Serines 260 and 288 Are Involved in Sulfate Transport by hNaSi-1*

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The low affinity Na+/sulfate cotransporter, NaSi-1, belongs to the SLC13 family that also includes the Na+/dicarboxylate cotransporters, NaDC. Two serine residues in hNaSi-1, at positions 260 and 288, are conserved in all of the sulfate transporters in the family whereas the NaDC contain alanine or threonine at those positions. Therefore, the functional roles of serines 260 and 288 in substrate and cation binding by hNaSi-1 were investigated. These two serine residues were first mutated to alanine and the mutants were characterized in Xenopus oocytes. Alanine substitution of Ser-260 resulted in increased \( K_m \) values for both substrate and \( \text{Na}^+ \) whereas alanine replacement at Ser-288 resulted in a broadened cation selectivity, indicating that these two serines might play important roles in cation and/or substrate binding of hNaSi-1. The two serines and 12 surrounding residues were further mutated to cysteine and studied using a thiol-reactive compound, [2-(trimethylammonium)ethyl]methane-thiosulfonate (MTSET). Four mutants surrounding Ser-260 (T257C, T259C, T261C, and L263C) were sensitive to MTSET inhibition. The sensitivity to MTSET was dependent on the presence of substrate, suggesting that the accessibility of these substituted cysteines depends on the conformational state of the transporter. Because the four residues are located in transmembrane domain 5, this transmembrane domain is likely to participate in the conformational movements during the transport cycle of hNaSi-1.

Sulfate is an essential inorganic anion because of its involvement in many physiological and pathophysiological processes (1, 2). The low affinity Na+/sulfate cotransporter from kidney, NaSi-1, reabsorbs sulfate across the luminal membrane of renal proximal tubule cells and plays an important role in maintaining sulfate homeostasis (3). NaSi-1 belongs to the SLC13 (Solute Carrier 13) gene family, which also includes the high affinity Na+/sulfate cotransporter (SUT-1) and the Na+/dicarboxylate cotransporters (ex. NaDC-1-3) (4). The transport of sulfate by NaSi-1 is electrogenic involving the cotransport of 3 \( \text{Na}^+ \) ions with one divalent sulfate anion (5). Although the function of NaSi-1 is relatively well characterized, there is little known about the structure or location of substrate and cation binding sites.

The NaSi-1 transporters share about 43% sequence identity with NaDC-1 transporters, whose structure-function relationships have been studied in more detail. Previous chimera studies of rabbit NaDC-1 and rat NaSi-1 showed that the substrate recognition site of these two transporters is located in the C-terminal portion of the protein, from transmembrane domains (TMD) \(^5\) to \(^11\) (6). Furthermore, at least one of the three \( \text{Na}^+ \) binding sites in NaDC-1 is located close to the substrate-binding site in TMDs \(^7\), \(^10\), and \(^11\) (7). Functionally important residues have been identified in TMDs \(^7\), \(^8\), and \(^9\) of NaDC-1, and cysteine-scanning mutagenesis showed that TMD-9 may be involved in transducing conformational changes between the cation-binding sites and the substrate-binding site (8–10).

In this study, the functional role of two serine residues in hNaSi-1 was investigated. Serines 260 and 288 are conserved in all of the sulfate transporters in the SLC13 superfamily, but the dicarboxylate transporters have alanine or threonine at those positions. Alanine replacement at Ser-260 resulted in increased \( K_m \) values for both sulfate and \( \text{Na}^+ \) whereas the S288A mutant had broadened cation selectivity, indicating that these two serine residues are important for cation and/or substrate binding. The residues surrounding Ser-260 are also functionally important residues. Four cysteine-substituted mutants surrounding Ser-260 (T257C, T259C, T263C, and L263C) were sensitive to [2-(trimethylammonium)ethyl]methane-thiosulfonate (MTSET) inhibition, depending on the conformational state of the transporter. Sensitivity was greatest in the presence of \( \text{Na}^+ \) and all four residues showed substrate protection. Since these residues are located near the extracellular face of TMD-5, the results suggest that TMD-5 is likely to move during the conformational changes associated with the transport cycle of hNaSi-1.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The mutants in this study were prepared using the oligonucleotide-directed mutagenesis method of Kunkel (11). The wild-type hNaSi-1 was used to prepare single-stranded DNA, which was rescued using M13K07 helper phage and then used as a template for mutagenesis using the Mutagene \( \text{in vitro} \) mutagenesis kit (BioRad), according to manufacturer’s instructions. The sequence of the mutants was verified by the Sealy Center for Molecular Science (University of Texas Medical Branch).

Xenopus Oocyte Injections—The wild-type or mutant hNaSi-1 cDNA in pSPORT1 plasmid was linearized with XbaI, purified using RNase-free Chroma-spin 1000 columns (Clontech), and used as a template for \( \text{in vitro} \) cRNA transcription using the T7 \text{mMessage mMachine kit} (Ambion) (12). The cRNA was resuspended in RNase-free water to a final concentration of 1 \( \mu \text{g/\mu l} \). Stage V and VI oocytes were prepared from adult female \( \text{Xenopus laevis} (\text{Xenopus I}) \) and injected with 50 nl of cRNA the following day as described previously (13). Oocytes were then stored at \( 18^\circ \text{C} \) in Barth’s solution supplemented with 100 \( \mu \text{g/\mu l} \) gentamycin sulfate, 50 \( \mu \text{g/ml} \) tetracycline, 2.5 \( \mu \text{m} \) sodium pyruvate, and 5% heat-inactivated horse serum. The medium and culture vials were changed daily.

Transport Measurements—Uptakes of \(^{35}\text{S}\)sulfate (PerkinElmer Life

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1 The abbreviations used are: TMD, transmembrane domain; PBS, phosphate-buffered saline; MTSET, [2-(trimethylammonium)ethyl]methane-thiosulfonate; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.

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were measured in *Xenopus* oocytes 3 or 4 days after cRNA injection as described previously. Each group of 5 oocytes was rinsed with room temperature choline buffer (in mM: 100 choline Cl, 2 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH adjusted to 7.5 using 1M Tris). Uptakes were initiated by adding 0.4 ml of transport buffer containing [³⁵S]sulfate in Na/H₁₅₀₀₀₀ buffer (same as choline buffer with 100 mM NaCl in place of choline Cl). After 10- or 15-min incubation, 3/₄ ml ice-cold choline buffer were added to stop the reaction and wash the oocytes. Individual oocytes were dissolved in 0.25 ml of 10% SDS and the radioactivity was counted in a scintillation counter. For most of the experiments, sulfate uptakes in uninjected oocytes have been subtracted from uptakes in cRNA-injected oocytes.

In kinetic experiments, 10-min uptakes were measured with increasing concentrations of sodium sulfate in the presence of 100 mM Na⁺. Previous experiments showed that uptakes of 1 mM sulfate by wild-type hNaSi-1 were linear to at least 60 min. The uptake rates were fitted to the Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]

where \([S]\) is the concentration of sulfate, \(V_{\text{max}}\) is the maximum uptake rate and \(K_m\) is the concentration of sulfate that produces 0.5 \(V_{\text{max}}\). In Na⁺ activation experiments, 10-min sulfate uptakes (as the potassium salt) were measured with different concentrations of Na⁺, prepared by replacing NaCl with choline. The data were fitted to the Hill equation:

\[ v = \frac{V_{\text{max}} [Na^+]^n}{K_{Na}^n + [Na^+]^n} \]

where \([Na^+]\) is the concentration of Na⁺, \(K_{Na}\) is the half-saturation constant for Na⁺ and \(n\) is the Hill coefficient. Data were fitted by non-linear regression to the Michaelis-Menten and Hill equations using Sigma Plot 2000 (SPSS).

For the cation selectivity experiments, uptakes of sulfate (potassium salt) in Li⁺, Cs⁺, K⁺, or choline buffer were measured by replacing 100 mM NaCl with 100 mM of LiCl, CsCl, KCl, or choline Cl while keeping other components in the buffer the same. For substrate specificity experiments, uptakes of 10 μM [³⁵S]sulfate were measured in the absence or presence of 1 mM test inhibitors. Sulfate uptakes in the presence of...
ence of inhibitors are presented as a percentage of uptakes in the absence of inhibitors. Statistical analysis of one-way analysis of variance or Student’s t test was performed using SigmaStat software (Jandel, Version 1.0).

MTSET Experiments—Oocytes were washed three times with room temperature choline buffer and then preincubated in [2-(trimethylammonium)methyl]methane-thiosulfonate (MTSET) in Na⁺ or choline buffer with or without 5 mM sulfate added. Control groups were preincubated in the same buffers without MTSET. The MTSET solution was prepared immediately prior to use. After 10 min incubation at room temperature, the MTSET solution was washed away with 4 × 4 ml washes of choline buffer. The uptake of 100 μM [35S]sulfate was measured as described above. The data are presented as a percentage of uptake by oocytes preincubated in the absence of MTSET.

MTSET is an irreversible inhibitor and the data were modeled according to reversible non-competitive inhibition, which resembles irreversible inhibition kinetically. The data were fitted to %

\[ \%i = \left( \frac{i_{\text{max}} \times [I]}{IC_{50} + [I]} \right) \]

where \( \%i \) is the % inhibition at a given inhibitor concentration, \( i_{\text{max}} \) is the maximal percent inhibition, \( IC_{50} \) is the concentration of inhibitor that produces half-maximal inhibition, and [I] is the concentration of inhibitor. Pseudo first-order rates of inactivation, \( k \) (in min⁻¹ mM⁻¹), were estimated from the IC₅₀ values where 0.5 = \( e^{-k \times [IC_{50}]^{-1}} \) where 0.5 represents 50% activity remaining after MTSET treatment and \( t \) is the time of MTSET incubation in minutes.

Biotinylation of Oocytes and Western Blots—Oocytes were biotinylated using the membrane impermeant reagent, Sulfo-NHS-LC-Biotin (Pierce), and then precipitated with ImmunoPure Streptavidin (Pierce) at 4 °C overnight as described previously. The biotinylated proteins were then separated by SDS-PAGE on 6% Tricine gels and transferred to nitrocellulose membranes (0.45 μm, Schleicher & Schuell Inc.) (14). The blots were blocked with PBS-TM (PBS containing 0.5% Carnation instant dried milk and 0.05% Tween 20) at room temperature for 1 h or at 4 °C overnight and then incubated for 2 h with 1:1000 dilution of primary antibodies in PBS-TM at room temperature and then washed three times with PBS-TM. The primary antibodies recognize a 65 amino acid peptide of hNaSi-1. The secondary antibody, horseradish peroxidase-linked anti-rabbit IgG from donkey (Amersham Biosciences), was diluted to 1:5000 in PBS-TM and applied to the blots at room temperature for 1 h, and then the blots were washed three times with PBS. Immunoreactive protein signals were detected using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce) and then precipitated with Immunopure Immobilized Streptavidin (Pierce) at 4 °C and visualized using a Kodak Image Station 440CF (Eastman Kodak Company) with 6 captures of 5 min to prevent saturation of the image. The mass of hNaSi-1 was estimated by comparison with chemiluminescent protein size standards (MagicMark Western Standard, Invitrogen).

RESULTS

Expression of S260A and S280A Mutants—The sequence alignment of hNaSi-1 with the other members of the SLC13 superfamily shows that serine residues 260 and 288 (numbered according to the hNaSi-1 sequence) are conserved in the Na⁺/sulfate transporters, but alanine or threonine is found in the Na⁺/dicarboxylate transporters (Fig. 1A). Therefore, alamines were substituted for serines 260 and 288 in hNaSi-1. These serines are located near the extracellular face of TMD-5 and -6 (Fig. 1B). As verified in a single Western blot experiment, both mutant proteins were expressed in Xenopus oocytes and appeared more abundant than the wild-type. However, the activity of S260A was greatly reduced compared with wild-type hNaSi-1 and S288A (Fig. 2A).

Kinetic Properties of S260A and S288A—The mean values of \( K_{\text{Na}} \) and \( V_{\text{max}} \) in wild-type hNaSi-1 are 340 ± 50 μM and 4153 ± 362 pmol/oocyte-hr (means ± S.E., \( n = 4 \)), respectively, similar to what had been reported previously (15). The mean \( K_{\text{Na}} \) for sulfate was significantly increased (\( p < 0.05 \)) to 1123 ± 223 μM in S260A compared with wild-type (mean ± S.E., \( n = 3 \)). The sulfate \( K_{\text{Na}} \) in S288A, 429 ± 144 μM (\( n = 3 \)), was not significantly different from that of wild-type. The two mutants had a similar or lower value of \( V_{\text{max}} \) compared with the wild-type, 2539 ± 252 pmol/oocyte-h for S260A and 3651 ± 492 pmol/oocyte-h for S288A. Fig. 3A shows sulfate kinetics in wild-type and mutant hNaSi-1 with the mean uptake values from three experiments normalized to \( V_{\text{max}} \). The results suggest that alanine replacement at Ser-260 produces a decreased apparent affinity of hNaSi-1 for substrate, whereas alanine replacement at Ser-288 does not affect the apparent affinity.

Na⁺ Activation of Sulfate Uptakes—As seen previously, there was a sigmoidal dependence of sulfate uptake on Na⁺ concentration (Fig. 3B), consistent with a transport mechanism involving three sodium ions (5). The \( K_{\text{Na}} \) for wild-type hNaSi-1 was 26 ± 3 mM and the Hill coefficient was 1.8 ± 0.4 (mean ± range, \( n = 2 \)). In a single experiment, the S260A mutant showed a greatly decreased sodium affinity but it was not possible to obtain an accurate kinetic constant because the kinetic curve did not show saturation at the highest concentration of sodium (100 mM) tested whereas the S288A mutant appeared to have an increase in Na⁺ affinity with a \( K_{\text{Na}} \) of 15 ± 2 mM (mean ± S.E. of regression).

Cation and Substrate Specificity—The cation selectivity of the two mutants and hNaSi-1 was measured by replacing the 100 mM Na⁺ buffer with Li⁺, Cs⁺, K⁺, or choline.
Both the wild-type hNaSi-1 and S260A mutant exhibited a strict cation selectivity with transport activity only in the presence of Na\(^+\) but not the other monovalent ions. Interestingly, the S288A mutant appeared to have a broadened cation selectivity. Relative uptakes in cations other than Na\(^+\) were significantly higher in S288A than in the wild-type or buffer (Fig. 4).

![Graph A](image1.png)

**Fig. 3.** Kinetic properties of wild-type hNaSi-1 or S260A and S288A mutants expressed in *Xenopus* oocytes. A, sulfate kinetics. 10-min uptakes were measured with increasing concentrations of [\(^{35}\)S]sulfate in the presence of 100 mM Na\(^+\). Each data point represents the mean ± S.E. of uptake rates of experiments with three separate batches of oocytes. The data have been normalized to the *V* \(_{\text{max}}\) values for each group. B, sodium activation of sulfate uptakes. 10-min uptakes of 100 \(\mu\)M [\(^{35}\)S]sulfate were measured in the presence of increasing concentrations of Na\(^+\). Each data point represents the mean ± S.E. of uptake rates of 5 oocytes. The data were fit to the Hill equation. The \(K_Na\) values are 30 mM (wild-type), 80 mM (S260A), and 15 mM (S288A). The maximum uptake rates, \(V_{\text{max}}\), are (in pmol/oocyte-h): 1921 (wild-type), 905 (S260A), and 1736 (S288A). The Hill coefficient is 1.37 for wild-type, 1.59 for S260A, and 1.35 for S288A.
S260A (p < 0.05). The rate of sulfate uptake measured in Li+ in S268A was ~28% of that measured in sodium buffer (Fig. 4).

Substrate specificity was determined by measuring the inhibition of uptakes of 10 μM sulfate by 1 mM concentrations of test inhibitors. There were no differences in substrate specificity between wild-type hNaSi-1 and the S260A and S288A mutants (results not shown). Similar to previous findings (15), our studies showed that hNaSi-1 has a broad substrate specificity for anions with structures that are similar to sulfate, such as chromate (CrO4^{2-}), thiosulfate (S2 O3^{2-}), selenate (Se O4^{2-}), and sulfite (S O3^{2-}). There was no inhibition by phosphate, probenecid, or an inhibitor of Na+/dicarboxylate transport, fursemide (16). There was minor inhibition by a second NaDC-1 inhibitor, flufenamate (16). Interestingly, we saw no inhibition by succinate although prior studies suggested that succinate could inhibit hNaSi-1 (15).

Cysteine-scanning Mutagenesis around Ser-260 and Ser-288—Wild-type hNaSi-1 is sensitive to inhibition by the membrane-permeant cysteine reagent, (2-aminomethyl) methanethiosulfonate (MTSEA), but insensitive to the membrane-impermeant reagents (2-sulfonatoethyl)methanethiosulfonate (MTSES) and MTSET (data not shown). MTSEA and MTSET both add positive charges and MTSES adds a negative charge to the protein (17). The related protein, NaDC-1, loses activity in proportion to the number of endogenous cysteines that are removed (18). Our previous study showed that there were no differences in MTSET sensitivity between cysteine mutants prepared in a wild-type NaDC-1 and cysteine-reduced (3 cysteines) background (9). Therefore, we used wild-type hNaSi-1 as the parental protein for cysteine scanning mutagenesis around Ser-260 (residues 257–263) and Ser-288 (residues 285–291). A total of 14 residues, including Ser-260 and Ser-288, were mutated to cysteine one at a time (Fig. 1). As shown in Fig. 5, all of the cysteine mutant proteins were expressed in Xenopus oocytes, but all except A291C had greatly reduced or no activity. Five of the mutants, G258C, S260C, N262C, F285C, and P290C were completely inactive.

MTSET Sensitivity of Cysteine Mutants—The cysteine-substituted mutants were incubated with a relatively high 1 mM concentration of MTSET to identify sensitive residues. Only four mutants surrounding Ser-260 (T257C, T259C, T261C, and L263C) were sensitive to MTSET (results not shown). In contrast, S288C and the surrounding active mutants (T286C, F287C, F289C, and A291C) were all functionally insensitive to chemical modification by MTSET (results not shown).

The concentration dependence of MTSET inhibition for the four sensitive mutants (T257C, T259C, T261C, and L263C) was tested by measuring the sulfate uptake activity of each mutant after 10 min preincubation with increasing concentrations of MTSET (Fig. 6). T259C had the highest IC50 of about 900 μM, followed by T261C with an IC50 of 63 μM, T257C with an IC50 of 3 μM and L263C with an IC50 of 0.2 μM. The reactivity with MTSET is related to the accessibility of the cysteine, which

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**Fig. 4.** Cation selectivity of wild-type hNaSi-1 or S260A and S288A mutants expressed in Xenopus oocytes. 15-min uptakes of 100 μM [35S]sulfate were measured in 100 mM Na+, Li+, Cs+, K+, or choline buffer. The data are shown as a percentage of uptakes in Na+ buffer. The data are means ± S.E. (n = 4 or 5 oocytes). The asterisk indicates significant difference from wild-type within a given treatment (p < 0.05).

**Fig. 5.** Western blots and transport activity of Xenopus oocytes expressing the cysteine mutants surrounding Ser-260 and Ser-288 in hNaSi-1. A, Western blots of oocytes biotinylated with Sulfo-NHS-LC biotin. The biotinylated proteins were separated on 6% Tricine SDS gels and transferred to nitrocellulose membranes. The blots were probed with anti-hNaSi-1 antibodies as described under “Experimental Procedures.” B, uptakes of 100 μM [35S]sulfate in Na+ buffer were measured for 15 min. The uptake values were corrected by subtracting counts in control, uninjected oocytes. The data are presented as a percentage of uptakes by oocytes expressing wild-type hNaSi-1. The data shown in the figure are means ± range or S.E. of two or three experiments.
may be modified by the local environment surrounding the residue (19). For comparison, the pseudo first-order rate constants of inhibition, $k$ (in min$^{-1}$ mM$^{-1}$), were also estimated: 345 (L263C), 23 (T257C), 1 (T261C), 0.08 (T259C). The value of $k$ for T259C is probably the least accurate since there seems to be some activation at low MTSET concentrations and the maximal inhibition was not reached at the highest concentration of MTSET. However, the results suggest that the substituted cysteines at positions 263 and 257 are likely to be more accessible to MTSET, whereas the substituted cysteines at positions 259 and 261 are less accessible to MTSET.

### Cation and Substrate Dependence of MTSET Inhibition

The effect of cations and substrate on the MTSET modification of substituted cysteines is shown in Fig. 7. The concentration of MTSET was selected for each mutant based on the relative IC$_{50}$ values (T257C, 2.5 μM; T259C, 500 μM; T261C, 250 μM; L263C, 1 μM). All four mutants were equally sensitive to inhibition after preincubation with MTSET in Na$^+$ or choline buffer ($p < 0.05$). Although there appears to be a difference between Na$^+$ and choline in the L263C mutant, this was not significantly different ($p = 0.08$). All four mutants showed significant substrate protection in Na$^+$ but not choline buffer.

### Effect of Na$^+$ on Sulfate Kinetics in hNaSi-1

The changes in MTSET accessibility with different cations or substrate likely reflect changes in conformation in hNaSi-1. We assume that binding is ordered, with sodium binding before sulfate, which then triggers a conformational change that allows sulfate binding. Previous kinetic studies with rabbit renal brush border membrane vesicles have suggested that sodium binding precedes sulfate binding (20), but this model had not been tested using the cloned NaSi-1. The relationship between sulfate kinetic constants and sodium concentration in hNaSi-1 expressed in *Xenopus* oocytes was measured (results not shown). The sulfate $K_m$ was lowest in 100 mM Na$^+$ buffer (379 ± 66 μM, $n = 6$) compared with 20 mM Na$^+$ (707 ± 70 μM, $n = 3$) or 40 mM Na$^+$ (687 ± 23 μM, $n = 2$) (mean ± S.E.). The maximum uptake rates, $V_{max}$, of uptakes in 20 mM, 40 mM and 100 mM Na$^+$ buffer are (in nmol/oocyte-hr): 2.7 ± 1.5, 5.5 ± 2.4, and 3.1 ± 0.7, respectively. There was no significant difference in $V_{max}$ values but there was also considerable variation within groups.

### DISCUSSION

The goal of this study was to determine the functional roles of two serines, at positions 260 and 288, in the human Na$^+$/sulfate cotransporter, hNaSi-1. These residues are conserved in all of the sulfate transporters of the SLC13 superfamily but alanine or threonine is found at those sites in the dicarboxylate transporters. Serine 260 appears to be important for both substrate and cation binding. Furthermore, the residues surrounding Ser-260 at the outer surface of TMD-5 appear to change their accessibility to the extracellular medium depending on the conformational state of the protein. In contrast, Ser-288 and the surrounding amino acids are probably required to maintain the structure of the protein and substitution of these
residues results in decreased uptake activity. Ser-288 does not appear to be involved directly in substrate or cation binding, and there are no functional consequences of chemical modification of cysteines in TMD-6 with MTSET.

Serines were chosen for mutagenesis because previous studies have suggested that hydroxyl groups of serines can participate in substrate or cation binding in transport proteins (21–24). Although hNaSi-1 contains a total of 42 serine residues, only two of these, Ser-260 and Ser-288, are conserved in the sulfate but not the dicarboxylate transporters of the SLC13 superfamily. Since the transport mechanism in all members of this family is similar, involving three sodium ions and one divalent anion substrate, differences in sequences might identify residues responsible for differences in substrate selectivity. Our previous study showed that the substrate recognition domain in NaDC-1 and NaSi-1 is found in the C-terminal half of these transporters, past amino acid 141 (6). Therefore, we hypothesized that serines 260 and 288 might be involved in determining substrate selectivity or binding.

The results of this study suggest that Ser-260 is likely to be involved in substrate and/or cation binding. Replacement of Ser-260 by alanine resulted in decreases in apparent affinity for both substrate and cations. One possible explanation is that Ser-260 is directly involved in the structure of the substrate and cation binding sites or it is located close to these binding sites. Our previous results with NaDC-1 have shown a close relationship between the cation and substrate binding sites, and single mutations often affect both (8). Another possibility is that Ser-260 participates only in sodium binding, but since sodium is an essential activator of transport, changes in sodium affinity are reflected as changes in substrate affinity. The sodium activation experiment with S260A showed that uptakes were measured at subsaturating sodium concentration, since the curves did not show saturation even at the highest sodium concentration tested. In hNaSi-1, we found that changes in sodium concentration affect the \( K_m \) for substrate with an increased \( K_m \) at subsaturating concentrations. The finding that the S260A mutation produces a change in sodium binding was a bit surprising since we would expect that the dicarboxylate and sulfate transporters would share sodium binding sites. But since binding information is transduced between sodium and sulfate binding sites, these sites should be located close together.

Three of seven mutants around Ser-260 (G258C, S260C and N262C) completely lost transport activity, whereas the other four mutants (T257C, T259C, T261C, and L263C) exhibited

![Fig. 7. Cation and substrate dependence of MTSET inhibition of sulfate uptakes by T257C, T259C, T261C, or L263C.](image-url)
Serines 260 and 288 of hNaSi-1 transporter. T represents the transporter with cation and substrate binding sites accessible from the outside (T/) or from the inside (T). The binding of 3 Na\(^+\) ions triggers a conformational change that increases the affinity for sulfate. Sulfate binding to the transporter results in another conformational change in which the fully loaded transporter translocates cations and substrate to the inside of the cell. The cations and substrate are released inside the cell (note that there is no experimental evidence supporting any particular order of release) and the transporter reorient to the outward-facing conformation.

Serine 288 does not play as critical a role in transport in hNaSi-1 compared with Ser-260. Alanine substitution of Ser-288 resulted in an increased apparent affinity for cations and the ability to transport sulfate in the presence of lithium. It is not likely that Ser-288 directly forms part of the cation binding sites because of the increased sodium affinity with alanine replacement. Therefore, it is possible that the hydroxyl group in the side chain of Ser-288 may form a hydrogen bond with another residue to support the structure of the cation-binding sites, rather than directly interacting with cations. Interestingly, all of the members of this family have the capacity to interact with lithium with very low affinity (4). Therefore, the fact that lithium can substitute for sodium probably reflects an increased binding affinity for all cations. Previous studies of the Na\(^+\)/K\(^+\)-coupled glutamate transporter, GLT-1, showed that glycine replacement at Ser-440 produced a broadened ion specificity but did not affect the $K_m$ for transportable substrates (24). Since Ser-440 in GLT-1 is likely to be near the substrate-binding site, it was suggested that the changed ion specificity may also be related to a small change in substrate binding produced by the mutation.

Sodium is an essential activator of sulfate transport in NaSi-1 and appears to bind prior to sulfate (see transport model, Fig. 8). In previous studies with renal brush border membrane vesicles, sodium affected the $K_m$ for sulfate but did not affect $V_{max}$ (20). Our studies here with the cloned hNaSi-1 expressed in oocytes also showed decreased $K_m$ with increasing sodium, with no significant change in $V_{max}$. In an ordered, rapid equilibrium system in which the activator binds prior to substrate binding, the $V_{max}$ is not affected by concentrations of the activator but the apparent $K_m$ for substrate increases with decreasing concentrations of the activator (26). The $V_{max}$ is not affected because it is possible to overcome the low concentration of activator with high enough substrate concentrations. In this study, exposure of hNaSi-1 to the presence or absence of sodium is likely to produce different conformational states in the transporter. In the absence of sodium (choline buffer) the transporter is most likely to be in the unloaded state with binding sites exposed either to the inside or outside of the cell (Fig. 8). When the cells are incubated in sodium buffer without substrate, the transporter is most likely to be in state 2 (Fig. 8).
Finally, in the presence of sodium and substrate, the transporter is likely to immediately translocate so that the binding sites are exposed to the inside of the cell and not accessible to MTSET (Fig. 8). A further supporting observation is the fact that choline + substrate produces the same results as choline alone. If the binding order allowed substrate to bind before sodium, it would be possible to see substrate protection in the absence of sodium. An alternate explanation for substrate protection that we cannot rule out at this point is that the substrate-binding site is located near the substituted cysteine, thus preventing access of MTSET.

MTSET modification of substituted cysteines in the four mutants, T257C, T259C, T261C, and L263C, was dependent on the presence of substrate. All four substituted cysteines appear to be accessible to MTSET at the same conformational states. These mutants were equally sensitive to MTSET modification in Na⁺ or choline buffer, and there was evidence of substrate protection. Therefore, accessibility of these substituted cysteines to MTSET is high in multiple conformational states, such as 1 (or 6) and 2 (Fig. 8). All four substituted cysteines in TMD-5 exhibited substrate protection. The simplest explanation for this is that substrate binding triggers a conformational change in the protein and the cysteine is no longer accessible to the MTSET. Alternately, the substituted cysteine is located near the substrate-binding site and substrate binding prevents MTSET accessibility.

The four substituted cysteines sensitive to MTSET are located near the extracellular face of TMD-5, on opposite sides of the helix (Fig. 9). The apparent changes in accessibility of these residues to MTSET modification suggest that TMD-5 is likely to be involved in the conformational changes during the transport cycle, either because it moves itself or other parts of the protein move to expose or occlude the residues. There is growing evidence from studies with lac permease that conformational changes involving the rotation and tilting of helices occur during the transport cycle (12). We previously reported similar conformationally-sensitive residues in TMD-9 of NaDC-1 (9).

In conclusion, the main findings of this study are that the conserved serines at positions 260 and 288 and the surrounding amino acids in TMDs 5 and 6 are functionally important in hNaSi-1. Serine 280 may be involved in indirectly maintaining the structure of the cation-binding site. Serine 260 at the outer face of TMD-5 appears to be more important functionally. It determines the $K_m$ for both substrate and cations. Furthermore, the residues surrounding Ser-260 appear to have different accessibilities to the extracellular medium depending on the presence or absence of substrate. Therefore, it is likely that TMD-5 is involved in the conformational changes during transport, either directly or indirectly.

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