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Polar residues in a conserved motif spanning helices 1 and 2 are functionally important in the SulP transporter family

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ABSTRACT

The SulP family (including the SLC26 family) is a diverse family of anion transporters found in all domains of life, with different members transporting different anions. We used sequence and bioinformatics analysis of helices 1 and 2 of SulP family members to identify a conserved motif, extending the previously defined 'sulfate transporter motif'. The analysis showed that in addition to being highly conserved in both sequence and spacing, helices 1 and 2 contain a significant number of polar residues and are predicted to be buried within the protein interior, with at least some faces packed closely against other helices. This suggests a significant functional role for this region and we tested this by mutating polar residues in helices 1 and 2 in the sulfate transporter, SHST1. All mutations made, even those removing only a single hydroxyl group, had significant effects on transport. Many mutations abolished transport without affecting plasma membrane expression of the mutant protein, suggesting a functional role for these residues. Different helical faces appear to have different roles, with the most severe effects being localised to two interacting faces of helices 1 and 2. Our results confirm the predicted importance of conserved polar residues in helices 1 and 2 and suggest that transport of sulfate by SHST1 is dependent on a network of polar and aromatic interactions between these two helices.

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1. Introduction

The SulP family is an ancient and diverse family of anion transporters, with members identified by sequence homology in prokaryotes and eukaryotes. Human members comprise the SLC26 family and the properties of a number have been characterised in detail (reviewed in (Kere, 2006; Sindic et al., 2007)). Most are anion exchangers and transport a wide range of anions both organic and inorganic, with individual transporters showing different specificities. Several have been recognised to be involved in disease states such as diastrophic dysplasia (SLC26A2), congenital chloride diarrhoea (SLC26A3) and congenital deafness (SLC26A5) (Kere, 2006). Most plant and fungal members of the family that have been characterised are sulfate transporters, with different members showing different affinities for sulfate and different expression patterns, suggesting specialised roles in the movement of sulfate into and within the plant (Hawkesford, 2003; Smith et al., 1995b). Recently two distantly related SulP members were identified as molybdate transporters (Tejada-Jimenez et al., 2007; Tomatsu et al., 2007). Many bacterial genomes also contain SulP family members but very few have been characterised, one exception being a bicarbonate transporter in photosynthetic bacteria (Price et al., 2004). Despite the fact that the physiological roles of some members of the family are well understood, there is very little knowledge of the structure and function of any transporter within the family.

Hydropathy plot analysis of SulP family members suggests that they contain 12 transmembrane α-helices with

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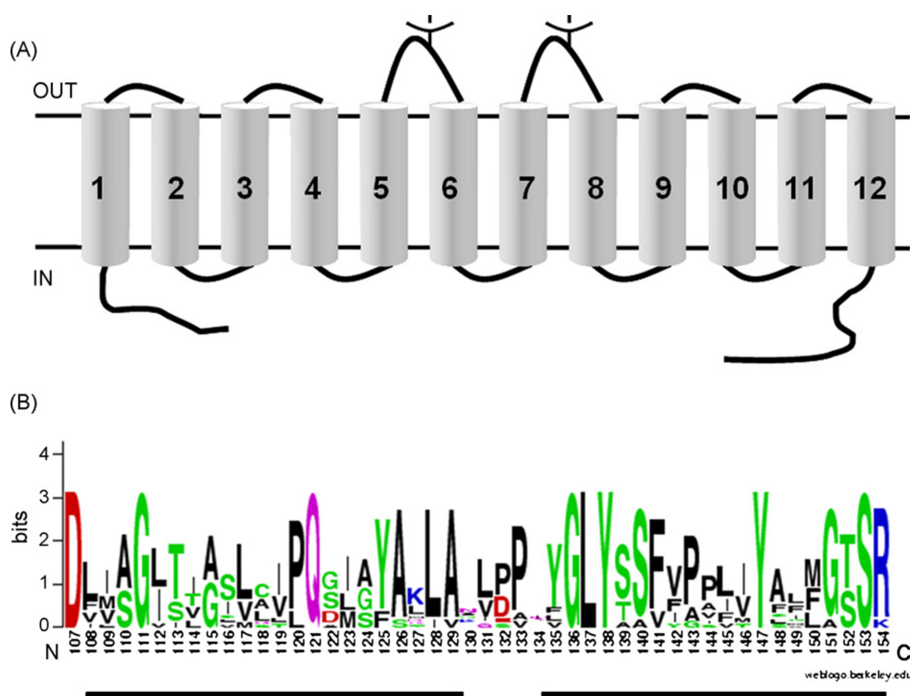

Fig. 1. (A) Predicted topology of SHST1. Two putative glycosylation sites in extracellular loops are indicated. (B) Sequence logo for helices 1 and 2 of selected SulP family members, using numbering for SHST1. The height of each stack indicates the level of conservation and the height of each letter within the stack indicates the frequency it is found in that position. The consensus sequence (shown in the text) was determined as the most frequently found residue if the stack height was 50% or greater than the maximum height. Predicted transmembrane α-helices of SHST1 are indicated by black lines below the plot. The transporters used for the alignment are as follows (NCBI protein ID number shown in parentheses): SHST1, *Stylosanthes hamata* high affinity sulfate transporter 1 (CAA57710); TtSultr1.1, *Triticum tauschii* high affinity sulfate transporter 1 (CAB42985); *Arabidopsis thaliana* sulfate transporter 1.1 (BAA33932); SHST3, *S. hamata* lower affinity sulfate transporter (CAA57831); *A. thaliana* sulfate transporter 2.1 (BAA20085); *A. thaliana* sulfate transporter 3.1 (BAA21657); *A. thaliana* sulfate transporter 4.1 (BAA23424); SLC26A3 CLD, human chloride anion exchanger (P40879); SLC26A4 pendrin, human sodium-independent chloride/iodide transporter (O43511); SLC26A2 DTDST, human diastrophic dysplasia transporter protein (P50443); SLC26A5 prestin, human prestin protein (AAP43685); SUL1, *Saccharomyces cerevisiae* sulfate transporter (CAA85259).

an intracellular N- and C-terminus (Fig. 1A). SulP transporters also contain a C-terminal domain, the STAS domain, which is thought to be intracellular and has been shown to be important for function in several members of the family (Ko et al., 2004; Shibagaki and Grossman, 2006). The first two putative transmembrane α-helices of SulP family members show a significantly higher level of conservation than that observed for the entire protein and include one of the two 'sulfate transporter motifs' that have been used to define the SulP family (Saier et al., 1999). The loop between putative α-helices 1 and 2 is short and this short length is conserved between quite diverse members of the family. Previous mutagenesis studies have found that residues in helices 1 and 2 are important for function of two SulP members, a plant sulfate transporter, SHST1 (Khurana et al., 2000; Shelden et al., 2001, 2003; Loughlin et al., 2002) and prestin, a molecular motor protein responsible for cochlear amplification in the inner ear (Rajagopalan et al., 2006) but there has been no systematic investigation of the role and function of these helices.

We are using SHST1, a high affinity sulfate transporter from the forage legume *Stylosanthes hamata* as a model member of the SulP family due to the ability to express it in the yeast heterologous expression system YSD1 (Smith et al., 1995a). Expression of SHST1 in the yeast strain YSD1 which is deficient in its native sulfate transporters, provides a useful system in which to test the function of mutants. The high conservation of helices 1 and 2 led us to hypothesize that this region may have an important functional role. The aim of this study was to test this hypothesis, using both bioinformatics analysis and site-directed mutagenesis to investigate the function of helices 1 and 2.

2. Materials and methods

2.1. Molecular biology

Standard procedures were used for bacterial plasmid isolation and transformation into *E. coli*. SHST1 was expressed using the yeast expression vector pYES3 (Smith et al., 1995b) under a galactose inducible promoter. Polymerase Chain Reaction (PCR) was completed using Pfu polymerase (Promega) and a standard PCR protocol. All mutants were generated by site-directed mutagenesis using the QuikChange method from Stratagene. Mutagenic primer pairs included the base-pair change required to introduce the desired alteration in the shst1 sequence. The shst1 cDNA for each mutant was fully sequenced to ensure no PCR derived errors had been introduced. PCR was used to construct a haemaglutinin (HA)-tagged variant of SHST1,

with a new start codon and the HA tag sequence in frame with shst1 being incorporated into the 5′ primer, resulting in an N-terminally tagged SHST1.

2.2. Yeast growth and transformation

YSD1, a *Saccharomyces cerevisiae* strain, with a deletion in the yeast's native sulfate transporter gene, Sul1 (Smith et al., 1995b), was used for the expression of SHST1 and all mutant derivatives. YSD1 was grown in sulfur-free growth media supplemented (uracil free) with either 76.52 mg/L of homocysteine thiolactone or 100 μM sulfate, and either 2% galactose (inducing conditions) or 2% glucose (non-inducing conditions). Yeast transformation were carried out using the LiCl/PEG method (Gietz et al., 1995).

2.3. Sulfate uptake assays

[^{35}S] Sulfate uptake assays were performed as described previously (Shelden et al., 2001). Uptake for YSD1 transformed with pYES3 was subtracted from the data for wild-type and each mutant. The Michaelis–Menten equation was fitted to the data to give a K_m and a V_{max}. Unpaired two tailed *t*-tests were used to compare each mutant value for V_{max} with the wild-type value with $p < 0.01$ being considered significant.

2.4. Yeast cell membrane fractionation and Western blotting

Yeast cells of control or mutant strains were grown to mid-log phase in sulfur-free media supplemented with homocysteine thiolactone and 2% galactose. Intracellular and plasma membranes were separated by running cell lysates on a sucrose density gradient following the procedure of Katzmann et al. (1999) as modified by Khurana et al. (2000). The separation of endoplasmic reticulum fractions from plasma membrane fractions has been previously confirmed using antisera against marker proteins (Shelden et al., 2003). Fractions 9–12 were combined to obtain the plasma membrane fraction. Equivalent amounts of plasma membrane protein from each strain were separated on an SDS-PAGE gel (NuPAGE 4–12% Bis-Tris, Invitrogen), and then transferred to nitrocellulose using a semidry transfer protocol. The blot was probed with a monoclonal anti-hemagglutinin (anti-HA) antisera. Immunoreactive SHST1 was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (ICN) and enhanced chemiluminescence (Pierce, SuperSignal substrate).

2.5. Bioinformatics

Two programs were used to examine the propensity of helices, and particular faces of helices, to be buried within the protein interior. The RANTS program (Adamian and Liang, 2006a) was accessed at: http://gila.bioengr.uic.edu/lab/larisa/rants.html.

The LIPS program (Adamian and Liang, 2006b) was accessed at: http://gila.bioengr.uic.edu/lab/larisa/lips.html Fig. 1B was created with WebLogo (Crooks et al., 2004): http://weblogo.berkeley.edu/logo.cgi.

3. Results

3.1. Bioinformatic analysis of helix packing in the SulP family

The SulP family is identified by a motif that extends across putative helix 2 and a second conserved motif in putative helix 9 (Saier et al., 1999), the 'sulfate transporter motifs'. However, an alignment including a broad cross section of eukaryotic family members shows that there are also positions in helix 1 showing equivalent high levels of conservation (displayed as a sequence logo in Fig. 1B). In addition to conservation of the residue at each position, the spacing between them, including a short loop between the first two helices, is maintained throughout the eukaryotic members of the family. We have identified an extended conserved motif spanning this region:

DLx**AGLT**x**A**x**L**x**IPQ**x**IAYA**x**LA**x**L**x**P**x**YGLYSSF**x**P**xx **IY**xx**MGTSR**

The motif can also be identified in prokaryotic members of the family. A higher level of sequence variability is evident but the size of the loop between helices 1 and 2 is completely conserved (results not shown). The conservation of spacing suggests that positioning of the two helices relative to each other is crucial for function and therefore that the position, as well as the identity, of the conserved residues is functionally important. It is evident from Fig. 1B that the extended SulP motif includes a significant number of polar residues, especially those containing hydroxyl groups. This is despite the fact that most of this sequence is predicted to occur within transmembrane helices.

We examined the SulP family using the RANTS program (Adamian and Liang, 2006a) which analyses sequence alignments to determine the propensity of predicted helices within a family to be buried in the protein interior or exposed to the lipid. The program has a prediction accuracy of 78% so two separate sequence alignments were analysed. The SulP family exhibits significant functional diversity; while all characterised members, except perhaps the motor protein prestin, are anion transporters, there is considerable diversity in substrates transported. Even within the plant branch of the SulP family, five phylogenetically and functionally distinct groups have been identified with Groups 1–4 being involved in sulfate transport (Hawkesford, 2003), and Group 5 transporting molybdate (Tejada-Jimenez et al., 2007; Tomatsu et al., 2007). Therefore the two alignments used for analysis by RANTS were firstly, a diverse set of sequences from plants, yeast and mammals and secondly, the complete set of sequences comprising Groups 1–4 of SulP family members found in Arabidopsis. A topology of 12 transmembrane helices was predicted, as this is consistent with the hydropathy plot and the need to have both the N- and C-terminus intracellular which has been experimentally demonstrated for prestin (Zheng et al., 2001) and SHST1 (unpublished observations). Both analyses gave the

Table 1
Output from RANTS prediction of propensity for each helix in SulP family members to be buried

TM number	RANTS output[a]	
	Eukaryotic SulP family member alignment	*Arabidopsis thaliana* SulP family member alignment (subgroups 1–4)
TM1	0.3423	0.1882
TM2	0.3591	0.2110
TM3	0.8168	0.6839
TM4	0.7932	0.4099
TM5	0.3280	0.2058
TM6	1.1984	0.8719
TM7	0.6203	0.4872
TM8	0.9085	0.5636
TM9	0.4190	0.3360
TM10	0.4999	0.3817
TM11	0.6853	0.4212
TM12	0.5143	0.3857

[a] Lower values indicate a greater propensity for the helix to be buried in the protein interior. Values can be compared only within outputs from the same alignment but not between outputs for different alignments.

same results, with helices 1, 2 and 5 having the lowest propensity to be exposed to the lipid, indicated by the lowest scores (Table 1). This indicates that these helices are most likely to be buried within the protein interior and that therefore the residues in the SulP motif, in addition to helix 5, might be important for either the transport pathway or helix packing.

To obtain some information about the positioning of the residues of the extended SulP motif, helices 1 and 2 were displayed as helix plots, with the motif highlighted (Fig. 2). In both helices, polar residues cluster in patches on particular faces. Sequence alignments for helices 1 and 2 were also analysed by the LIPS program, which predicts helical faces most likely to be exposed to the lipid bilayer with an accuracy of 88% (Adamian and Liang, 2006b). Helix 2, in which SulP motif residues are found on all faces, was found to have overlapping low scoring faces (Fig. 2), suggesting that it is unlikely to be exposed to the lipid and therefore may be completely buried within the protein interior. One face of helix 1, which includes some residues of the extended SulP motif is less strongly predicted to be exposed to lipid than the other faces. In summary, we have identified an extended motif characterising the SulP family which includes a significant number of polar residues and is predicted to be buried within the protein. These analyses would suggest an important functional role for these residues.

3.2. Construction of mutants affecting polar residues in helices 1 and 2 of SHST1

We tested the prediction that the polar SulP motif residues were functionally important by using site-directed mutagenesis of the sulfate transporter, SHST1. We aimed to identify the importance of the hydroxyl group at each position by mutating conservatively to remove the hydroxyl group (tyrosine residues were replaced by phenylalanine, and serine and threonine by alanine). Tyrosine residues were also mutated to serine, phenylalanine and/or alanine to investigate the roles of the aromatic and polar groups. Two other polar residues, Gln121 and Asp122, were also mutated. Gln121 is part of the extended SulP motif while Asp122 is conserved only within the plant Group 1 sulfate transporter subfamily.

The point mutations of residues in helices 1 and 2 of SHST1 were generated using site-directed mutagenesis (Table 2). To characterise the wild-type and mutant transporters each was transformed into the sulfate transport deficient yeast strain YSD1 (Smith et al., 1995b). SHST1 and each of the mutants was characterised for their ability to transport sulfate, and for expression of the mutant protein. To enable detection of the mutant protein, a hemagglutinin (HA) tag was introduced at the N-terminus of SHST1 as described in the methods. The presence of the HA tag did not affect function of SHST1 ($K_m = 5.59 \pm 0.9$ μM, $V_{max} = 5.00 \pm 0.50$ nmol/min/mg protein for HA-tagged SHST1 compared to $K_m = 4.96 \pm 0.82$ μM, $V_{max} = 3.74 \pm 0.15$ nmol/min/mg protein for wild-type SHST1, mean plus standard error from at least three

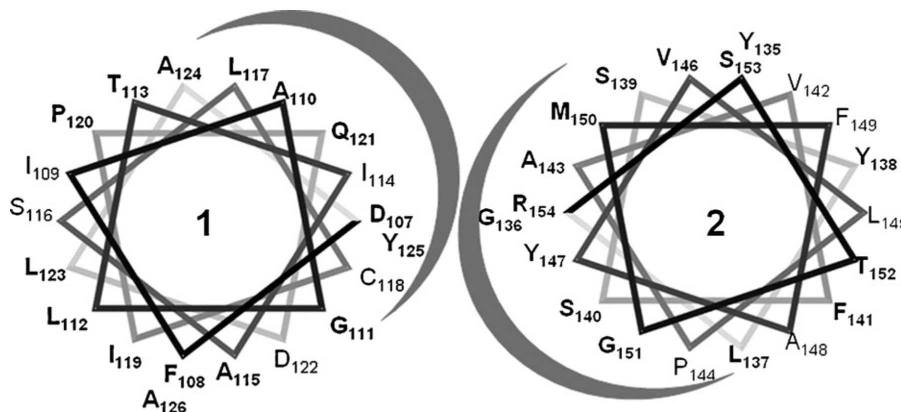

Fig. 2. Helical wheel plots of helices 1 and 2 from SHST1. Darker to lighter lines indicates progression from cytoplasmic (Asp107 in helix 1 and Arg154 in helix 2) to extracellular (Ala126 in helix 1 and Gly136 in helix 2) side. Residues forming the extended SulP motif are shown in bold. The helices are aligned to position Asp107 and Arg154 adjacent, consistent with a suggested charge pair between them (Shelden et al., 2003). LIPS analysis (Adamian and Liang, 2006b) using data from the multiple alignment used to generate Fig. 1B, was used to predict faces of these two helices that are most likely to face the lipid bilayer. Seven individual faces are scored. The lowest scoring faces have the greatest likelihood of being packed against other helices. Grey crescents indicate the faces most likely to be packed against another helix (determined as being where at least two of the three lowest scoring faces overlap).

Table 2
Growth and transport characteristics of all polar residue mutants of SHST1

Mutation	Complementation on 100 μM SO_4^{2-}/galactose[a]	V_{max} (nmol of sulfate/min/mg protein)[b]	K_m (μM)[b]
Wild-type SHST1	+++	3.74 ± 0.20	4.96 ± 0.82
pYES	–	ND	ND
Helix 1 mutants			
T113A	+++	1.11 ± 0.04	5.32 ± 1.23
S116A	+++	1.38 ± 0.11	5.00 ± 0.83
D122S	–	ND	ND
Y125A	–	ND	ND
Y125S	–	ND	ND
Y125F	–	ND	ND
Y135 and Y138 mutants			
Y135A/Y138A	–	ND	ND
Y135A	+++	0.33 ± 0.07	3.35 ± 0.63
Y135S	+++	0.49 ± 0.09	4.03 ± 1.52
Y138A	–	ND	ND
Y138S	–	ND	ND
Y135F/Y138F	+++	0.98 ± 0.10	8.61 ± 0.41
Helix 2 mutants			
S139A	+++	1.06 ± 0.14	3.14 ± 0.35
S140A	–	ND	ND
Y147S	–	ND	ND
Y147F	+++	0.70 ± 0.08	2.69 ± 0.38
T152A	+++	1.29 ± 0.30	5.51 ± 1.36
S153A	+++	1.58 ± 0.24	4.34 ± 0.17

[a] Key: (+++) wild-type levels of growth and (–) no growth. All mutants were tested for complementation in at least 3 experiments.

[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.

experiments) but allowed detection of the wild-type and mutant proteins in the yeast plasma membrane. As has been observed previously (Shelden et al., 2001), SHST1 appears on a Western blot at approximately double the predicted molecular mass (Fig. 5) in addition to a band slightly smaller than the expected size of 73 kDa. The higher band appears to represent some post-translational modification, possibly a dimer.

3.3. Characterisation of helix 1 mutants

The roles of Thr113, Ser116, Gln121, Asp122 and Tyr125 from helix 1 were investigated in this study. All mutants were tested for their ability to complement YSD1 on minimal media where 100 μM sulfate was the sole source of sulfur in the presence of either galactose or glucose. Complementation on galactose, but not glucose, plates confirms that the observed transport activity is due to expression from the GAL promoter. Sample complementation results are shown in Fig. 3. No mutant complemented in the presence of glucose. The ability of mutants to complement on galactose is summarised in Table 2. The mutants Q121A, D122S, and all three substitutions for Tyr125 (Y125A, Y125S and Y125F) resulted in a complete loss of transporter function, with each unable to complement YSD1. The lack of function for these mutants was confirmed with sulfate uptake assays showing no detectable transport of sulfate (Fig. 4A, Table 2). Only the T113A and S116A mutants were able to complement YSD1 when sulfate was the sole sulfur source (Table 2). An examination of the transport kinetics for the T113A and S116A mutants showed they both had dramatically reduced activity, at about one third of the wild-type SHST1 value (Fig. 4A and Table 2). The affinity for sulfate was unaltered in both mutants (Fig. 4B, Table 2).

These results suggested that all residues mutated had some role in sulfate transport as every mutant showed a reduction in sulfate transport activity. Reduced function could be either because the mutation directly affects transport function or because it affects folding of the mutant protein. Misfolded proteins will not be accepted by the quality control system of the endoplasmic reticulum and will not be trafficked to the plasma membrane (Ellgaard

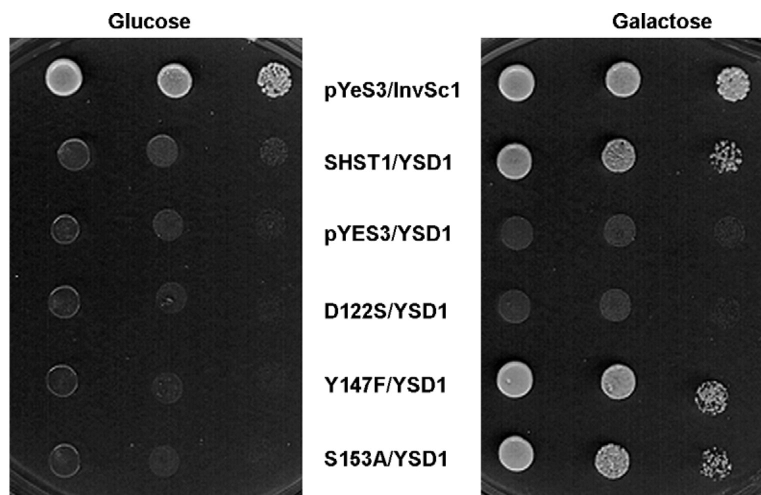

Fig. 3. Growth of selected mutant strains on 100 μM sulfate with galactose or glucose as sole carbon source. Cells were resuspended in sterile distilled water, the cell density adjusted to A_{600} = 0.5, followed by serial dilutions. Dilutions shown are: undiluted, 1 in 10 and 1 in 100 from left to right. All mutants were tested for complementation on both glucose and galactose, typical growth patterns for two complementing and one non-complementing mutant are shown. InvSc1, the parent strain of YSD1, is included as a positive control for growth on glucose/sulfate.

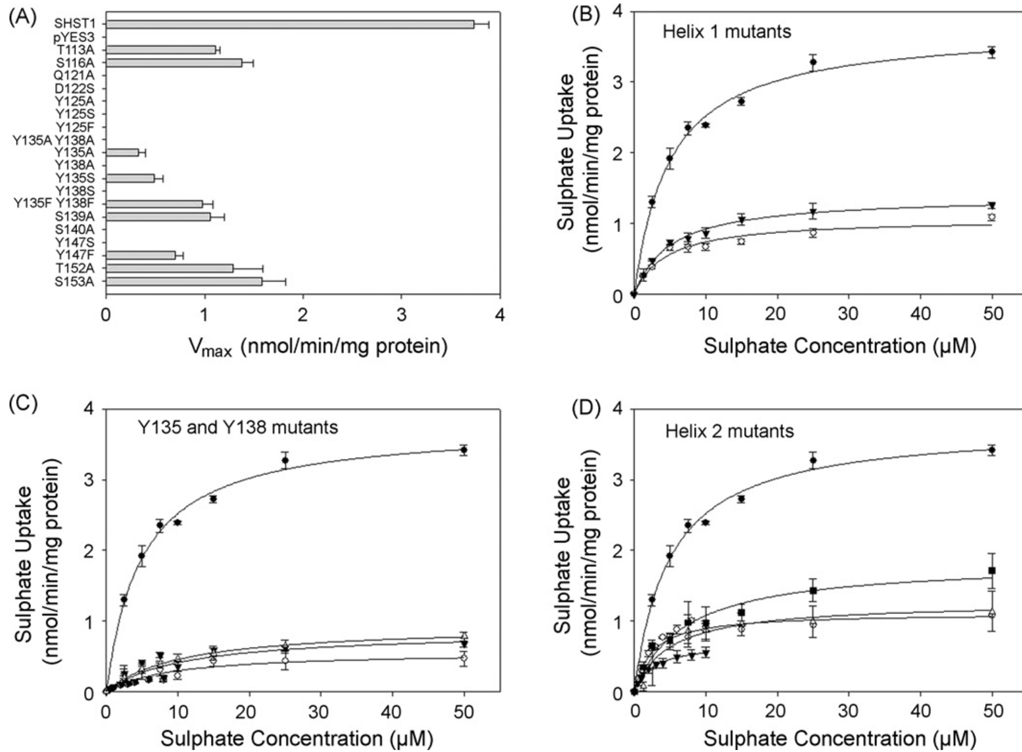

Fig. 4. Sulfate transport properties of each SHST1 mutant. (A) The maximum rate of [^{35}S] sulfate uptake for all mutants (data shown is the mean and standard error of at least three experiments). All values were significantly lower than the wild-type as determined by an unpaired *t*-test ($p < 0.01$). (B–D) For each mutant that had measurable sulfate uptake, [^{35}S] sulfate uptake was measured over a range of concentrations and the K_m and V_{max} determined by applying the Michaelis–Menten equation to the data. Each of the data points (with standard error) is the result of a minimum of three experiments. (B) *Helix 1 mutants*: SHST1 (●); T113A (○) and S116A (▼). (C) *Y135 and Y138 mutants* SHST1 (●); Y135A (○); Y135S (▼) and Y135F/Y138F (△). (D) *Helix 2 mutants* SHST1 (●); S139A (○); Y147F (▼); T152A (△) and S153A (■).

et al., 1999). These two alternatives can be distinguished by examining the amount of mutant protein present in the plasma membrane. After cell lysis and fractionation on a sucrose gradient, the plasma membrane fraction (Shelden et al., 2003) was examined by Western blotting to determine the level of trafficking and expression of each of the helix 1 mutants (Fig. 5A). Levels of plasma membrane expression similar to the HA-tagged wild-type SHST1 were observed for all mutants except those at position 125 and S116A. This suggests that most of these mutations affected function, rather than folding, of SHST1. The reduced expression of S116A probably accounts for the reduced V_{max} observed. For mutants affecting Tyr125, the nature of the substitution affected the level of trafficking to

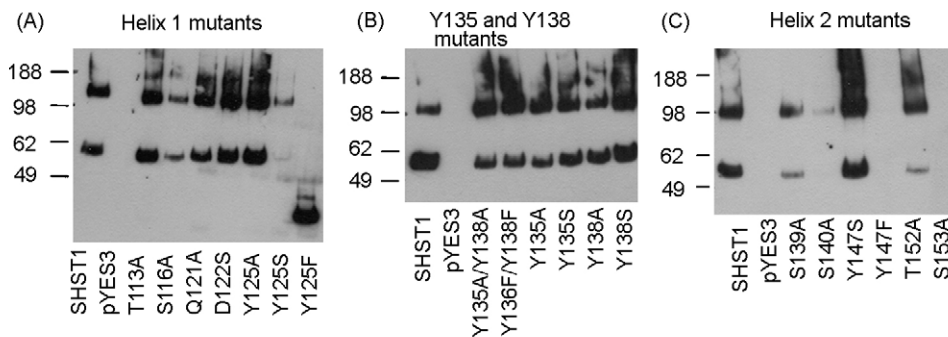

Fig. 5. Western blots of plasma membrane samples from all SHST1 mutants. Cells were grown and 2 mg cell lysate from each strain used for fractionation to separate membranes as described in Section 2. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, probed with a monoclonal antisera to the HA tag, and detected with enhanced chemiluminescence. Fractionation and Western blotting was done at least twice for each mutant and representative results are shown. Sizes of markers in kDa are shown. (A) *Helix 1 mutants*. Lane 1, HA-SHST1; Lane 2, pYES3; Lane 3, HA-T113A; Lane 4, HA-S116A; Lane 5, HAQ121A; Lane 6, HA-D122S; Lane 7, HA-Y125A; Lane 8, HA-Y125S; Lane 9, HA-Y125F. (B) *Y135 and Y138 mutants*. Lane 1, HA-SHST1; Lane 2, pYES3; Lane 3, HA-Y135A Y138A; Lane 4, HA-Y135F Y138F; Lane 5, HA-Y135A; Lane 6, HA-Y135S; Lane 7, HA-Y138A; Lane 8, HA-Y138S. (C) *Helix 2 mutants*. Lane 1, HA-SHST1; Lane 2, pYES3; Lane 3, HA-S139A; Lane 4, HA-S140A; Lane 5, HA-Y147S; Lane 6, HA-Y147F; Lane 7, HA-T152A; Lane 8, HA-S153A.

the plasma membrane, with normal expression observed for Y125A and reduced expression for Y125S. The Y125F mutant protein is visible as two bands, both smaller than wild-type SHST1 (Fig. 5A). It is possible that this mutation causes slight misfolding that allows trafficking but leaves the resultant protein sensitive to proteolysis, which may occur *in vivo* or after lysis of the yeast cells.

3.4. Characterisation of helix 2 mutants

The roles of two tyrosine residues predicted to be on the same face of helix 2 at the membrane–water interface were investigated by mutagenesis. Tyr135 and Tyr138 form part of the SulP family motif and, as aromatic residues near the ends of transmembrane helices are often important for helix localisation and/or orientation (Killian and von Heijne, 2000) they were mutated separately to produce the single mutants, Y135A, Y135S, Y138A, Y138S and together to give the double mutants, Y135A/Y138A and Y135F/Y138F. Both single mutants altered at position 135 and the double mutant Y135F/Y138F were able to complement YSD1 when sulfate was the sole sulfur source while the single mutants affecting position 138 and the double mutant Y135A/Y138A were not (Table 2). Preliminary results for the Y135A/Y138A mutant have previously been reported (Loughlin et al., 2002). Transport assays with [^{35}S] sulfate confirmed these results, with the non-complementing mutants showing no transport. Those that retained the ability to complement also demonstrated the ability to transport sulfate but this was reduced to between 10% and 25% of the wild-type activity (Fig. 4). Although there were significant effects on transporter activity, there was no impact on the transporter's affinity for sulfate for any mutant (Fig. 4, Table 2). Western blotting of plasma membrane fractions from YSD1 expressing each mutant showed that the reduction in transport activity to low or zero values was not a reflection of decreased expression of the mutant transporter proteins in the plasma membrane (Fig. 5B), indicating that all mutant proteins were correctly folded and trafficked.

Several other polar residues in helix 2 were mutated including three serine residues (at positions 139, 140 and 153) and Thr152, each of which was replaced by alanine, and Tyr 147, which was replaced by either serine or phenylalanine. The mutants S140A and Y147S resulted in abolition of transporter function as indicated by the failure to complement YSD1 on low sulfate media while all other mutants were able to complement (Table 2). However, sulfate transport assays showed that all mutations reduced the level of transport to 50% or below wild-type levels (Fig. 4A). The affinity for sulfate of each mutant was not adversely affected although the Y147F mutant did show increased affinity for sulfate (Fig. 4D, Table 2). Western blotting of plasma membrane fractions of yeast was used to determine if the reduced transport was due to a failure of the mutant protein to traffic to the plasma membrane. In the case of S140A, the lack of transport was due to the absence of the mutant protein from the yeast plasma membrane (Fig. 5C) but for S139A and T152A expression in the plasma membrane was similar to wild-type SHST1. Different results were obtained for the two substitutions of Tyr147. For Y147S there was no reduction in plasma membrane expression of the transport protein (Fig. 5C) indicating that the absence of transport activity was not due to a lack of normal trafficking. An unexpected result was obtained for the Y147F mutant in that no protein was detected in the plasma membrane even though this mutant had measurable sulfate transport activity. A similar result was obtained for the S153A mutant. As the complementation on 100 μM sulfate plates of these mutants is dependent on galactose and absent when glucose is present (Fig. 3), it is clear that a functional SHST1 protein is expressed for these mutants. Failure to detect protein on a Western blot may be due to sensitivity to proteolysis of the mutant protein (for example loss of the HA tag) or perhaps high turnover rate. In either case, this result is consistent with both mutations affecting folding and stability of the protein.

4. Discussion

We have investigated the role of polar residues in helices 1 and 2 of the sulfate transporter, SHST1. Using bioinformatics tools to analyse the sequences of a range of eukaryotic members of the SulP family (in which the human members comprise the SLC26 family), we identified an extended conserved motif which spans transmembrane helices 1 and 2 and contains a significant number of polar residues (Fig. 1B). Both helices are predicted to be buried within the protein interior and to form helix packing interactions (Table 1, Fig. 2). Consistent with this analysis, the results of our mutagenesis study support a functional role in transport for the extended SulP motif. Firstly, every polar residue within the motif has been mutated either in this or earlier studies (Khurana et al., 2000; Shelden et al., 2001, 2003; Loughlin et al., 2002) and all mutants showed a significant reduction in sulfate transport, in many cases to undetectable levels (Table 2, Fig. 4). Secondly, differential effects were observed for residues on different helical faces in both helices 1 and 2. We conclude that the packing arrangement of these two helices is important for sulfate transport and that the different helical faces play different roles.

Bioinformatic analysis of structures of membrane proteins has resulted in the identification of polar networks that appear to stabilise helix packing and mediate interactions, including polar clamps and polar zippers (Adamian and Liang, 2002) and other motifs (Curran and Engelman, 2003; Eilers et al., 2002; Wan and Milner-White, 1999). In model systems involving screens for motifs driving oligomerisation of single transmembrane helices, it has been found that multiple polar interactions allow tight interactions between transmembrane helices, probably by forming a cooperative hydrogen bonded network (Dawson et al., 2002). It was also observed that, while a single polar interaction mediated by serine or threonine residues was insufficient to cause a tight interaction, mutational disruption of just one interaction within a more extended motif could prevent the helices from interacting (Dawson et al., 2002). This may be as a result of leaving another polar group without a hydrogen bonding partner, which has been found to have a destabilizing effect in the interior of soluble proteins (Blaber et al., 1993).

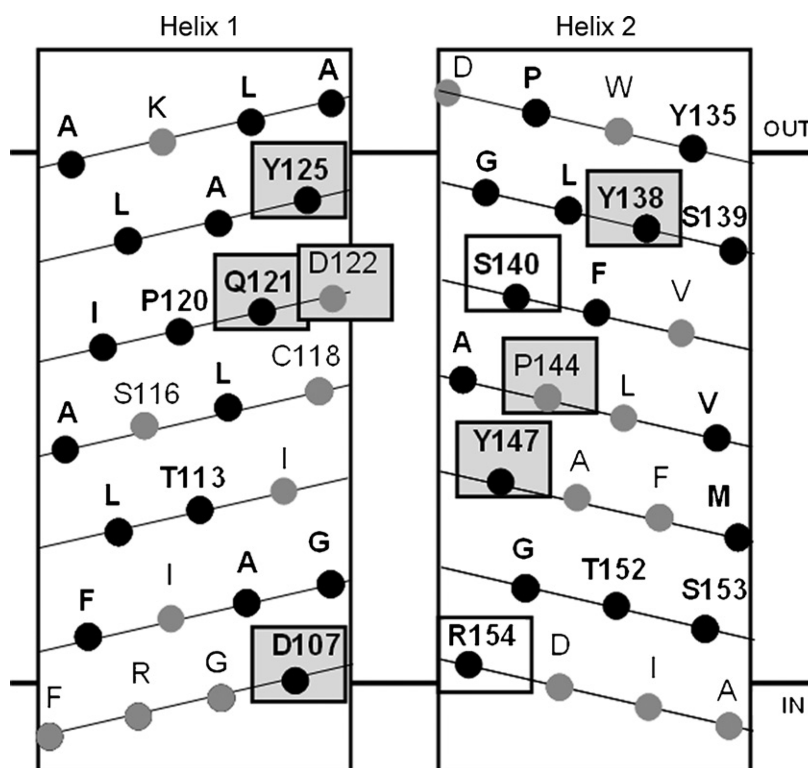

Fig. 6. Helix projections for α-helices 1 and 2 of SHST1 indicating the faces where mutations abolish transport activity. Start and end of α-helices is predicted based upon presence of charged residues (D107 and R154) and hydropathy plot analysis of SHST1 and other SulP family members. The residues that form the extended SulP motif are shown in bold and indicated by black circles while other residues are indicated by grey circles. Numbered residues were mutated in this, or earlier studies. Residues predicted to have a functional role in the transport pathway (as indicated by loss of function but normal trafficking for at least one mutation at that position) are shown in shaded boxes. Residues where mutation reduces activity to less than 20% of wild-type SHST1 due to the failure of the protein to be trafficked to the plasma membrane are shown in white boxes.

Our results reflect what has been observed in model systems and support the prediction that such polar networks are important in stabilising membrane protein structures *in vivo*. We have shown that mutation of polar residues in the SulP motif, even the loss of a single hydroxyl group, has severe effects on sulfate transport. As SHST1 is a polytopic membrane protein, as opposed to the single helices described above to study polar interactions, it is likely that each helix is stabilised by multiple contacts to other helices as well as the interhelix loops. Loss of a single polar group could easily be enough to disrupt a specific interaction on one helical face without having major effects on folding and trafficking. Our observation that the major effects of most mutations were on V_{max}, rather than K_m, is consistent with such small structural perturbations. Remarkably, the mutants for which sulfate transport was completely abolished all map to one face of helix 1 and one face of helix 2 (Fig. 6), except for a pair of tyrosine residues, Tyr135 and Tyr138. These faces are those most likely to interact with other helices as determined by the LIPS and RANTS analyses (Fig. 2). Furthermore, it has previously been proposed that these two faces interact with each other, based on evidence supporting a charge pair between Asp107 at the cytoplasmic end of helix 1 and Arg154 in the equivalent position of helix 2 (Shelden et al., 2003). Our results are consistent with multiple polar interactions supporting tight packing between these two helices, with the loss of polar groups through mutations disrupting the interaction and hence affecting function The effects of polar mutations on other faces were, in general, less severe and these residues may be involved in interactions with other helices.

It is significant that for a number of mutants, particularly those on the interacting faces of helices 1 and 2, the loss of transport activity cannot be attributed to reduced trafficking to the plasma membrane. A correctly folded and trafficked mutant protein that fails to function implies that the mutation has a direct effect on transport but several explanations for how this might occur are possible. As described above, disruption of specific polar interactions that are important in positioning residues in the transport pathway of SHST1 is a likely explanation. This has been observed in other proteins: for example, conformational changes leading to inactivation of HERG potassium channels are thought to involve altered interactions within a network of polar residues close to the pore (Bian et al., 2004). Alternatively, the residues may participate directly in the transport pathway; if this were the case, mutants might be expected to show changes in K_m indicating a reduced affinity for sulfate. We hypothesized that Asp122, which is found only in Group 1 of the plant SulP members, may contribute to the much higher affinity for sulfate seen in members of this subgroup (micromolar K_m) compared

to that of most other family members. To test this hypothesis, we replaced Asp122 with serine, the equivalent residue in other plant sulfate transporters as well as some other SulP members (Fig. 1B). However, as the D122S mutation resulted in the loss of sulfate transport activity, our data are not able to support or preclude this hypothesis.

The hypothesis that polar residues on these two interacting helical faces contribute to the transport pathway is an attractive one for several reasons. It is consistent with the high conservation of this region throughout the SulP/SLC26 family (Fig. 1B), the bioinformatics data suggesting that these helices are buried on the interior of the protein (Table 1, Fig. 2) and our finding that mutation of residues on these faces had the most severe effects on transport activity but not trafficking (Fig. 5). Studies on other transporters have identified polar residues that function directly in transport, for example, in the sulfate binding protein of unrelated ABC-type sulfate transporters, sulfate is bound through polar interactions (Pflugrath and Quiocho, 1988) and in a Na^+-coupled iodide transporter, polar patches on transmembrane helices similar to those formed by the SulP motif appear to contribute to the Na^+ transport pathway (De la Vieja et al., 2007). However, as those SHST1 mutants that did retain some function did not show significant shifts in K_m (Table 2), confirmation of this hypothesis awaits further studies. It should be noted, however, that a similar conclusion about the role of helices 1 and 2 was drawn for another SulP member, prestin (Rajagopalan et al., 2006).

For the four tyrosine residues mutated, it is clear that the aromatic moiety is just as important for function as the hydroxyl group. Aromatic residues at the end of α-helices, particularly tryptophan and tyrosine, are implicated in anchoring helices in the membrane (Killian and von Heijne, 2000). We tested whether the aromatic or hydroxyl group of each tyrosine residue in the SulP motif was important for function by making different substitutions at each position. We found significant, but different, effects on both function and trafficking with different mutations (Figs. 4 and 5). These results are consistent with the tight packing we have proposed for helices 1 and 2 and it can be concluded that aromatic interactions, in addition to polar networks, are important in positioning these helices. Substitution of Tyr125 and Tyr147 with a phenylalanine residue resulted in proteolysis of the mutant protein and reduced trafficking, respectively. In both cases this suggests a structural alteration to the protein and therefore that polar interactions formed by these residues are important for normal folding. However, this was not the case for Tyr135 and Tyr138 which are one helical turn apart at the extracellular end of helix 2 (Fig. 6), and are among the most highly conserved residues in the SulP family. Substitution of either or both these residues with phenylalanine reduced transport activity but did not affect trafficking. Substitution of each of the four residues with serine had a more severe effect on transport, although at least some protein was observed in the plasma membrane for all mutants (Fig. 5), suggesting that the aromatic moieties are more important for transport function. It is possible that these tyrosine residues play an indirect functional role, such as enabling the structural changes necessary for substrate translocation, as previously observed for the KirBac1.1 potassium channel (Domene et al., 2006). Aromatic–aromatic stacking interactions have also been suggested to be important for helix packing (Hong et al., 2007) and this is a likely role for the pair of tyrosine residues at positions 135 and 138.

Although our study has focused on the polar residues in the SulP motif, our conclusion that the SulP motif is important for helix packing is supported by a mutational study of hydrophobic residues in another, distantly related, member of the SulP family, prestin (Rajagopalan et al., 2006). The function of prestin was severely compromised by a number of mutations affecting conserved hydrophobic residues in helices 1 and 2. As both the size and hydrophobicity of the introduced residues determined the severity of the effect, it was suggested that the reduction in function was due to disruption of helix packing. In addition, it was suggested that helices 1 and 2 may be involved in the binding of chloride ions, forming part of the voltage sensor, and that this role was equivalent to the substrate binding site for transport through other SulP members (Rajagopalan et al., 2006). Since several of the mutated residues form part of the extended SulP motif we have identified, this provides further support for its role in the close packing of helices 1 and 2. Taken together with the results of our study on SHST1, this suggests a similar role for helices 1 and 2 throughout the entire SulP/SLC26 family.

In conclusion, we have demonstrated the importance of polar residues in helices 1 and 2 in the structure and function of the model SulP family member, SHST1, using bioinformatics and mutational approaches. Our results suggest an important role for the extended SulP motif in mediating helix packing and that residues on different helical faces have different functional roles. It appears that networks of polar and aromatic interactions in helices 1 and 2 are important for function of SHST1, either by contributing directly to the transport pathway or by influencing the position of crucial residues in these two helices.

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