial equilibration in CHARMM (using gradually decreasing restraints) 60-ns production runs were performed using NAMD Version 2.7b1. The final system size was 98.39 $\times$ 98.39 $\times$ 98.39 Å$^3$. Additional details of the simulation and the systems setup have been discussed elsewhere.

Data Analysis—Studies were carried out at least three times in triplicate, and bars represent the S.E. for $n \geq 3$. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for data analysis using one-way analysis of variance with Dunnett’s post hoc test to determine statistical significance.

RESULTS

Cysteine Scanning of TM2—According to a 7TM topology model (Fig. 1A), residues spanning Leu$^{73}$ to Val$^{98}$ form the TM2 helix. High sequence conservation in TM2 was observed among various species (Fig. 1B). To elucidate the role of TM2 on hASBT expression, function, and stability, each individual residue of TM2 (except Cys$^{74}$) was mutated to cysteine. The mutations were generated using the hASBT C270A scaffold, which is largely insensitive to thiol-specific chemical reagents. Consequently, the expression and function of the mutants were compared with that of the C270A.

Transport Activity and Membrane Expression of TM2 Mutants—Each of the TM2 mutants was transiently transfected into COS-1 cells, which have extremely low background expression of ASBT. The cell surface localization of these mutants was determined with the membrane-impermeable agent, sulfo-NHS-SS-Biotin followed by immunoblotting and densitometric analysis of the developed bands. Total cellular and membrane-localized (surface) expression of these mutants was compared with the expression of the C270A scaffold.

The total expression of each TM2 mutants is comparable to the expression of the C270A control, except P94C, L95C, and V98C (see Fig. 3A); these three mutants had detectable levels of total cellular hASBT expression, albeit with low cell surface localization. Membrane localization of the majority of TM2 mutants is similar to that of C270A (Fig. 2B). Interestingly, mutations made to the residues in the center of the TM2 helix showed relatively higher cell surface expression than C270A, especially T82C. The surface expression of each mutant was first semiquantified by normalizing to cadherin, a plasma membrane expression marker; then $[^3]$H]TCA uptakes were normalized to quantified cell surface hASBT expression (Fig. 2C). Significant changes in TCA uptake rates were observed for the majority (64%) of the mutants compared with the C270A control, suggesting a functional role for these residues. Uptake activity of P80C and P94C mutations were almost abolished. A severe loss of activity (<10% activity remaining) for Q75C, F76C, M79C, P80C, G83C, P94C, and Q96C mutations, yet measurable cell surface transporter expression, suggests that the reduction in activities did not result from a loss of protein at the plasma membrane. These residues may play a significant role in bile acid substrate or Na$^+$ translocation.

Solvent Accessibility via Sulfhydryl Modification—To explore whether TM2 residues are involved in substrate binding and/or translocation, a membrane-impermeable, sulphydryl-reactive labeling agent, MTSET, was used to probe solvent accessibility. Residues involved in bile acid translocation are likely exposed to the aqueous phase; therefore, cysteine mutation of this residue would make it available for thiol modification by MTSET. Once the residue is chemically modified, substrate binding or translocation will be impeded. No significant change in TCA uptake rate was observed for any of the TM2 mutants upon chemical modification (Fig. 3), indicating that TM2 residues may not be directly involved in bile acid binding and/or translocation. Control experiments with WT ASBT revealed significant changes in TCA uptake rate upon MTSET modification, confirming previous findings (19).

Na$^+$ Sensitivity of TM2 Residues—hASBT relies on the physiological sodium gradient to transport bile acids, with an approximate 2:1 Na$^+$ to bile acid co-transport stoichiometry (8). To identify residues of TM2 that may be involved in sodium interactions, TCA uptake was studied at equilibrating (12 mM) and physiological (137 mM) sodium concentrations. Previous studies have demonstrated minimal sodium sensitivity for the C270A control (16, 17, 19, 30). Therefore, the ratio of TCA uptake at 12 mM versus 137 mM Na$^+$ for each mutant was compared with that of the C270A control. Several TM2 mutants exhibited sensitivity toward changes in Na$^+$ concentration following a clear pattern of $\alpha$-helical periodicity, i.e. mutants of every third or fourth residue were significantly affected (Fig. 4); Q75C, M79C, G83C, and L86C showed a significant decrease, whereas I92C and Q96C exhibited a significant increase in TCA transport activity.
uptake. Interestingly, residues of adjacent TM3 were also found to be \( \text{Na}^+ \)-sensitive and may participate in sodium binding and translocation. Based on their close proximity, it is tempting to speculate that TM2 and TM3 may cooperate during sodium translocation.

**Sodium and TCA Kinetics of Select TM2 Mutants**—To further delineate the underlying mechanism for the observed changes in TCA uptake rates and sodium sensitivities, a subset of residues were selected for TCA and \( \text{Na}^+ \)/H11001 kinetic analysis. Only Q75C and G83C showed a significant change in TCA affinity (\( K_{\text{TCA}} \)) compared with C270A control (Table 1). Because both mutants have minimal TCA uptake compared with control (<10%, Fig. 1), it is difficult to draw conclusions from kinetic parameters alone. The other mutations (F76C, M79C, G83C, D91C, I92C, and Q96C) did not affect bile acid binding affinity (\( K_{\text{TCA}} \)) but demonstrated significantly diminished maximal turnover rates (\( J_{\text{max}} \)), suggesting that these mutations affected hASBT structure or \( \text{Na}^+ \) coupling rates. Thus, \( \text{Na}^+ \) kinetics for this subset of residues was further evaluated. M79C and L86C showed a significant increase in \( K_{\text{Na}^+} \) as well as a significant decrease in \( J_{\text{max}} \) compared with that of C270A control (Table 2). Interestingly, mutants L73C, D91C, I92C, and Q96C demonstrated a significant increase in sodium affinity that coincided with a significant decrease in maximal TCA transport rate (\( J_{\text{max}} \)). We hypothesize that the concomitant changes in sodium affinity and maximal transport rates (Table 2) may, at least in part, provide evidence for either direct or indirect involvement of these residues in sodium translocation.

**Mutation of Critical Residues on a Wild Type Background**—To exclude the possibility that the C270A scaffold affects the functional changes observed above, conservative and non-conservative mutations were performed. Only Q75C and G83C showed a significant change in TCA affinity (\( K_{\text{TCA}} \)) when compared with C270A control (Table 1). Because both mutants have minimal TCA uptake compared with control (<10%, Fig. 1), it is difficult to draw conclusions from kinetic parameters alone. The other mutations (F76C, M79C, G83C, D91C, I92C, and Q96C) did not affect bile acid binding affinity (\( K_{\text{TCA}} \)) but demonstrated significantly diminished maximal turnover rates (\( J_{\text{max}} \)), suggesting that these mutations affected hASBT structure or \( \text{Na}^+ \) coupling rates. Thus, \( \text{Na}^+ \) kinetics for this subset of residues was further evaluated. M79C and L86C showed a significant increase in \( K_{\text{Na}^+} \) as well as a significant decrease in \( J_{\text{max}} \) compared with that of C270A control (Table 2). Interestingly, mutants L73C, D91C, I92C, and Q96C demonstrated a significant increase in sodium affinity that coincided with a significant decrease in maximal TCA transport rate (\( J_{\text{max}} \)). We hypothesize that the concomitant changes in sodium affinity and maximal transport rates (Table 2) may, at least in part, provide evidence for either direct or indirect involvement of these residues in sodium translocation.
Involvement of hASBT TM2 in Sodium Translocation

Conservative mutations were made against a WT_ASBT background to residues with significantly affected TCA uptake rates (Fig. 5). When Gln75 was mutated to Asp/Glu, TCA uptake was almost abolished. Similarly, a non-conservative mutation Q75A resulted in loss of function, indicating that both the size and physicochemical properties of the Gln75 side chain are critical for hASBT function. Non-conservative mutation F76A severely dampened hASBT function, whereas the more conservative mutations, F76Y and F76W, preserved >10% bile acid uptake activity. These data suggest that the phenyl moiety on the Phe76 side chain may play an important role in hASBT function, perhaps by providing a scaffold for \( \pi \)-charge interactions with \( \text{Na}^+ \). A complete loss of function for the P80A and P94A mutations was expected as proline mutations in transmembrane domains may lead to structural instability (31) by disrupting the hinge movement of Pro residues provide to \( \alpha \)-helicies (32). The conservative and non-conservative mutations Q96N and Q96A resulted in complete loss of function; however, Q96E did not affect TCA uptake compared with control, indicating that the size of the side chain is critical and that charged moieties are tolerated at this position. Gln96 is located at the border between plasma membrane and extracellular space and may provide a role in stabilizing membrane localization.

FIGURE 2. [\( ^{3} \text{H} \)]TCA uptake by TM2 mutants and transporter expression. A, COS-1 cells were transfected with TM2 mutants, pCMV5 and C270A controls as described under “Experimental Procedures.” 40–48 h after transfection, [\( ^{3} \text{H} \)]TCA uptake was carried out for 12 min before cells were washed 3 times and lysed in 1 n NaOH, and the lysates were subjected to radioactive counter analysis. TCA uptake rates were calculated as pmol [\( ^{3} \text{H} \)]TCA/min/mg of protein. The final results were expressed as the percentage of the uptake rate of the C270A control. B, total (whole cell homogenate) and cell surface expression of each cysteine mutant were subjected to 12.5% polyacrylamide gel analysis followed by transferring to PVDF membrane. hASBT bands were confirmed with anti-ASBT antibody (1:1000 dilution). Cadherin was used as a cell surface expression marker; calnexin was used as an ER protein marker. C, uptake for each mutant was normalized to surface expression level of hASBT mutants and expressed as a percentage of Control (C270A). *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).
Asp91 is Na⁺-sensitive—Asp91 is the sole residue in TM2 with a polar and possibly charged side chain. Cysteine substitution of Asp91 and its adjacent residues Phe90 and Ile92 resulted in significantly decreased TCA uptake rates (Fig. 2C), suggesting that this region is critical to hASBT function. Solvent accessibility data ruled out the direct involvement of this region in bile acid substrate translocation (Fig. 3), prompting the hypothesis that altered sodium interactions might be responsible for the observed change in TCA uptake. I92C resulted in a significantly increased sodium affinity ($K_{Na^+} = 4.5 \pm 1.32$ mM) compared with the C270A control ($K_{Na^+} = 14.22 \pm 3.03$ mM). However, the increased sodium binding significantly reduced TCA uptake activity for the mutant, indicating that either Na⁺ translocation was hindered or that tighter binding of Na⁺ to the mutant transporter did not result in a conformational change that promotes bile acid binding and, consequently, translocation.

### Table 1

| Mutations | $K_{TCA}$ (mM) | $I_{max}$ (pmol/mg protein) |
|-----------|----------------|-----------------------------|
| C270A     | 17.80 ± 2.365  | 2623 ± 106.1                |
| Q75C      | 1097.0 ± 688.0* | 584.9 ± 319.6*              |
| P76C      | 24.05 ± 5.58   | 8072 ± 4.79*                |
| M79C      | 60.45 ± 11.35  | 1579 ± 12.48*               |
| G83C      | 478.8 ± 199.4* | 676.1 ± 213.6*              |
| D91C      | 17.75 ± 1.83   | 442.1 ± 13.89*              |
| I92C      | 13.38 ± 2.92   | 479.5 ± 29.37*              |
| Q96C      | 18.6 ± 3.58    | 640.1 ± 3.79*               |

* $p < 0.001$.  
* $p < 0.05$.  

### Table 2

| Mutations | $K_{Na^+}$ (mM) | $I_{max}$ (pmol/mg protein) |
|-----------|----------------|-----------------------------|
| C270A     | 14.22 ± 3.03   | 544.3 ± 33.08               |
| L73C      | 6.52 ± 1.52    | 375.7 ± 19.09*              |
| G77C      | 16.01 ± 2.99   | 529.9 ± 29.2                |
| M79C      | 162.9 ± 35.59* | 26.25 ± 6.43*               |
| L86C      | 32.9 ± 5.65    | 328.5 ± 20.16*              |
| F90C      | 18.51 ± 6.06   | 215.5 ± 22.15*              |
| D91C      | 5.197 ± 1.98   | 68.8 ± 5.20*                |
| I92C      | 4.50 ± 1.32    | 195.8 ± 11.43*              |
| Q96C      | 3.30 ± 2.16    | 27.07 ± 3.11*               |

* $p < 0.001$.  

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**Involvement of hASBT TM2 in Sodium Translocation**

![Graph showing TCA kinetics constants for selected TM2 mutants](image1)

![Graph showing Sodium kinetics parameters for selected TM2 mutants](image2)

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![Figure 3. Probing solvent accessibility of TM2 residues.](image3)

![Figure 4. Sodium sensitivity of TM2 residues.](image4)
Next, conservative and non-conservative mutations were made to Asp91 using WT_ASBT as the scaffold. D91A resulted in a total loss of function, whereas conservative mutations, D91E and D91N, preserved 20% activity (Fig. 6A). Asp91 is not fully conserved across species, and rASBT and mASBT feature a glycine in position 91. Here, a D91G mutation preserved 15% uptake activity of hASBT. Mutations at Asp91 neither significantly altered the total expression or the cell surface presence of the mutant protein (Fig. 6B). To further evaluate the role of Asp91 in hASBT function, we determined Na⁺/H⁺ kinetics of these mutants (Fig. 7). The Na⁺ binding affinity for D91E and D91N was similar to that of the WT transporter with similar Hill coefficients (Table 3), yet mutations resulted in a significant decrease in transporter activity. Interestingly, the Hill coefficient of D91G was nearly 1, suggesting a possible loss of one of the two sodium binding sites or translocation pathways.

**TM2/TM3 May Form a Na⁺ Translocation Pathway**—To gain insight into the structural arrangement of the residues and D91N, preserved ~20% activity (Fig. 6A). Asp91 is not fully conserved across species, and rASBT and mASBT feature a glycine in position 91. Here, a D91G mutation preserved ~15% uptake activity of hASBT. Mutations at Asp91 neither significantly altered the total expression or the cell surface presence of the mutant protein (Fig. 6B). To further evaluate the role of Asp91 in hASBT function, we determined Na⁺/H⁺ kinetics of these mutants (Fig. 7). The Na⁺ binding affinity for D91E and D91N was similar to that of the WT transporter with similar Hill coefficients (Table 3), yet mutations resulted in a significant decrease in transporter activity. Interestingly, the Hill coefficient of D91G was nearly 1, suggesting a possible loss of one of the two sodium binding sites or translocation pathways.
involved in sodium binding, an analysis was performed on the explicit membrane molecular dynamics (MD) simulations of the hASBT dimer. The structural model of the hASBT dimer was developed using an elaborate homology modeling scheme followed by refinement using both implicit and explicit protein-membrane MD simulations. The functional significance of the dimer model was evaluated by comparing it with mutagenesis results. Cross-linking experiments verified the presence of the functional dimer predicted by in silico modeling. In this study we perform additional analysis on the trajectory obtained from dimer MD simulations. The MD simulation was able to capture two sodium binding sites, one in each monomer. High sodium distribution was observed around residue Asp91 (Fig. 8). This observation correlated well with the experimental results. In addition, the sodium-sensitive residues Gln75/Phe76, Met79, Thr82, Leu86, and Phe90/Asp91 in TM2 are in line and are facing previously reported sodium-sensitive residues, Thr134, Leu138, and Thr149, from TM3 (Fig. 9). Therefore, a consortium of these residues may form the sodium translocation pathway.

**Conservation of TM2 Sodium-sensitive Residues in ASBT**

Despite low sequence identity (24%) between hASBT and recently crystallized ASBT, sequence alignment indicates 60% overlap between TM2 of hASBT in a 7TM mammalian topology model and TM3 of ASBT (Fig. 10A). Both TMs span the plasma membrane in the same direction (intracellular to exofacial). Furthermore, the sodium-sensitive residues in TM2 of hASBT, Gln75/Phe76, Met79, Thr82, Leu86, are conserved in ASBT and reveal clear α-helical periodicity, facing TMs 5 and 8 (Fig. 10B). The ASBT crystal structure captured two bound Na+ ions, one of which interacts with five coordinating amino acids (Fig. 10C), including Gln75 (corresponding to residue Gln75 in hASBT). Combining these data, we propose that sodium-sensitive residues may play a role in sodium translocation either directly, by interacting with sodium, or indirectly, by perturbing the conformation of adjacent TM helices.

**DISCUSSION**

In the absence of a mammalian crystal structure, various biochemical approaches have been utilized to characterize hASBT...
at the molecular level. However, there is paucity of information on the role of TM2 and its constituent amino acids on the expression, structure, and function of hASBT. This study applied a targeted and systematic analysis of TM2 using the substituted cysteine accessibility method. Our findings suggest that TM2 may not be directly involved in bile acid substrate translocation, but we clearly demonstrate that several residues of TM2 are sensitive to changes in Na\(^+\)/H\(^+\) concentration; in particular, Asp\(^91\) may provide a Na\(^+\)/H\(^+\) binding/interaction site. Sodium-sensitive residues are positioned on one face of the membrane helix, indicating a putative Na\(^+\)/H\(^+\) translocation pathway. In silico simulations reveal high sodium distribution around residue Asp\(^91\), which further corroborates experimental findings.

TM2 has a high degree of sequence conservation among species (Fig. 1B), suggesting an important role in ASBT function and/or structure. It is noteworthy that a GXXP motif exists within TM2 that has been reported to provide a hinge-bending motion in the transmembrane regions of integral membrane proteins (32, 33). In fact, cysteine substitution of Pro\(^80\) resulted in a severe loss of function (Fig. 2C), suggesting a structural role for TM2 in hASBT function.

Conservative and non-conservative mutations were utilized to evaluate the functional importance on a WT_ASBT background of residues that were either sodium-sensitive or had loss of function. We found that residues Cys\(^74\), Gln\(^75\), Phe\(^76\), Pro\(^80\), Phe\(^89\), Asp\(^91\), Ile\(^92\), and Pro\(^94\) (mutated using WT_ASBT as the template) are critical for hASBT function (Fig. 5A). To further delineate the mechanism underlying altered transport activity of these residues, we performed detailed kinetic analysis. Mutants F76C, M79C, D91C, and I92C had decreased \(J_{\text{max}}\), yet only M79C appeared to have significantly altered TCA binding affinity (\(K_m\)) compared with C270A control (Table 1). Even though TCA has affinity for these mutant transporters, they reveal significantly lowered turnover rates, perhaps by mutation-induced conformational changes leading to a perturbed
transport cycle. We next hypothesized that reduced uptake rates could be caused by altered Na\(^+\) binding (affinity). M79C and L86C had significantly increased \(K_{Na^+}\), accompanied by significant decreases in \(J_{\text{max}}\). Interestingly, L73C, F76C, I92C, and Q96C all demonstrate higher affinity to sodium than C270A yet significantly lower \(J_{\text{max}}\) (Table 2), suggesting that these residues are involved in Na\(^+\) interactions. Mutating Asp\(^{91}\) to either glutamate or asparagine (conservative mutations) using WT_ASBT as the scaffold did not affect \(K_{Na^+}\) or the Hill coefficient; however, mutation resulted in significantly lower \(J_{\text{max}}\) values (Table 3). A Hill coefficient of 1 or lower for D91A and D91G suggests that mutations have affected Na\(^+\) binding to the transporter, whereas charge and size of the side chain at position 91 appears to be critical for interaction with Na\(^+\).

Analysis of sodium distribution profiles on our 7TM homology model revealed sodium binding regions in the N terminus (Asp\(^{9}\)), EL1 loop (Asp\(^{120}\), Asp\(^{122}\)), EL3 loop (Glu\(^{261}\)), and TM2 (Asp\(^{91}\)) regions. In agreement with experimental studies, MD simulations were able to capture the binding of sodium ions at Asp\(^{91}\) in one of the monomers. In view of the dimer model, this translates to sodium binding at two Asp\(^{91}\) sites, one for each monomer. The presence of two binding sites agrees with the 2:1 stoichiometric ratio. Although MD simulations were able to identify the possible initiation of the sodium translocation event, they could not capture the transfer of sodium ions from the extracellular to intracellular milieu. However, this information can be gathered from mapping the sodium-sensitive residues onto the dimer model. Seven additional cysteine mutants in TM2 (Q75C, F76C, M79C, G83C, L86C, F90C, and D91C) exhibited substantial sodium sensitivity, revealing a possible pathway for sodium translocation that faces the interior of the 7TM assembly. It is interesting to note that none of the residues face the membrane-bound side of the 7TM assembly, again in accordance with experimental data. The identification of two other key residues, Thr\(^{134}\) and Thr\(^{149}\), in TM3 that also exhibited sodium sensitivity strengthens the hypothesis of the predicted translocation pathway via the 7TM assembly. Thus, although the negative charge on Asp\(^{91}\) provides the focal point to initiate the uptake of sodium from the extracellular milieu, the subsequent translocation is governed by possible cation-\(\pi\) (Phe\(^{30}\), Phe\(^{76}\)) and cation-polar residue (Met\(^{79}\) and Gln\(^{75}\) in TM2, Thr\(^{134}\) and Thr\(^{149}\) in TM3) interactions and supported by few non-polar residues (Gly\(^{83}\), Lys\(^{86}\)) to complete the translocation pathway (Fig. 9).

Recently, a crystal structure of a putative bacterial homologue of ASBT from Neisseria meningitides, ASBT\(_{\text{NM}}\), was solved at 2.2 Å resolution (10). The crystal structure reveals a 10TM topology contrary to the systematically validated mammalian 7TM model used in this study. It has been argued that ASBT\(_{\text{NM}}\) may not be a member of the SLC10A family (34) due to its relatively low amino acid identity (~24%) compared with evolutionarily closer homologues (SLC10A3-6) that do not transport bile salts. Regardless, TM2 of hASBT shares ~60% sequence similarity with its proposed counterpart (TM3) in ASBT\(_{\text{NM}}\) (Fig. 10A), most notably the sodium-sensitive residues Gln\(^{75}\)/Phe\(^{76}\), Met\(^{79}\), Thr\(^{82}\), and Leu\(^{83}\) are conserved in ASBT\(_{\text{NM}}\). In addition, the corresponding residues in ASBT\(_{\text{NM}}\) line a discrete face along TM3 facing TM5 and TM8. The side chain of residue Gln\(^{77}\), corresponding to residue Gln\(^{75}\) in hASBT, was shown in the crystal structure to accommodate one of the two Na\(^+\) ions (Fig. 10C), in agreement with our hypothesis. Combining these observations, we conclude that the sodium translocation mechanism proposed here is conserved between hASBT and ASBT\(_{\text{NM}}\) despite their remote evolutionary distance and highly divergent membrane topologies.

In conclusion, we report the central role of TM2 in hASBT function. Both experimental and computational analyses converge on the same understanding that residues of TM2, in particular Phe\(^{80}\)/Asp\(^{91}\) and Gln\(^{75}\)/Phe\(^{76}\), may interact with Na\(^+\) ions. Integrating the present data with our previous structure-function studies as well as structural information from its remote bacterial homolog, we speculate that the negative charge on Asp\(^{91}\) may initiate long-range electrostatic interactions with Na\(^+\) ions in cooperation residues from EL1 and EL3. Furthermore, the interface between TM2 and TM3 may form a Na\(^+\) translocation pathway. Because sodium-sensitive residues are conserved across species and are present in the alleged bacterial homolog ASBT\(_{\text{NM}}\), we conclude that the proposed sodium translocation mechanism may be universal to the SLC10A transporter family.

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