Interactions between Charged Amino Acid Residues within Transmembrane Helices in the Sulfate Transporter SHST1†

Megan C. Shelden, Patrick Loughlin, M. Louise Tierney, and Susan M. Howitt*

School of Biochemistry and Molecular Biology, Faculty of Science, Australian National University, Canberra, ACT 0200, Australia

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ABSTRACT: The aim of this study was to identify charged amino acid residues important for activity of the sulfate transporter SHST1. We mutated 10 charged amino acids in or near proposed transmembrane helices and expressed the resulting mutants in a sulfate transport-deficient yeast strain. Mutations affecting four residues resulted in a complete loss of sulfate transport; these residues were D107 and D122 in helix 1 and R354 and E366 in helix 8. All other mutants showed some reduction in transport activity. The E366Q mutant was unusual in that expression of the mutant protein was toxic to yeast cells. The R354Q mutant showed reduced trafficking to the plasma membrane, indicating that the protein was misfolded. However, transporter function (to a low level) and wild-type trafficking could be recovered by combining the R354Q mutation with either the E175Q or E270Q mutations. This suggested that R354 interacts with both E175 and E270. The triple mutant E175Q/E270Q/R354Q retained only marginal sulfate transport activity but was trafficked at wild-type levels, suggesting that a charge network between these three residues may be involved in the transport pathway, rather than in folding. D107 was also found to be essential for the ion transport pathway and may form a charge pair with R154, both of which are highly conserved. The information obtained on interactions between charged residues provides the first evidence for the possible spatial arrangement of transmembrane helices within any member of this transporter family. This information is used to develop a model for SHST1 tertiary structure.

Plants are able to take up sulfate from the soil using specific, high-affinity transporters, which are energized by cotransport of the sulfate molecule with protons. The first sulfate transporters to be identified and cloned from a plant were from the tropical legume *Stylosanthes hamata* (*1*). *S. hamata* possesses three homologous sulfate transporters, SHST1 and SHST2, which are 95% identical at the amino acid level, and SHST3, which is 55% identical to SHST1 and SHST2 (*1*). All three transporters have been expressed and characterized in a sulfate transport-deficient yeast mutant. In this system, SHST1 and SHST2 have K_m values for sulfate of around 10 μM, and SHST3 has a 10-fold lower affinity. The different transporters have different expression patterns and are thought to have different roles within the plant (*2*). SHST1 and/or SHST2 are (is) strongly expressed in roots under conditions of sulfate deprivation, suggesting a major role in sulfate uptake under these conditions.

These transporters belong to the SulP family, members of which are found throughout the prokaryotic and eukaryotic kingdoms. Mutations in human homologues are responsible for the diseases Pendred syndrome, diastrophic dysplasia, and congenital chloride diarrhea (*3*). There is, however, little known about the structure and function of any member of this family. The expression of the plant sulfate transporter, SHST1, in yeast provides a useful system with which to study the properties of one member of the SulP family. Charged amino acid residues have been implicated in the functioning of a number of different transporters (*4*−*8*). Charged amino acids within transmembrane helices are likely to be required for structure or function because of the energy cost required to bury them within the membrane. For example, in the lactose permease from *Escherichia coli*, all charged amino acids within transmembrane helices have functional roles, having either a direct involvement in the transport mechanism or a structural role in forming charge pairs (*9*). Interactions between the charged amino acids in the lac permease have been proposed on the basis of studies involving mutagenesis and isolation of second site revertants (*9*). These interactions have recently been confirmed by the crystal structure of the lac permease (*10*), validating the mutagenesis approach. SHST1 contains a number of charged amino acid residues in putative transmembrane helices (Figure 1). The role of charged residues in members of the SulP family has not yet been analyzed, except in prestin, a molecular motor protein that is homologous to SulP transporters (*11*). However, this mutagenesis focused on nonconserved charges that may act as the voltage sensor, a function that is unique to prestin within the SulP family. It has been suggested that arginine residues may have an essential role in sulfate transporters from barley roots, based on chemical modification studies (*1*, *12*).

We have previously shown that the first three helices of SHST1 play an important role in sulfate transport (*13*). In addition, the first three transmembrane helices are more

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* Corresponding author. E-mail: Susan.Howitt@anu.edu.au. Phone: 61 2 6125 4356. Fax: 61 2 6125 0313.

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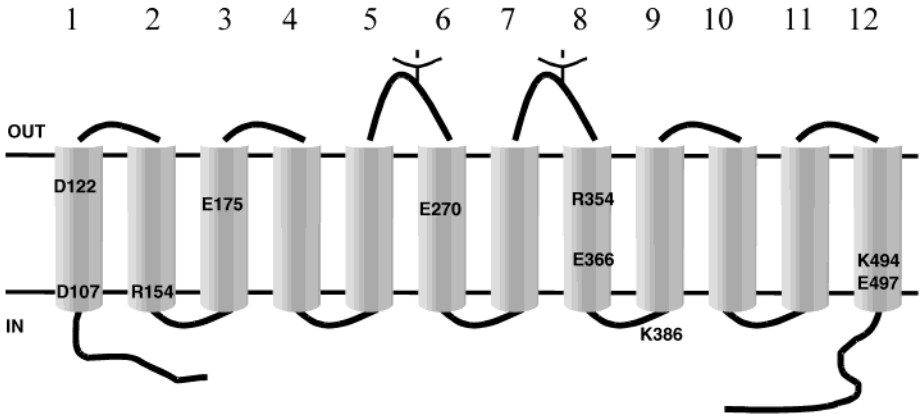

FIGURE 1: Proposed topology of SHST1 indicating the positions of charged amino acid residues altered in this study. Two putative glycosylation sites are also shown.

highly conserved than the protein as a whole. Charged amino acid residues are distributed throughout the putative transmembrane helices of SHST1, with a greater number of negative charges found. The predicted ends of many helices are also defined by charged amino acids. The aim of the present study was to use site-directed mutagenesis to investigate the roles of charged amino acid residues within transmembrane helices and to identify the interactions between them. We also mutated some conserved charged residues at the ends of helices in addition to those predicted to be within transmembrane helices. Previously, D107, a highly conserved amino acid located at the cytoplasmic border of helix 1 (Figures 1 and 2), had been mutated, resulting in the complete loss of sulfate transport activity (*14*). R154 at the cytoplasmic end of helix 2 was targeted because this residue, like D107, is conserved throughout the SulP family (Figure 2). K386, at the cytoplasmic end of helix 9, was mutated because a previous study had found that mutation of the adjacent residue, E387, had a severe effect on trafficking of SHST1, suggesting that loss of this charged residue resulted in misfolded protein (*15*).

MATERIALS AND METHODS

Molecular Biology. Standard procedures were used for bacterial plasmid isolation and transformation into *E. coli*. SHST1 in the yeast expression vector pYES3 (*16*) was used for site-directed mutagenesis, using the QuikChange method from Stratagene. Mutagenic oligonucleotides were 25−35 bases long and included the codon changes shown in Table 1. The entire *shst1* cDNA was sequenced following mutagenesis to confirm that no PCR-derived errors had arisen.

Yeast Growth and Transformation. *Saccharomyces cerevisiae* strain YSD1, which has a deletion in the native sulfate transporter gene, Sul1 (*16*), was used for expression of SHST1 and the mutants derived from it. Sulfate-free growth medium was prepared as previously described (*16*) and supplemented with either 38.26 mg/L homocysteine thiolactone or 100 μM sulfate and either 2% galactose or 2% glucose. Yeast transformation was by a LiCl/PEG method (*17*).

Sulfate Uptake Assays. [^{35}S]Sulfate uptake assays were based on those described by Smith et al. (*1*, *16*). Yeast cells were grown to mid log phase in sulfate-free media minus uracil and supplemented with homocysteine thiolactone and 2% galactose. Cells were then harvested by centrifugation at 5000*g* for 15 min, washed and resuspended in sulfur-free growth medium supplemented with galactose, and incubated at 30 °C for 15 min prior to the uptake measurements. Cells were then incubated with different concentrations of [^{35}S]sodium sulfate. Samples were withdrawn after 2 min, and the reaction was stopped by rapid centrifugation through a silicone oil layer into 5 μL of 40% perchloric acid. Radioactivity in the pellet was determined by liquid scintillation counting. Uptake by YSD1 transformed with pYES3 was subtracted from the data for each mutant. The Michaelis−Menten equation was fitted to the data obtained to give a K_m and V_{max}.

Methylammonium Uptake Assays. Uptake of [^{14}C]methylammonium was performed as previously described (*18*). Yeast cells were grown to mid log phase in nitrogen-free YNB media minus uracil, supplemented with 38.26 mg/L homocysteine thiolactone, 1% proline, and 2% glucose. Two aliquots of cells were collected by centrifugation and resuspended in either the same medium, as an uninduced control, or a similar medium containing 2% galactose instead of glucose to induce expression of SHST1. After 6 h, cells were harvested, resuspended in 0.1 M sodium phosphate buffer, pH 7, with 2% glucose, and incubated at 30 °C for 15 min. Cells were then incubated with 500 μM [^{14}C]methylammonium. Samples were withdrawn every minute for 4 min, and the reaction was stopped by rapid centrifugation through a silicone oil layer into 5 μL of 40% perchloric acid. Radioactivity in the pellet was determined by liquid scintillation counting.

Preparation of Plasma Membranes for Western Blotting. Yeast cells for membrane preparation were grown to mid log phase in sulfate-free media minus uracil and supplemented with homocysteine thiolactone and 2% galactose. Plasma membranes were separated from internal membranes by running cell lysates on a sucrose density gradient following the procedure of Katzmann et al. (*19*), as modified by Khurana et al. (*15*). Western blots of different fractions probed with antisera against marker proteins from different organelles demonstrated that there was good separation between the ER and the more dense plasma membrane (*19*). We confirmed that the plasma membrane fractions were separated from the endoplasmic reticulum by using antisera against BiP, an endoplasmic reticulum chaperone protein from yeast (*20*) (Figure 3). Most BiP was located in fractions 6−9, indicating the presence of ER in these fractions.

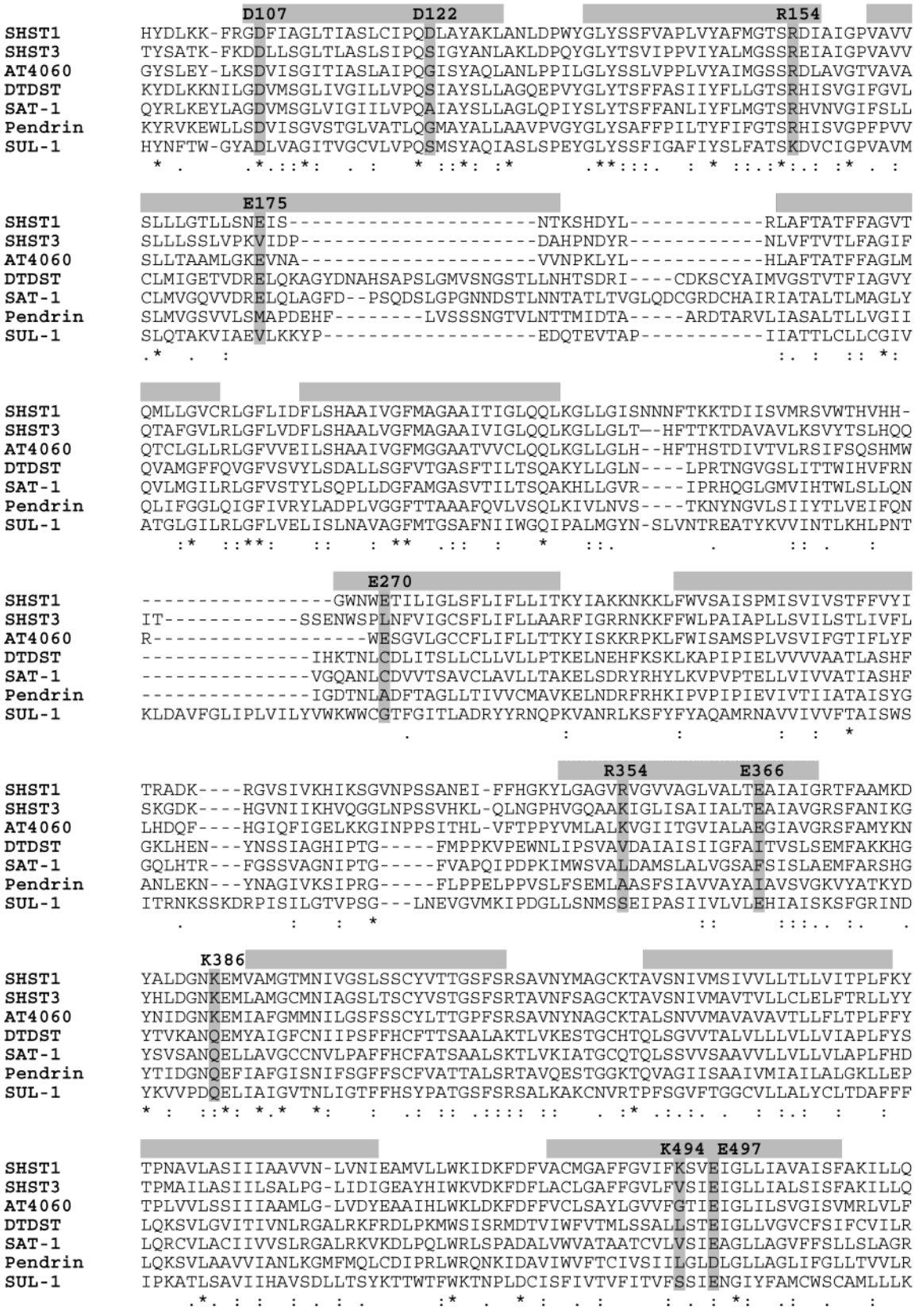

FIGURE 2: Alignment of selected SulP transporters. The transporter sequences were chosen to reflect different subgroups with the SulP family, and Clustal W was used to generate the alignment. Only the transmembrane regions are shown as the N- and C-terminal tails are quite divergent. Invariant residues are indicated by an asterisk, conserved residues by a colon, and semiconserved residues by a period. The residues mutated in this study are highlighted, and the number of the SHST1 residue is given. Predicted transmembrane helices for SHST1 are shown by a gray bar above the sequence; these may not correspond exactly to transmembrane helices for all of the other transporters shown. The transporters shown are as follows (GenBank accession number in parentheses): SHST1, *S. hamata* high-affinity sulfate transporter (P53391); SHST3, *S. hamata* low-affinity sulfate transporter (P53393); AT4060, *Arabidopsis thaliana* putative sulfate transporter (O04289); DTDST, human diastrophic dysplasia sulfate transporter (P50443); SAT-1, rat sulfate anion transporter (P45380); pendrin, human sodium-independent chloride/iodide transporter (O43511); SUL-1, *S. cerevisiae* high-affinity sulfate transporter (P38359).

Table 1: Mutations Introduced into SHST1

amino acid change	codon change	amino acid change	codon change
D107N	G(379)AT → AAT	R354Q	A(1120)GA → CAA
D122N	G(454)AT → AAT	E366Q	G(1156)AA → CAG
R154Q	A(520)GA → CAG	K386Q	A(1216)AA → CAG
E175Q	G(583)AG → CAA	K494Q	A(1540)AG → CAA
E270Q	G(868)AG → CAA	E497Q	G(1549)AA → CAA

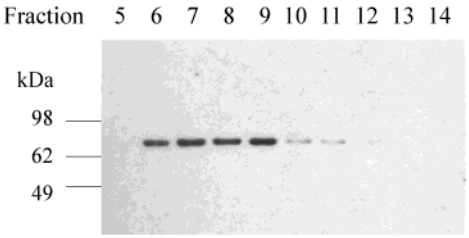

FIGURE 3: Western blot of fractions from a sucrose gradient probed with antisera against yBiP, an ER-specific chaperone (*19*). Yeast cell extracts were loaded onto a sucrose density gradient and separated by centrifugation as described in Materials and Methods, based on the procedure of Katzmann et al. (*18*). Fractions 5−14 are shown (5 being the least dense). Plasma membranes are found in the more dense fractions (*18*), which are shown here to be relatively free of ER.

Fractions 10−12 were combined to obtain plasma membrane proteins for Western blotting to detect the level of cell surface expression of SHST1 in each mutant. Equivalent amounts of plasma membrane protein were loaded on an SDS−PAGE gel. Proteins were resolved by SDS−PAGE, blotted onto nitrocellulose using a semidry transfer protocol, and probed with polyclonal antisera raised against SHST1 (*15*). Immunoreactive SHST1 was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN) and enhanced chemiluminescence (Pierce, SuperSignal substrate).

RESULTS

Construction and Functional Analysis of Single Charge Mutants. Site-directed mutagenesis was used to introduce the mutations shown in Table 1 into SHST1. All charged amino acid residues predicted to be within the membrane, as well as two at the ends of helices (Figure 1), were replaced by polar residues in order to investigate the role of the charge group at each position. Each mutant plasmid was transformed into the sulfate uptake deficient yeast strain, YSD1, and analyzed for sulfate transporter function. The results for the mutant D107N have been published previously (*14*) but are included here for comparison. Of the ten mutants, four were unable to complement YSD1 on growth media containing 100 μM sulfate and galactose, indicating that these mutants had lost the ability to take up sulfate (Table 2). Interestingly, all four of the nonfunctional mutants involved amino acid residues localized to either helix 1 (D107 and D122), which has previously been suggested to be involved in transport (*13*, *14*), or helix 8 (E366 and R354). Consistent with the failure of these mutants to show complementation of growth, we were unable to measure significant levels of sulfate transport for the D107N, D122N, and R354Q mutants (Table 2). We found that it was not possible to grow the E366Q mutant in defined growth media where the carbon source was galactose, regardless of the source of sulfur present. This suggested that expression of the mutant transporter is toxic to yeast. This toxicity was investigated further as described below.

Table 2: Properties of SHST1 Mutants

mutant strain	complementation on 100 μM sulfate[a]	K_m (μM)[b]	V_{max} [nmol of sulfate min^{-1} (mg of protein)$^{-1}$][b]
SHST1	+++	9.0 ± 2	3.03 ± 0.28
pYES	−		<0.05
D107N	−		<0.05
D122N	−		<0.05
R154Q	+++	4.1 ± 0.1	0.58 ± 0.02
E175Q	+++	11.1 ± 0.6	1.05 ± 0.01
E270Q	+++	11.1 ± 0.7	0.40 ± 0.04
R354Q	−		<0.05
E366Q	−	ND	ND
K386Q	+++	6.9 ± 1.2	2.19 ± 0.09
K494Q	+++	2.6 ± 0.3	1.0 ± 0.04
E497Q	+++	9.8 ± 0.8	1.88 ± 0.21
D107N/R154Q	−		<0.05
D122N/R354Q	−		<0.05
E175Q/R354Q	+	2.0 ± 0.4	0.21 ± 0.02
E270Q/R354Q	++	2.7 ± 0.3	0.28 ± 0.04
E175Q/E270Q/R354Q	−	15.1 ± 3.0	0.15 ± 0.04

[a] Key: +++, wild-type growth; ++, moderate growth; +, poor growth; −, no growth. [b] Uptake of [^{35}S]sulfate was measured at different concentrations of sulfate, and the Michaelis−Menten equation was fitted to the data to determine the K_m and V_{max}. Each value is the mean (with standard error) of at least three independent experiments. ND = not determined.

The other six mutants were able to complement YSD1, as indicated by growth when sulfate was the sole source of sulfur (Table 2). The ability of each mutant strain to take up [^{35}S]sulfate was determined, and the kinetic parameters of sulfate transport are shown in Table 2. The mutants R154Q and E270Q both had substantially reduced levels of sulfate transport, with the V_{max} values being less than 20% of the wild type. R154Q also showed a 2-fold increase in affinity for sulfate, while the K_m for sulfate of E270Q was similar to wild type. The remaining mutants, E175Q, K386Q, K494Q, and E497Q, all retained some sulfate transport activity, although none was fully wild type in character. K494Q also showed an increase in K_m. Thus, all of the mutations had some effect on sulfate transport activity, suggesting roles for these charged amino acid residues.

Construction and Analysis of Double and Triple Mutants. Of the four mutations that abolished function, three affected negatively charged amino acids (D107N, D122N, and E366Q) and only one affected a positively charged amino acid (R354). The high energy cost of burying an arginine residue within the lipid bilayer makes it very unlikely that this residue is not either charge paired or hydrogen bonded to other residues. We were interested to see if R354 was paired with one of the negatively charged residues. The predicted positions of charges within the membrane (Figure 1) suggest that likely partners for R354 are D122, E175, or E270. We therefore constructed double mutants in which R354 and either D122, E175, or E270 were neutralized. Both E175Q/R354Q and E270Q/R354Q were able to complement, allowing YSD1 to grow on sulfate media, but D122N/R354Q was not (Table 2). The sulfate transport activity of both E175Q/R354Q and E270Q/R354Q was very low, and the K_m was lower than wild type (Figure 4, Table 2). The activity of both double mutants was lower than that of the single

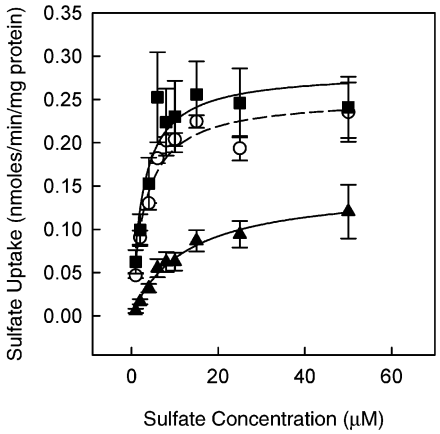

FIGURE 4: Uptake of [^{35}S]sulfate by the E175Q/R354Q, E270Q/R354Q, and E175Q/E270Q/R354Q mutants. Uptake of radioactive sulfate was measured at different concentrations of sulfate. Each value is the mean (with standard error) of at least three independent experiments. The lines represent the theoretical fit of the Michaelis−Menten equation to the data points. The K_m and V_{max} values are also given in Table 2 for comparison to wild-type SHST1. Key: E175Q/R354Q (○, dotted line), E270Q/R354Q (■, solid line), and E175Q/E270Q/R354Q (▲, solid line).

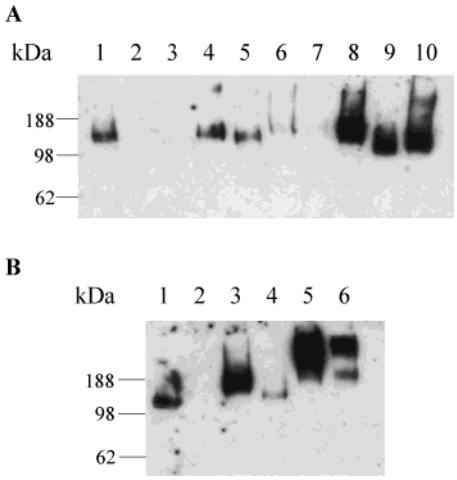

FIGURE 5: Western blot of plasma membrane fractions from selected mutants. Cells were grown and plasma membranes prepared as described in Materials and Methods. Plasma membrane proteins were separated by SDS−PAGE, transferred to nitrocellulose, probed with polyclonal antisera to SHST1, and detected by ECL. Panel A: lane 1, SHST1; lane 2, pYES3; lane 3, D122N; lane 4, E175Q; lane 5, E270Q; lane 6, R354Q; lane 7, D122N/R354Q; lane 8, E175Q/R354Q; lane 9, E270Q/R354Q; lane 10, E175Q/E270Q/R354Q. Panel B: lane 1, SHST1; lane 2, pYES3; lane 3, D107N; lane 4, R154Q; lane 5, D107N/R154Q; lane 6, K494Q.

mutants, E175Q and E270Q. However, the fact that both double mutants do have some sulfate transport activity indicates that mutation of either glutamate residue is able to compensate for the loss of R354. This could occur if R354 interacted with both E175 and E270. Mutation of R354 alone would then result in the presence of two negative charges unpaired within the membrane, and repulsion between them may prevent normal folding of SHST1. Mutation of either glutamate residue would still allow R354 to interact with the remaining glutamate, and therefore some assembly could occur. We also mutated all three charged residues together, but the E175Q/E270Q/R354Q mutant was unable to complement YSD1. However, we could measure marginal levels of sulfate transport activity, with a V_{max} lower than that of all other mutants and a K_m similar to that of wild type (Table 2, Figure 4).

Plasma Membrane Expression of Mutant Proteins. Many mutations to membrane proteins result in inefficient trafficking of the altered protein, and therefore a reduced V_{max} could result from a reduction in the amount of transporter present in the plasma membrane. The quality control mechanism of the endoplasmic reticulum ensures that misfolded proteins undergo degradation, rather than continuing through the secretory pathway (*21*). Inefficient trafficking of a mutant protein therefore indicates that the protein is misfolded, and the effects of that mutation are largely structural. Conversely, mutations that affect function but not trafficking are likely to have interfered with functioning of the protein. To identify such mutations, plasma membranes were prepared from mutant strains that had significantly reduced V_{max} values. Plasma membrane was separated from internal cell membranes by sucrose density gradients as described in Materials and Methods. The expression of the mutant sulfate transporters in the plasma membrane was investigated by Western blotting, and the results are shown in Figure 5. D107N, E175Q, E270Q, and K494Q had similar levels of sulfate transporter protein in the plasma membrane to the wild type. The reduced V_{max} values for these mutants therefore suggest that the mutant transporters function less well than wild type. The D122N mutant protein was not detectable, and the R154Q and R354Q mutant proteins were present in only trace amounts (Figure 5), indicating that these mutations result in misfolded proteins that are not trafficked efficiently to the plasma membrane. The double mutants, E175Q/R354Q and E270Q/R354Q, were interesting in that they showed higher levels of plasma membrane expression than the corresponding single mutants (Figure 5A). In contrast, plasma membrane protein for the D122N/R354 mutant was barely detectable. The triple mutant, E175Q/E270Q/R354Q, showed recovery of protein expression in the plasma membrane compared to the single mutants. These results provide further evidence for an interaction between these three residues, suggesting that the reduced trafficking of the single mutant, R354Q, is due to misfolding of the protein arising from unpaired or mispaired glutamate residues. The high level of expression in the plasma membrane of the D107N/R154Q mutant is also interesting (Figure 5B) because it suggests that there may be a charge pair between these two residues; the R154Q mutant may be misfolded and not trafficked because D107 forms an inappropriate interaction with another residue when R154 is not present.

Many of the mutant proteins showed aberrant mobility on the SDS−PAGE gel, with some forming higher molecular weight species, e.g., K494Q and D107N/R154Q. Wild-type SHST1 in plasma membrane fractions has an apparent molecular mass approximately double the predicted value of 73 kDa, an effect likely to result from incomplete denaturation (*13*). Extensive analysis of different sample preparation conditions did not resolve this discrepancy (*13*). The different mobilities observed for the various mutants are consistent with incomplete denaturation, which may be affected by misfolding and by the amount of SHST1 in the sample. Although there were equivalent amounts of total protein in each sample, the amount of SHST1 present in the plasma membrane varied considerably between mutants. For those mutants with much higher mobility, it is likely that

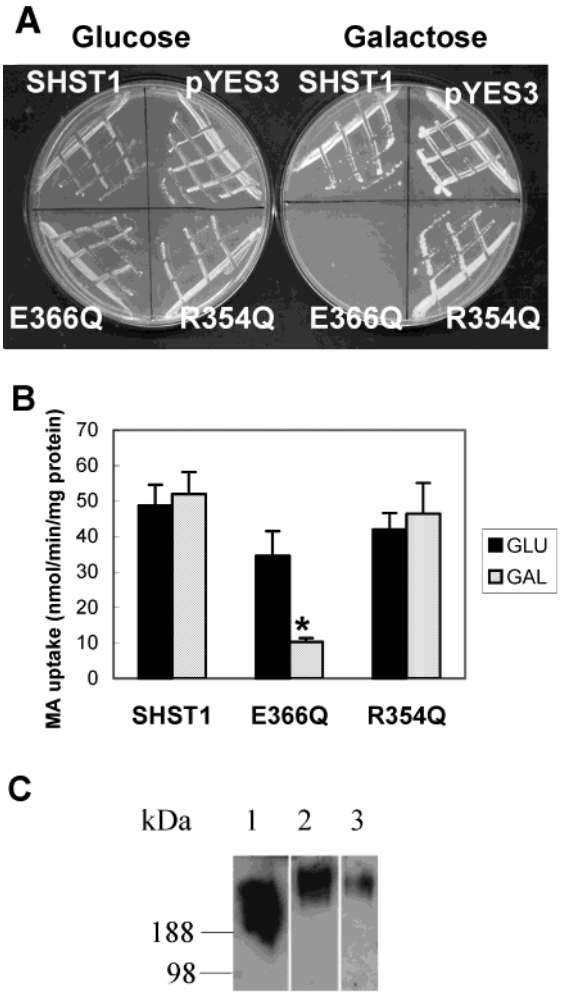

FIGURE 6: (A) Growth of E366Q and R354Q on SC$^-$ medium supplemented with galactose or glucose and homocysteine thiolactone. (B) Uptake of [^{14}C]methylammonium (MA) by yeast cells expressing wild-type SHST1, the E366Q mutant, or the R354Q mutant. Yeast cells were grown as described in Materials and Methods, and SHST1 expression was induced by 6 h growth in galactose (gray bars), or cells were grown for 6 h in glucose as a noninduced control (black bars). Values are the mean (with standard error) of at least four independent experiments. The asterisk indicates that the E366Q (Gal) value is significantly different from each other value at $p < 0.03$, as indicated by paired t tests. (C) Western blot of plasma membrane fractions of E366Q, R354Q, and wild-type SHST1. Cells were grown and plasma membranes prepared as described in Materials and Methods. Plasma membrane proteins were separated by SDS−PAGE, transferred to nitrocellulose, probed with polyclonal antisera to SHST1, and detected by ECL. The blot was overexposed in order to detect small amounts of the E366Q mutant protein. SHST1 and R354Q can be compared to Figure 5 where a blot with normal exposure is shown. Lanes: 1, SHST1; 2, R354Q; 3, E366Q.

aggregates composed of misfolded protein are present. These problems are not uncommon with highly hydrophobic proteins (*22*).

Toxicity of the E366Q Mutant. As described above, it was not possible to grow E366Q in defined media with galactose as the carbon source. The lack of growth of this mutant on SC$^-$ plates supplemented with galactose and homocysteine thiolactone is shown in Figure 6A. Under these conditions, expression of the transporter is induced by galactose, but its activity is not required for growth. Another mutant which is also unable to support growth complementation of YSD1 (R354Q) is shown for comparison. All other mutants behaved similarly to R354Q in that growth on galactose and homocysteine thiolactone was unaffected by expression of the mutant transporter. No toxicity was observed when galactose was replaced by glucose (Figure 6A), indicating that expression of E366Q was required to prevent growth of the yeast cells. One potential explanation for toxicity is that the E366Q mutation results in a transporter that is leaky to protons or other ions. Uncoupled movement of protons down their concentration gradient would have the effect of collapsing the proton gradient across the plasma membrane, and this would be expected to inhibit growth. This has been observed for point mutations in the lac permease of *E. coli* (*23*−*25*). We attempted to test whether this was the case for the E366Q mutant by indirectly assessing membrane potential.

If the E366Q mutant protein is leaky to protons, the failure of the mutant strain to maintain its membrane potential should have an effect on other secondary transport systems. Such an effect has previously been observed for proton-leaky mutants of the *E. coli* lactose permease (*23*). We tested this by measuring the activity of the native yeast ammonium transporters. Yeast has three ammonium transporters, MEP1, MEP2, and MEP3, and their activity has been demonstrated to be dependent on the membrane potential (*26*). Expression of these transporters is induced by nitrogen starvation (*26*, *27*). YSD1 transformed with a plasmid encoding either the E366Q mutant, the R354Q mutant, or wild-type SHST1 was grown on YNB media containing proline as the sole nitrogen source to induce maximal expression of the MEP proteins and glucose to repress expression of SHST1. The media were supplemented with homocysteine thiolactone. Cultures were grown to mid log phase and split into two aliquots, and the cells were harvested by centrifugation. The two aliquots were resuspended in identical growth media supplemented with either galactose, to induce expression of the wild type or mutant SHST1, or glucose, as a control. After 6 h, cells were assayed for their ability to take up [^{14}C]methylammonium.

The cells expressing wild-type SHST1 and the R354Q mutant behaved similarly. Uptake of [^{14}C]methylammonium was similar in the glucose-grown and galactose-grown yeast, indicating that expression of wild-type SHST1 or the R354Q mutant does not affect [^{14}C]methylammonium uptake. In contrast, cells expressing the E366Q mutant had significantly lower [^{14}C]methylammonium uptake in galactose than in glucose (Figure 6B). The level of uptake in glucose-grown cells was similar to that by wild-type SHST1 and the R354Q mutant. These data indicate that transport by the native yeast ammonium transporters is inhibited by expression of the E366Q mutant. These results are consistent with the toxicity of the E366Q mutant being due to an ion leak through the mutant transporter that collapses the membrane potential, although other explanations are possible.

If an ion leak in the plasma membrane is the explanation for the toxicity of the E366Q mutant, we would expect to see the mutant protein in the plasma membrane. Using Western blotting, we could detect traces of the E366 mutant protein in the plasma membrane 12 h after induction. The protein was visible only after overexposing the blot (Figure 6C). The low level of expression is indicated by a comparison with both the wild type and the R354Q mutant, another nonfunctional mutant that was not trafficked normally (see above). After 6 h of induction, neither wild-type SHST1 nor the E366Q mutant was detectable by Western blotting, using

the same conditions. At this time we could measure sulfate transport by wild-type SHST1 but not by the E366Q mutant (results not shown), which serves to illustrate the relative sensitivity of the two different techniques. We can conclude from these results that E366Q is not trafficked normally, but small amounts of the E366Q mutant protein do reach the plasma membrane.

DISCUSSION

The goal of this study was to identify charged amino acid residues in transmembrane helices that were required for function of SHST1. The two major findings of this study are as follows: first, we have obtained evidence consistent with a charge interaction between amino acid residues in helices 3, 6, and 8 and, possibly, also between helices 1 and 2, and second, we have confirmed the functional importance of the first three transmembrane helices. Our results provide the first evidence for possible spatial relationships between transmembrane helices in the SulP family.

We have proposed a charge interaction between E175 in helix 3, E270 in helix 6, and R354 in helix 8, and this proposal is based on the properties of single, double, and triple mutants affecting these residues. Of the three single mutants, only R354Q is completely nonfunctional. The lack of function is due to dramatically reduced trafficking to the plasma membrane of the mutant protein. The defect in both trafficking and function can be overcome by combining the R354Q mutation with either of the mutations, E175Q or E270Q. In contrast, the R354Q mutation could not be suppressed by the D122N mutation. Both single mutants, E175Q and E270Q, did retain some sulfate transport activity. These results suggest that E175, E270, and R354 participate in a charge network and loss of either glutamate residue can be tolerated because R354 can still interact with the remaining one. Loss of R354 cannot be tolerated because it is the only positively charged residue in the interaction; neutralization of this residue would result in repulsion between the two glutamate residues remaining. Significantly, although the recovery of activity in the double mutants is very low, trafficking is restored to wild-type levels, and the K_m values are wild type or lower, suggesting that the misfolding problem can be overcome by removing either of the two remaining negative charges. It appears that one unpaired glutamate residue can be tolerated in the folding pathway, perhaps because it can interact with the introduced glutamine residues. The triple mutant, E175Q/E270Q/R354Q, is also trafficked normally, indicating that the charge network is not required for folding or trafficking of SHST1.

Although the presence of all three charged residues is not absolutely essential for sulfate transport, all mutants affected in these three residues function considerably less well than wild-type SHST1. Of those mutants that show significant levels of protein in the plasma membrane, the single mutants (E175Q and E270Q) function better than the double mutants (E175Q/R354Q and E270Q/R354Q), with the triple mutant showing marginal sulfate transport activity that does not support complementation. Thus, the greater the disruption to the charge network, the greater the effect on transport activity. Although the double and triple mutants have V_{max} values less than 10% of the wild type, they show wild-type levels of protein in the plasma membrane and have K_m values similar to or lower than that of the wild type. This suggests a functional role in transport for the charge network between E175, E270, and R354, rather than a role in folding. The low levels of activity observed in the mutants may arise because the polar glutamine residues can substitute for the charged residues to a limited degree. Potential roles for this charge network in SHST1 can be suggested from recent studies describing three-way interactions between charged residues in other membrane proteins. These are two homologous bacterial multidrug transporters, exemplified by MexB from *Pseudomonas aeruginosa* (*7*) and AcrB from *E. coli* (*28*), and the mammalian $GABA_A$ receptor (*29*). Interactions in both proteins involve two negatively charged residues and one positively charged residue, similar to SHST1. In the case of MexB, mutagenesis data initially suggested an interaction between D407, D408, and K939 (*7*), all of which are located within transmembrane helices. This interaction was confirmed by the crystal structure of the homologous transporter, AcrB (*28*), and it was proposed that these residues participate in the proton translocation pathway. In the $GABA_A$ receptor, K239, located in the loop between transmembrane helices 2 and 3, interacts with two aspartate residues in the extracellular substrate-binding domain. Mutagenesis and cross-linking experiments have indicated that electrostatic interactions between these three residues mediate gating, i.e., coupling the conformational change due to GABA binding to the opening of the transmembrane channel (*29*). In SHST1, the interaction between E175, E270, and R354 could be involved directly in the transport pathway or in mediating the conformational changes associated with the transport of sulfate.

We have also obtained data that confirm the importance of the first two helices of SHST1 for sulfate transport and raise the possibility of an interaction between D107 in helix 1 and R154 in helix 2. We have previously described other mutations in the first two helices of SHST1 that significantly affect function. P144 in helix 2 is essential for function (*13*), and mutations in the highly conserved PXYGLY motif in the loop between helices 1 and 2 either abolish function or result in slightly increased affinity for sulfate (*14*). These data, in addition to the high conservation of this region, led to the hypothesis that these two helices may form part of the transport pathway. In the present study, three charged residues in this region (D107, D122, and R154) were mutated to further test this idea. Loss of any one of the three had severe effects on sulfate transport. Replacement of D107 by asparagine resulted in a complete loss of SHST1 function but did not affect trafficking of the transporter, suggesting that D107 may be essential to the transport mechanism. D107 is completely conserved throughout the SulP family (Figure 2). A positive charge in the equivalent position to R154 is also conserved; replacement of this residue by glutamine resulted in loss of 80% of transport activity, but this was due to reduced trafficking of the mutant protein. The double mutant, D107N/R154Q, had regained normal trafficking, consistent with an interaction between these two residues. However, sulfate transport activity was not recovered in the double mutant. Recovery of activity would not necessarily be expected if D107 was essential for the transport mechanism. The predicted location of D107 and R154 at the cytoplasmic ends of helices 1 and 2, respectively (Figure 1), and the high conservation of these two residues (Figure

2) are also consistent with these two residues forming a charge pair. Substitution of D122 in helix 1 by asparagine also resulted in a complete loss of activity, but this was due to an effect on trafficking, suggesting that the mutant protein is misfolded. D122 is not conserved in other members of the SulP family, but it is located within a cluster of polar residues in helix 1. This suggests that D122 itself is unlikely to be important. It is possible that mutating this residue may disrupt the interaction between helices 1 and 2.

The role of E366 in normal SHST1 function is not clear from our results although it is potentially important for sulfate transport. Trace levels of the mutant protein are present in the plasma membrane (Figure 6C), and the observed inhibition of ammonium transporter activity (Figure 6B) could be due to the collapse of the membrane potential mediated by a leak of protons or other ions through the mutant protein. Other explanations for toxicity are, of course, possible, and more direct measurements of membrane potential are required to test this hypothesis. It is interesting to note that proton-leaky mutants of the lac permease of *E. coli* have also been isolated (*23*−*25*, *30*, *31*). It is thought that they maintain an inappropriate conformation, allowing them to transport protons into the cell in the absence of lactose binding, or that they transport protons with lactose but then recycle the lactose back out of the cell, resulting in the net import of protons. Different categories of mutants have substrate-dependent leaks or leaks that can be blocked by substrate (*24*, *25*). The toxicity of E366Q was not dependent on the presence of sulfate, and we could not block the toxicity of the E366Q mutant (as measured by either growth or methylammonium uptake) by the presence of sulfate at up to millimolar concentrations (results not shown). Different substitutions at this position may throw more light on the role of this residue.

Three other charged residues were mutated and were not essential for sulfate transport activity. These were K386, K494, and E497. K386 is at the end of helix 9 adjacent to the highly conserved E387. Mutation of E387 was previously shown to have a severe effect on trafficking of SHST1 (*15*). In contrast, mutation of K386 had very little effect on SHST1 function, suggesting different roles for these two residues. If this region is helical, K386 would be on a different face of the helix from E387. Mutation of either K494 or E497 was only slightly detrimental to SHST1 function. These two residues are one turn of a helix apart and thus would be capable of interacting with each other to form a charge pair. However, E497 is highly conserved throughout the SulP family, with glutamate or aspartate found in the equivalent position of all the transporters shown in Figure 2 whereas uncharged residues are found in the position equivalent to K494. This suggests that E497 is more likely to interact with other residues. Despite the conservation of this residue, our results indicate that this charge group is not essential for sulfate transport by SHST1. Haimeur et al. (*32*) have found that, in the multidrug resistance protein 1, mutations affecting two oppositely charged residues one helical turn apart have different effects. As is the case for our results with SHST1, this suggests that these two residues participate in different interactions, even though they could potentially interact with each other. Clearly, the interactions between transmembrane helices can be complex.

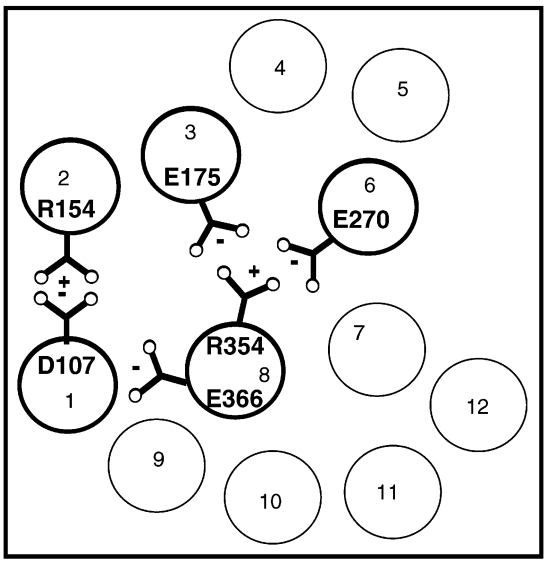

FIGURE 7: Proposed structural model of SHST1 based on the charge interactions described in this work. Helices about which we have information are shown in bold. The positions of the other helices are speculative. E366 is shown close to helix 1, but as we have not identified an interacting partner for this residue, other interactions are possible.

The results we have obtained in this study suggest interactions between helices 1 and 2 and between helices 3, 6, and 8 and can therefore be used to produce a spatial model for the arrangement of transmembrane helices in SHST1 (Figure 7). Although hydropathy plots show three distinct hydrophobic peaks corresponding to helices 1, 2, and 3, if each of these helices consists of the full 19 amino acids required to span the lipid bilayer, the interhelix loops would be very small. This spacing is conserved throughout the SulP family (Figure 2), making it likely that these three helices are adjacent in the three-dimensional structure of the transporter. Our data suggesting an interaction between E175 on helix 3 with E270 on helix 6 and R354 on helix 8 brings helices 6 and 8 close to the first three helices, as shown in Figure 7. The properties of mutants of charged and other residues in these helices are consistent with these helices forming the transport pathway for SHST1, although we cannot yet describe a detailed model for the transport process. E366 could potentially be located in or near the transport pathway. Like R354, it is located in helix 8 and thus may be able to interact with residues in helices 1 or 2 (Figure 7). Further substitutions at position 366 and a greater understanding of the cause of toxicity will be required to identify the role of this residue. The model described here will form the basis for further mutagenesis aimed at understanding the transport mechanism of SHST1.

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