Proline residues in two tightly coupled helices of the sulphate transporter, SHST1, are important for sulphate transport

Megan C. SHELDEN, Patrick LOUGHLIN, M. Louise TIERNEY and Susan M. HOWITT¹

Division of Biochemistry and Molecular Biology, The Faculties, Australian National University, Canberra, ACT 0200, Australia

The sulphate transporter SHST1, from *Stylosanthes hamata*, features three tightly coupled transmembrane helices which include proline residues that are conserved in most related transporters. We used site-directed mutagenesis and expression of the mutant transporters in yeast to test whether these proline residues are important for function. Four proline residues were replaced by both alanine and leucine. Only one of these proline residues, Pro-144, was essential for sulphate transport. However, mutation of either Pro-133 or Pro-160 resulted in a severe decrease in sulphate transport activity; this was due more to a

decrease in transport activity than to a decrease in the amount of mutant SHST1 in the plasma membrane. These results suggest that all three proline residues are important for transport, and that the conformation of the three tightly coupled helices may play a critical role in sulphate transport. We also show that SHST1 undergoes a post-translational modification that is required for trafficking to the plasma membrane.

Key words: post-translational modification, site-directed mutagenesis, transmembrane helix.

INTRODUCTION

Plants are able to take up sulphate from the soil using specific, high-affinity transporters, which are energized by the co-transport of the sulphate molecule with protons. The first sulphate transporters to be identified and cloned from a plant were from the tropical legume *Stylosanthes hamata* [1]. Expression in yeast has been used to verify their function as sulphate transporters [1]. *S. hamata* possesses three homologous sulphate transporters: SHST1 and SHST2, which are 95% identical at the amino acid level and have K_m values for sulphate of approx. 10 μ M, and SHST3, which has a 10-fold lower affinity [1]. The different transporters have different expression patterns, and are thought to have different roles within the plant [2].

Homologous high-affinity sulphate transporters have been identified in other plants, as well as in fungi and mammals, and are members of an expanding family of anion transporters [3]. This family has been designated SulP (T.C. #53) [3], although it is now clear that some mammalian members of the family do not transport sulphate, but are specific for other anions. Over the last few years, several of the human members of this anion transporter family have been implicated in various diseases. For example, the diseases diastrophic dysplasia (which results in skeletal deformities), Pendred syndrome (a congenital deafness) and congenital chloride diarrhoea all arise as a consequence of mutations in anion transporters from this family (reviewed in [4]). Despite the diversity of effects, these three transporters have significant identity with each other and with the plant sulphate transporters. This suggests that all may operate via a similar mechanism.

There is almost no information to date on the molecular operation of any member of the SulP family. We are using the *S. hamata* sulphate transporter SHST1 as a model member of this family with which to examine structure–function relationships. SHST1 is predicted to have twelve transmembrane helices (Figure 1). We have previously reproduced some mutations found

in patients with diastrophic dysplasia in SHST1 [5]. These had severe effects, consistent with their role in causing diastrophic dysplasia. Mutations affecting two charged residues in putative helix 9 of SHST1 were found to abolish sulphate transport by affecting the assembly and/or trafficking of SHST1, when expressed in yeast [5].

Proline residues have been suggested to play functional roles in membrane proteins, and are found more often than expected by chance in transmembrane helices of transporter proteins [6]. Potential mechanisms by which proline residues could be involved in transport are through *cis*–*trans* isomerization, by causing a kink in the α -helical backbone, or as a result of the unfulfilled backbone hydrogen bonds that arise when a proline residue is incorporated into an α -helix. Four proline residues are found in the first three predicted transmembrane helices of SHST1. These three helices are unusually close together, being connected by very small loops (Figure 1). The presence of proline residues in such tightly coupled helices suggests that they may be important for the correct structure or positioning of these helices. The aim of the present study was therefore to use site-directed mutagenesis to determine whether the four conserved proline residues are necessary for sulphate transport by SHST1. Modelling and experimental studies have indicated that replacement of a proline residue in an α -helix by alanine does not necessarily result in the loss of the kink [7–9], presumably because of packing constraints. We have used a strategy of replacing each proline residue by both alanine and leucine [7] to determine if proline-induced kinks are important for function.

EXPERIMENTAL

Molecular biology

Standard procedures were used for bacterial plasmid isolation and transformation into *Escherichia coli*. SHST1 in the yeast

¹ To whom correspondence should be addressed (e-mail Susan.Howitt@anu.edu.au).

Figure 1 Model for the transmembrane topology of SHST1

The positions of the residues mutated in the present study are indicated.

Table 1 Mutations introduced into SHST1 and the resulting mutant strains

Amino acid change	Codon change	Yeast strain
Pro-120 → Leu	C ⁴¹⁹ CT → CGT	YSD26
Pro-120 → Ala	C ⁴¹⁹ CT → GCT	YSD27
Pro-133 → Leu	C ⁴⁵⁷ CT → CGT	YSD28
Pro-133 → Ala	C ⁴⁵⁷ CT → GCT	YSD29
Pro-144 → Leu	C ⁴⁹⁰ CT → CGT	YSD30
Pro-144 → Ala	C ⁴⁹⁰ CT → GCT	YSD31
Pro-160 → Leu	C ⁵³⁸ CT → CGT	YSD32
Pro-160 → Ala	C ⁵³⁸ CT → GCT	YSD33

expression vector pYES3 [10] 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 sulphate transporter gene *Sul1* [10], was used for expression of SHST1 and the mutants derived from it. Sulphate-free growth medium was prepared as described previously [10] and supplemented with either 38.26 mg/l homocysteine thiolactone or 100 μ M sulphate. Yeast transformation was by an LiCl/polyethylene glycol method [11].

For the growth experiments, yeast cultures were grown at 28 °C, with shaking. Absorbance was measured using a Klett–Summerson calorimeter. The growth rate constant during the exponential phase of growth was calculated for each mutant strain, and this was used to obtain the doubling time [12].

Sulphate uptake assays

Sulphate uptake assays were based on those described by Smith et al. [1,10]. Yeast cells were grown to mid-exponential phase in sulphate-free medium lacking uracil and supplemented with homocysteine thiolactone and 2% (w/v) galactose. Cells were then harvested by centrifugation at 5000 *g* for 15 min, washed and resuspended in sulphur-free growth medium supplemented with galactose, and incubated at 28 °C for 30 min prior to the uptake measurements. Cells (50 μ l) were then incubated with sodium [³⁵S]sulphate for the times indicated, and the reaction

was stopped by rapid centrifugation through a silicone oil layer into 5 μ l of 40% (v/v) perchloric acid. The [³⁵S]sulphate concentration was 50 μ M unless otherwise indicated. Radioactivity in the pellet was determined by liquid-scintillation counting.

Preparation of membrane fractions and Western blotting

Yeast cells for membrane preparation were grown to mid-exponential phase in sulphate-free medium lacking uracil and supplemented with homocysteine thiolactone and 2% (w/v) galactose. After cell lysis, plasma membranes and endoplasmic reticulum were separated on a sucrose density gradient using the procedure of Katzmann et al. [13]. The concentration of protein in cell lysates from each mutant was determined by using the BCA[™] Protein Assay Reagent (Pierce), and the concentration was adjusted so that a similar amount of protein was loaded on to each sucrose gradient. Plasma membrane and endoplasmic reticulum fractions were harvested and protein was precipitated with trichloroacetic acid, as described by Katzmann et al. [13]. Protein was then resuspended in sample buffer (6.25 mM Tris/HCl, pH 6.8, 5% SDS, 6 M urea, 500 mM dithiothreitol, 10% glycerol and 0.002% Bromophenol Blue) and heated at 70 °C for 25 min prior to loading on to a polyacrylamide gel. Proteins were resolved by SDS/PAGE, blotted on to nitrocellulose using a semi-dry transfer protocol and probed with polyclonal antisera raised against SHST1 [5]. Immunoreactive SHST1 was detected with horseradish peroxidase-conjugated goat anti-(rabbit IgG) (ICN) and enhanced chemiluminescence (Pierce; SuperSignal[™] Substrate).

RESULTS

Alignment of three putative N-terminal helices and construction of mutants

Alignment of protein sequences of some members of the SulP family indicated that the first three putative transmembrane helices show a greater level of conservation than the proteins as a whole ([1]; results not shown). This suggests that this region may be important for the function of this family of transporters. Figure 2 shows the alignment of the protein sequences covering the first three putative helices of selected transporters, including members from plants, mammals and fungi. The transporters from plants represent four different functional groups, as defined by phylogenetic and functional analysis [2]. As outlined above, proline residues may be important in the functioning of membrane proteins, and all four proline residues identified in SHST1 are present in at least some other members of the SulP family.

Figure 2 Alignment of selected members of the SulP family showing the first three predicted transmembrane helices

Alignment was performed using ClustalW. Identical residues are shaded, and those mutated in the present study are indicated by asterisks above the sequence. Putative transmembrane helices are indicated by bars above the sequence. The sequences shown, with gi ('gene identifier') numbers in parentheses, are: SHST1 (607184) and SHST3 (607188), *Stylosanthes hamata*; ATD631 (2285884), *Arabidopsis thaliana*; NOD70 (730164), *Glycine max*; SUL1 (3334497), *Saccharomyces cerevisiae*; CYS14 (101874), *Neurospora crassa*; SAT-1 (1173364), *Rattus norvegicus*; DTDST (1706534), CLD (4557535) and pendrin (2654005), *Homo sapiens*.

Table 2 Growth of SHST1 mutants on low-sulphate media

Complementation of YSD1 by each mutant plasmid, as indicated by growth on minimal galactose plates supplemented with 100 μ M sulphate, was rated as follows: + + +, wild-type growth; + +, moderate growth; +, poor growth; —, no growth. Doubling times for growth in liquid minimal medium (supplemented with galactose and 100 μ M sulphate) were calculated as described in the Experimental section. Values are means $\pm$ S.E.M. from at least three independent experiments.

Mutant strain	Complementation on agar plates	Doubling time in liquid culture (h)
YSD7(SHST1)	+ + +	5.5 $\pm$ 0.1
YSD6(pYES)	—	—
YSD26(P120L)	+ + +	5.8 $\pm$ 0.8
YSD27(P120A)	+ + +	4.9 $\pm$ 0.6
YSD28(P133L)	+	16.5 $\pm$ 2.1
YSD29(P133A)	+ + +	5.3 $\pm$ 0.3
YSD30(P144L)	—	—
YSD31(P144A)	+	—
YSD32(P160L)	+ +	27.6 $\pm$ 0.8
YSD33(P160A)	+ +	26.8 $\pm$ 5.0

Two of these (Pro-120 and Pro-133 in SHST1) are conserved in at least eight of the ten sequences shown. A proline residue in an equivalent position to Pro-160 is found in six sequences, whereas the remaining proline, Pro-144, is present only in the four transporters from plants. Figure 1 shows a predicted topology

for SHST1, indicating the locations of these residues. Pro-133 occurs between putative transmembrane helices 1 and 2, and is predicted to participate in a β -turn [14], while the other three proline residues appear to be located within transmembrane helices. The spacing between the three more highly conserved proline residues (Pro-120, Pro-133 and Pro-160 in SHST1) is also conserved. In all sequences examined, the first three helices are separated by very small loops (Figure 2).

We tested whether this conservation was due to an essential functional role by using site-directed mutagenesis to make the amino acid changes shown in Table 1. All four proline residues were substituted with both alanine and leucine. The mutant plasmids were then transformed into the sulphate-uptake-deficient yeast strain YSD1 [10] to give the strains indicated in Table 1.

Growth properties of and sulphate uptake by mutant strains

The mutant plasmids were tested for their ability to complement the yeast strain YSD1 on plates containing 100 μ M sulphate, with galactose as the carbon source. One mutation, Pro-144 $\rightarrow$ Leu, abolished complementation on solid media, while all other mutants were able to grow on 100 μ M sulphate (Table 2). However, YSD28(P133L) and YSD31(P144A) grew very poorly. These results were confirmed by growth in liquid medium, except that YSD31(P144A) failed to grow at all (Table 2). It is apparent

Table 3 Kinetic parameters of SHST1 mutants

Uptake of radioactive sulphate was measured at different concentrations of sulphate, and the Michaelis–Menten equation was fitted to the data to determine K_m and V_{max} values. Values are means $\pm$ S.E.M. of at least three independent experiments.

Mutant strain	V_{max} (nmol/min per mg of protein)	K_m (μ M)
YSD7(SHST1)	3.1 ± 0.3	9 ± 2
YSD6(pYES)	< 0.2	—
YSD26(P120L)	1.3 ± 0.2	23 ± 3
YSD27(P120A)	1.1 ± 0.2	21 ± 1
YSD28(P133L)	0.4 ± 0.1	7 ± 2
YSD29(P133A)	1.5 ± 0.3	3 ± 1
YSD30(P144L)	< 0.2	—
YSD31(P144A)	< 0.2	—
YSD32(P160L)	0.6 ± 0.1	19 ± 2
YSD33(P160A)	0.6 ± 0.1	9 ± 2

that the growth rates of strains YSD28(P133L), YSD32(P160L) and YSD33(P160A) were significantly reduced, while the other strains grew at a rate similar to that of wild-type SHST1.

The ability of each mutant strain to take up sulphate was determined, and the results are shown in Table 3. All the mutations resulted in sulphate uptake activity being reduced by at least 50%. There was no measurable sulphate uptake activity in the mutant YSD30(P144L), consistent with its failure to grow with sulphate as the sole sulphur source (Table 2). Although slight growth of YSD31(P144A) could be seen on solid medium, sulphate uptake activity was not significantly different from that of the negative control. Both changes at Pro-160 and the Pro-133 $\rightarrow$ Leu mutation resulted in low but measurable sulphate uptake activity, while the remaining mutants had between one-third and one-half of the transport activity of wild-type SHST1. The K_m for sulphate was determined for those mutants that had retained some sulphate uptake activity (Table 3). For YSD26(P120L), YSD27(P120A) and YSD33(P160L), the K_m had approximately doubled, while YSD33(P160A) and YSD28(P133L) had K_m values similar to that of wild-type SHST1. YSD29(P133A) showed a 3-fold decrease in K_m , indicating that this mutation had increased the affinity of the transporter for sulphate.

Characterization of SHST1 by Western blotting

An important consideration in mutant studies is whether or not the mutant protein is normally expressed and trafficked. To resolve this question, polyclonal antisera had been raised previously against the C-terminal, hydrophilic portion of SHST1 [5]. Surprisingly, these antisera recognized a protein of 140 kDa in the plasma membrane of yeast expressing SHST1 [5], although the predicted molecular mass is 73 kDa. The reason for this aberrant running was investigated prior to characterization of the mutant transporters. Membranes were separated on a sucrose gradient, as described in the Experimental section, and fractions 5–14, which include the endoplasmic reticulum and the plasma membrane [13], were examined by Western blotting (Figure 3). It is clear from this experiment that SHST1 in the endoplasmic reticulum runs at the expected molecular mass of approx. 70 kDa, but that in the plasma membrane it runs at double this size. This suggests that SHST1 undergoes some post-translational modification in the endoplasmic reticulum which increases the apparent molecular mass and is required for trafficking to the plasma membrane.

Figure 3 Western blot of endoplasmic reticulum and plasma membranes from strain YSD7 (expressing wild-type SHST1)

Yeast membranes were separated on a sucrose gradient, as described in the Experimental section. Proteins from fractions 5–14 from the sucrose gradient were separated by SDS/PAGE, transferred to nitrocellulose and probed with polyclonal antisera to SHST1. The fractions enriched for the endoplasmic reticulum are indicated as ER, and those enriched for the plasma membranes as PM ([13]; M. C. Shelden and S. M. Howitt, unpublished work).

Figure 4 Western blot of plasma membrane proteins from mutant yeast strains

Cells were grown and plasma membranes prepared as described in the Experimental section. Plasma membrane proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with polyclonal antisera to SHST1. Lane 1, YSD7(SHST1); lane 2, YSD6(pYES); lane 3, YSD26(P120L); lane 4, YSD27(P120A); lane 5, YSD28(P133L); lane 6, YSD29(P133A); lane 7, YSD30 (P144L); lane 8, YSD31(P144A); lane 9, YSD32(P160L); lane 10, YSD33(P160A).

The doubling of the apparent molecular mass could be due to the formation of an SDS-stable dimer of two SHST1 molecules. To investigate this, different methods of sample preparation for SDS/PAGE were tested for their effects on the electrophoretic mobility of SHST1. Disulphide bridges are unlikely to be involved, as the sample buffers always included 500 mM dithiothreitol. In different experiments, we tested the effect of temperature in the range 37–100 °C, the inclusion of urea up to 6 M, SDS concentrations from 4–12%, dilution of protein in the sample and solubilization of SHST1 from plasma membranes with different detergents prior to incubation in sample buffer. None of these treatments, however, affected the migration of SHST1 on polyacrylamide gels (results not shown). We also investigated whether or not SHST1 was glycosylated, by growing YSD7(SHST1) in the presence of tunicamycin. However, we could not detect a difference in molecular mass between tunicamycin-grown cells and control cells for SHST1 in either the endoplasmic reticulum or the plasma membrane, suggesting that SHST1 is not glycosylated (results not shown).

Expression of mutant sulphate transporters in the plasma membrane

The low level of sulphate transport activity observed in all mutants could result from the loss of function of SHST1 or from a failure of SHST1 to be correctly trafficked to the plasma membrane. To distinguish between these two alternatives,

plasma membranes from each of the mutant strains were isolated on sucrose gradients and used for Western blotting, as described in the Experimental section. Figure 4 shows the results of this experiment. All of the mutant proteins were observed to run at 140 kDa, similar to wild-type SHST1. Only the Pro-144 → Leu mutation substantially reduced the level of expression of SHST1 in the plasma membrane. The mutations Pro-120 → Leu and Pro-133 → Ala both resulted in significantly less SHST1 in the plasma membrane. This lower expression correlates with a lower $V_{\max}$ (Table 3), suggesting that the major effect of these two mutations is on trafficking. Both YSD31(P144A) and YSD33(P160A) had protein levels in the plasma membrane that were similar to that of the wild type, so it can be concluded that the reduced $V_{\max}$ values observed are due to decreases in the sulphate transport activity of these mutant transporters. The remaining mutants, YSD27(P120A), YSD28(P133L) and YSD32(P160L), appear to show slightly lower expression of SHST1 in the plasma membrane. For both YSD28(P133L) and YSD32(P160L) there was still a significant amount of SHST1 in the plasma membrane, but the $V_{\max}$ values were extremely low (Table 3). These results indicate that these mutations affect the transport activity of the mutant transporter, as well as its trafficking.

DISCUSSION

We have shown that SHST1 undergoes post-translational modification in the endoplasmic reticulum, resulting in a doubling of the apparent molecular mass, and that this modification appears to be required for trafficking of the transported to the plasma membrane. However, we have been unable to determine the nature of the modification. Our results suggest that it is not due to glycosylation or the formation of disulphide bridges. SHST1 has two putative extracellular N-linked glycosylation sites, but both are predicted to be very close to the membrane boundary, which may prevent glycosylation. The large increase in the apparent molecular mass of SHST1 (from 70 kDa in the endoplasmic reticulum to 140 kDa in the plasma membrane) suggests that SHST1 is interacting with another protein. If this occurs, the interaction must be extremely stable, as we have been unable to disrupt it by quite harsh treatments, including heat, 6 M urea and 12% SDS. One possibility is that SHST1 is functional as a dimer. Glycophorin A, which is a membrane protein with a single transmembrane helix, forms an SDS-stable dimer [15]. Dimerization is mediated by a non-polar face of the transmembrane helix, and the dimer is resistant to boiling [16] and treatment with urea [17]. Although most secondary transporters are thought to function as monomers, this is not always the case. Glutamate transporters from the human brain have been shown to form dimers and trimers [18], and the sodium/proton exchanger, NhaA, has been crystallized as a dimer [19], although it is not known if this is the functional unit. None of the mutations we made in the present study affected the running of SHST1 at 140 kDa, but it is possible that mutagenesis could be used to explore the nature of the modification of SHST1. For example, the sequence GXXXG, which has been identified as a dimerization motif for transmembrane helices [20], occurs three times in the transmembrane helices of SHST1. Alternatively, SHST1 might not interact with another protein, but there may be some post-translational modification that dramatically alters the folding of SHST1. Given that SHST1 is a very hydrophobic protein, this could account for its aberrant running on SDS/PAGE.

The main aim of the present study was to investigate the effects of mutating proline residues on SHST1 function. Each proline

residue was replaced with both alanine and leucine. Proline causes a kink in a transmembrane helix and also results in disruption of the backbone hydrogen-bonding. In at least some cases, alanine can replace proline in an α -helix without removing the kink [7–9], but it does restore the backbone hydrogen-bonding. Leucine, however, is larger and will result in both the loss of the kink and the restoration of backbone hydrogen-bonding. Thus these two effects can be distinguished by comparing the effects of the two mutations at a single position.

The only position at which both substitutions abolished activity was at Pro-144, which occurs in putative transmembrane helix 2 (Figure 1). Surprisingly, this was the least conserved residue of those mutated, with proline present at this position only in sulphate transporters from plants. The Pro-144 → Leu mutation severely reduced trafficking to the plasma membrane and abolished sulphate transport activity, suggesting that this mutation causes a significant structural perturbation. The Pro-144 → Ala mutation also abolished activity, but allowed retention of normal trafficking, which indicates that Pro-144 is required for sulphate transport activity. If the alanine substitution did not disrupt the kink in this transmembrane helix, as has been observed previously [7,9], this would suggest that the unsatisfied backbone hydrogen bonds near the proline residue may play a role in the transport of sulphate. Interestingly, in members of the Sulp family in which Pro-144 is not conserved, there is either a polar residue at this position or a glycine or proline residue at the N-terminal adjacent position (Figure 2). This is consistent with a requirement for hydrogen-bonding capacity in this region. The hydrogen-bonding could arise either through a break in the helix backbone or from a polar residue. Alternatively, a requirement for proline at this position may be specific to plant members of the Sulp family.

The Pro-133 → Leu mutation also resulted in the almost complete loss of sulphate transport activity. There was some reduction in the amount of mutant SHST1 in the plasma membrane, but this was not sufficient to account for the decrease in transport activity. Therefore this mutation affects the functioning of the transporter. The Pro-133 → Leu mutation also affected function, in that it resulted in a 3-fold increase in affinity for sulphate. The $V_{\max}$ for sulphate of YSD28(P133A) was about half that of wild-type SHST1, and this could most probably be explained by the reduced plasma membrane expression of the mutant transporter. Pro-133 is predicted to occur within a β -turn [14] at the extracellular end of putative transmembrane helix 2 (Figure 1), and forms part of a highly conserved motif, PXYGLY (Figure 2). If Pro-133 does participate in a β -turn, it is likely that the conformation of the turn would be altered by the leucine substitution, and the subsequent repositioning of these conserved residues may explain the reduced activity. Another possible role for a proline residue at this position is in initiating helix 2. Statistical studies have found that proline has a preference for the second position at the N-terminal end of a helix [21], where it may have a role in stabilizing the helix.

Neither Pro-120 nor Pro-160 is essential for sulphate transport, as all mutants with substitutions at these positions were able to complement YSD1. However, our results suggest an important role for Pro-160 in sulphate transport. The effects of the mutations at position Pro-160 were more severe than those of mutations at Pro-120, as indicated by the significantly increased doubling times for growth in liquid medium with 100 μ M sulphate as the sole sulphur source. The $V_{\max}$ for sulphate transport was very low for both Pro-160 mutants. SHST1 carrying the mutation Pro160 → Ala was present in the plasma membrane at similar levels to wild-type SHST1. This is consistent with retention of the kink in this transmembrane helix so that

there is only a minor perturbation in structure. Nevertheless, this mutation is sufficient to dramatically reduce the sulphate transport activity of SHST1, indicating a functional role for Pro-160.

Pro-120, in contrast, is more likely to have a structural role. The effects of the two mutations at this position were similar, resulting in a decrease in $V_{\max}$ and an increase in K_m . These changes, however, were not sufficient to affect the growth rate of these mutants on sulphate. Both Pro-120 mutations were also observed to affect the migration of SHST1 on a polyacrylamide gel, as indicated by the smearing seen in Figure 4. This is also consistent with an alteration to the normal structure of SHST1.

Taken together, our results strongly suggest a major role in sulphate transport for helices 2 and 3 of SHST1. Pro-133 and Pro-144 are in helix 2, with Pro-133 predicted to be at the extracellular end and Pro-144 midway across the membrane (Figure 1). We have shown that Pro-144 is essential for activity and that Pro-133 also appears to play a role in sulphate transport, probably in maintaining the correct conformation of the loop between helices 1 and 2. Pro-160 is also important for function, as indicated by the severity of the effects of the alanine mutation. Pro-160 is in helix 3, which is connected to helix 2 by a very tight turn. The effects seen with the mutations at Pro-120 in helix 1 could result from a slight repositioning of helices 2 or 3 relative to each other, or to other residues required for transport activity. This would be likely to occur as proline residues often have structural roles, and the first three helices in SHST1 are separated by very small loops. Proline residues essential for function have been identified in transmembrane helices in a number of other transporters [7,22–25]. In both the *Pst* phosphate transport system in *E. coli* [22] and a mammalian dopamine transporter [23], proline residues in tightly coupled helices play a role in transport, similar to the situation we have observed in SHST1.

We thank Frank Smith (CSIRO Tropical Agriculture, Indooroopilly, Queensland, Australia) and Malcolm Hawkesford (IACR Rothamsted, Harpenden, Herts., U.K.) for the gift of the yeast strain YSD1 and the cDNA for *shst1*. We are grateful to Dr Dean Price for helpful discussions, and to Fiona Leves for technical assistance. This work was supported by a grant from the Australian Research Council.

REFERENCES

- Smith, F. W., Ealing, P. M., Hawkesford, M. J. and Clarkson, D. T. (1995) Plant members of a family of sulfate transporters reveal functional subtypes. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9373–9377.
- Hawkesford, M. J. (2000) Plant responses to sulphur deficiency and the genetic manipulation of sulphate transporters to improve S-utilization efficiency. *J. Exp. Bot.* **51**, 131–138.
- Saier, Jr., M. H., Eng, B. H., Fard, S., Garg, J., Haggerty, D. A., Hutchinson, W. J., Jack, D. L., Lai, E. C., Liu, H. J. and Nusinew, D. P. et al. (1999) Phylogenetic characterization of novel transport protein families revealed by genome analyses. *Biochim. Biophys. Acta* **1422**, 1–56.
- Everett, L. A. and Green, E. D. (1999) A family of mammalian anion transporters and their involvement in human genetic diseases. *Hum. Mol. Genet.* **8**, 1883–1891.
- Khurana, O. K., Coupland, L. A., Shelden, M. C. and Howitt, S. M. (2000) Homologous mutations in two diverse sulphate transporters have similar effects. *FEBS Lett.* **477**, 118–122.
- Brandl, C. J. and Deber, C. M. (1986) Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 917–921.
- Ri, Y., Ballesteros, J. A., Abrams, C. K., Oh, S., Verselis, V. K., Weinstein, H. and Bargiello, T. A. (1999) The role of a conserved proline residue in mediating conformational changes associated with voltage gating of Cx32 gap junctions. *Biophys. J.* **76**, 2887–2898.
- von Heijne, G. (1991) Proline kinks in transmembrane alpha-helices. *J. Mol. Biol.* **218**, 499–503.
- Yuan, H. S., Wang, S. S., Yang, W. Z., Finkel, S. E. and Johnson, R. C. (1994) The structure of Fis mutant Pro61Ala illustrates that the kink within the long alpha-helix is not due to the presence of the proline residue. *J. Biol. Chem.* **269**, 28947–28954.
- Smith, F. W., Hawkesford, M. J., Prosser, I. M. and Clarkson, D. T. (1995) Isolation of a cDNA from *Saccharomyces cerevisiae* that encodes a high affinity sulphate transporter at the plasma membrane. *Mol. Gen. Genet.* **247**, 709–715.
- Gietz, R. D., Schiestl, R. H., Willems, A. R. and Woods, R. A. (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11**, 355–360.
- Stanier, R. Y., Adelberg, E. A. and Ingraham, J. L. (1976) *General Microbiology*, 4th edn, MacMillan Press, London.
- Katzmann, D. J., Epping, E. A. and Moye-Rowley, W. S. (1999) Mutational disruption of plasma membrane trafficking of *Saccharomyces cerevisiae* Yor1p, a homologue of mammalian multidrug resistance protein. *Mol. Cell. Biol.* **19**, 2998–3009.
- Chou, P. Y. and Fasman, G. D. (1974) Prediction of protein conformation. *Biochemistry* **13**, 222–245.
- Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B. J., Dempsey, C. E. and Engelman, D. M. (1992) Glycophorin A dimerization is driven by specific interactions between transmembrane alpha-helices. *J. Biol. Chem.* **267**, 7683–7689.
- Fisher, L. E., Engelman, D. M. and Sturgis, J. N. (1999) Detergents modulate dimerization, but not helicity, of the glycophorin A transmembrane domain. *J. Mol. Biol.* **293**, 639–651.
- Marton, L. S. and Garvin, L. E. (1973) Subunit structure of the major human erythrocyte glycoprotein: depolymerization by heating ghosts with sodium dodecyl sulfate. *Biochem. Biophys. Res. Commun.* **52**, 1457–1462.
- Haugeto, O., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., Lehre, K. P. and Danbolt, N. C. (1996) Brain glutamate transporter proteins form homomultimers. *J. Biol. Chem.* **271**, 27715–27722.
- Williams, K. A., Geldmacher-Kaufer, U., Padan, E., Schuldiner, S. and Kuhlbrandt, W. (1999) Projection structure of NhaA, a secondary transporter from *Escherichia coli*, at 4.0 Å resolution. *EMBO J.* **18**, 3558–3563.
- Russ, W. P. and Engelman, D. M. (2000) The GxxxG motif: a framework for transmembrane helix-helix association. *J. Mol. Biol.* **296**, 911–919.
- Richardson, J. S. and Richardson, D. C. (1988) Amino acid preferences for specific locations at the ends of alpha helices. *Science* **240**, 1648–1652.
- Webb, D. C., Rosenberg, H. and Cox, G. B. (1992) Mutational analysis of the *Escherichia coli* phosphate-specific transport system, a member of the traffic ATPase (or ABC) family of membrane transporters. A role for proline residues in transmembrane helices. *J. Biol. Chem.* **267**, 24661–24668.
- Lin, Z., Itokawa, M. and Uhl, G. R. (2000) Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition, and expression. *FASEB J.* **14**, 715–728.
- Ferreira, T., Napias, C., Chevallier, J. and Brethes, D. (1999) Evidence for a dynamic role for proline376 in the purine-cytosine permease of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **263**, 57–64.
- Vilsen, B., Andersen, J. P., Clarke, D. M. and MacLennan, D. H. (1989) Functional consequences of proline mutations in the cytoplasmic and transmembrane sectors of the Ca²⁺(+)-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **264**, 21024–21030.

Received 2 January 2001/5 March 2001; accepted 28 March 2001