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The role of cysteine residues in the sulphate transporter, SHST1: Construction of a functional cysteine-less transporter

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Abstract

We investigated the role of cysteine residues in the sulphate transporter, SHST1, with the aim of generating a functional cysteine-less variant. SHST1 contains five cysteine residues and none was essential for function. However, replacement of C421 resulted in a reduction in transport activity. Sulphate transport by C205 mutants was dependent on the size of the residue at this position. Alanine at position 205 resulted in a complete loss of function whereas leucine resulted in a 3-fold increase in sulphate transport relative to wild type SHST1. C205 is located in a putative intracellular loop and our results suggest that this loop may be important for sulphate transport. By replacing C205 with leucine and the other four cysteine residues with alanine, we constructed a cysteine-less variant of SHST1 that has transport characteristics indistinguishable from wild type. This construct will be useful for further structure and function studies of SHST1.

Keywords: Sulphate transporter; Site-directed mutagenesis; Cysteine; Diastrophic dysplasia; Pendred syndrome; Congenital chloride diarrhoea

1. Introduction

Plants require sulphur for growth, obtaining it mainly as sulphate from the soil. Sulphate is taken up by specific transporters and is redistributed within the plant by processes involving other transporters [1–3]. The sulphate transporter gene family is extensive, consisting of 14 members in *Arabidopsis* [2] with homologous genes identified in a number of other plant species. This gene family has been divided into five groups, based on sequence homology, and it has been postulated that the different groups have different functional roles within the plant. There is some evidence to support this, with transporters from different groups having different affinities for sulphate, different regulation patterns and different distributions within the plant (for reviews see Refs. [1–3]). The kinetic properties of some transporters have been characterised by expression in yeast, using complementation of a sulphate transport deficient yeast strain [4–6]. However, characterisation of the family is far from complete and not all members have been demonstrated to transport sulphate.

Homologous sulphate transporters have been identified in other organisms, including fungi and mammals, and are members of an expanding family of anion transporters [7]. This family has been designated SulP 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 transporter family, known as the SCL26 family [8], have been implicated in different 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 Ref [9]). Despite the diversity of effects, these three transporters have significant homology to each other and to the plant sulphate transporters. This suggests that all may operate via a similar mechanism.

We are using SHST1, a high affinity transporter from the tropical legume, *Stylosanthes hamata*, as a model system to

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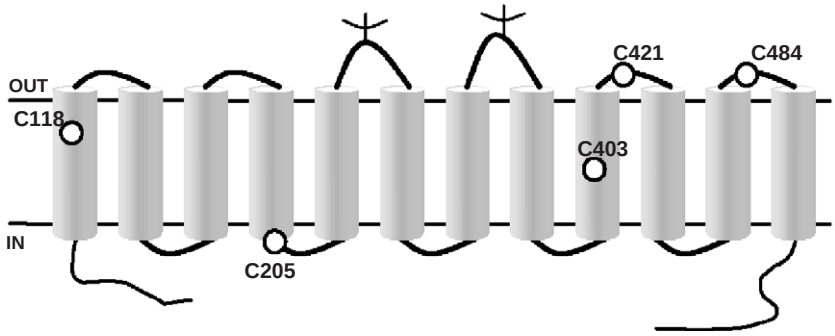

Fig. 1. A model for the transmembrane topology of SHST1. The positions of cysteine residues are indicated.

study structure and function of this family. SHST1 is a group 1 transporter, being involved in the high affinity of uptake from the soil into plant roots, and can be functionally expressed in yeast [6]. Previous studies have indicated that the first three helices of SHST1 play an important role in sulphate transport [10–12] and have identified interactions between charged amino acid residues that are functionally important. One very effective approach to structure and function of transporters is cysteine-scanning mutagenesis combined with labelling by sulfhydryl reagents. This would allow experimental determination of the topology of SHST1 as well as more sophisticated labelling experiments to confirm interhelical interactions and functional roles of residues as has been done, for example, for the *lac* permease from *E. coli* [13]. In order to do these experiments, it is first necessary to construct a mutant SHST1, which lacks all cysteine residues, and to demonstrate that this mutant transporter is still functional. SHST1 contains five cysteine residues, all of which are in, or close to, putative transmembrane helices (Fig. 1). The aim of the present work was to mutate each of the five cysteine residues in SHST1 to determine if any of them was necessary for function and to construct and characterize a cys-less SHST1.

2. Materials and methods

2.1. Molecular biology

Standard procedures were used for bacterial plasmid isolation and transformation into *E. coli*. The *shst1* cDNA in the yeast expression vector pYES3 [14] was used for site-directed mutagenesis, using the Quikchange method from Stratagene. Mutagenic oligonucleotides were 25–35 bases long. The entire *shst1* cDNA was sequenced following mutagenesis to confirm that no PCR-derived errors had arisen.

2.2. Yeast growth and transformation

Saccharomyces cerevisiae strain YSD1, which has a deletion in the native sulphate transporter gene, *Sul1* [14], was used for expression of SHST1 and the mutants derived from it. Sulphate-free growth medium was prepared as previously described [14] and supplemented with either 38.26 mg/l homocysteine thiolactone or 100 μM sulphate. Yeast transformation was by a Li acetate/PEG method [15].

2.3. Sulphate uptake assays

Sulphate uptake assays were based on those described by Smith et al. [6,14]. Yeast cells were grown to mid log phase in sulphate-free media minus uracil and supplemented with homocysteine thiolactone and 2% galactose. Cells were then harvested by centrifugation at 5000 ×*g* for 5 min, washed and resuspended in sulphur-free growth medium supplemented with galactose, and incubated at 30 °C for 15 min prior to the uptake measurements. Cells (50 μl) were then incubated with [^{35}S] sodium sulphate of the desired concentration. Samples were withdrawn at 1 min intervals and the reaction stopped by rapid centrifugation through a silicon oil layer into 5 μl 40% perchloric acid. Radioactivity in the pellet was determined by liquid scintillation counting.

2.4. Preparation of plasma membranes for Western blotting

Yeast cells for membrane preparation were grown to mid log phase in sulphate-free media minus uracil and supplemented with homocysteine thiolactone and 2% galactose. After cell lysis, plasma membranes were purified on a sucrose density gradient following the procedure of Katzmann et al. [16], as modified by Shelden et al. [11]. Proteins were resolved by SDS-PAGE, blotted onto nitrocellulose using a semidry transfer protocol and probed with polyclonal antisera raised against SHST1 [17]. Immunoreactive SHST1 was detected with a horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN) and enhanced chemiluminescence (Pierce, SuperSignal™ Substrate).

3. Results

3.1. Construction and properties of single cysteine mutants

Site-directed mutagenesis was used to individually replace each cysteine residue in SHST1 with alanine. The

mutant plasmids were tested for their ability to complement the yeast strain YSD1 on plates containing 100 μM sulphate with galactose as the carbon source. The mutation C205A abolished complementation while all other single cysteine replacements still allowed growth (Fig. 2A). The absence of complementation by the C205A mutant was surprising as cysteine residues in transmembrane helices are often found to be replaceable and C205 is not conserved within the SulP family. Other members of the SulP family commonly have a larger residue in this position, usually leucine or phenylalanine, and this larger residue is conserved in plant, fungal and mammalian transporters, except for SHST1 (Fig. 3). This suggested that the problem with the C205A mutant may have been a structural problem arising because alanine was smaller than cysteine rather than because the cysteine residue itself was required for SHST1 function. To test this idea, C205 was replaced by leucine, which is found in the related transporter, SHST3, and also by serine which is isosteric with cysteine. The resulting C205S and C205L single mutants were able to grow on minimal media containing 100 μM sulphate (Fig. 2A) providing evidence that C205 is not necessary for sulphate transport by SHST1. Thus, none of the cysteine residues in SHST1 is essential for function.

Sulphate transport activity was measured for each mutant (Fig. 2B). The C118A, C405A and C484A mutations had essentially wild type transport activity, while the C421A mutant had around 40% of wild type activity. The three mutations at position 205 had very different effects. The C205A mutant had no detectable sulphate transport activity, consistent with its growth phenotype, while the C205S mutant had transport activity at a level somewhat lower than wild type. Surprisingly, the C205L mutant had about three times the transport activity of the wild type transporter.

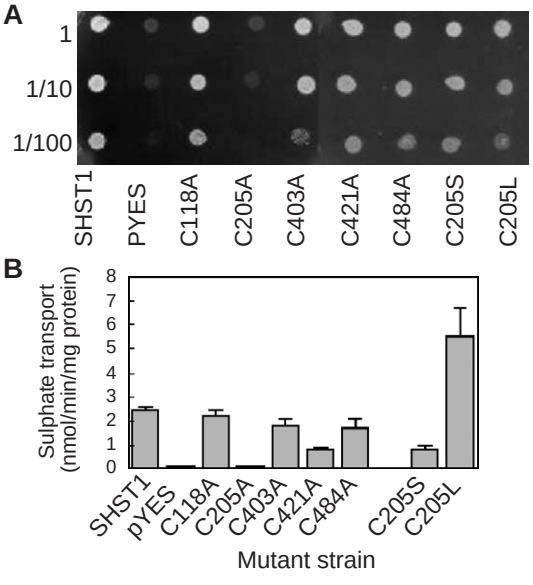

Fig. 2. A. Complementation of YSD1 by single cysteine mutants on 100 μM sulphate media. Cells were resuspended in water and diluted as shown. B. Sulphate transport activity of single cysteine mutants. Uptake of 50 μM sulphate was measured over time as described in Materials and methods. Each value is the mean (with standard error) of at least three independent experiments.

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SHST1   RLAFTATFFAGVTQMLLGVCRLGFLID
SHST3   NLVFTVTLFAGIFQTAFGVLRLGFLVD
AtST3.1 HLAFTATFFAGVLEASLGIFRLGFIVD
AtST4.1 ELAILLALLVGILECIMGLLRLGWLIR
CYS14   DIARTLAFISGAMLLFLGLIRFGFIVE
SUL1    IIATTLCLLCGIVATGLGILRLGFLVE
Pendrin LIASALTLLVGIIQLIFGGLQIGFIVR
CLD     AAAASVTVLSGIIQLAFGILRIGFVVI
DTDST   MVGSTVTFIAGVYQVAMGFFQVGFVSV
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Fig. 3. Alignment of selected SulP transporters showing putative transmembrane helix 4 and the following interhelix loop. The position of C205 is highlighted and putative helix 4 is indicated with a black bar above the sequence. The transporter sequences were chosen to reflect different subgroups with the SulP family and Clustal W used to generate the alignment. Invariant residues are indicated by '*', conserved residues as ':' and semi-conserved residues by '.'. The transporters shown are (accession number in parentheses): SHST1, *S. hamata* high affinity sulphate transporter, Group 1 (P53391); SHST3, *S. hamata* low affinity sulphate transporter, Group 2 (P53393); ATST1, *Arabidopsis thaliana* putative sulphate transporter, Group 3 (T48901); *A. thaliana* putative sulphate transporter, Group 4 (BAA23424); CYS14, *Neurospora crassa* sulphate permease II (P23622); SUL1, *Saccharomyces cerevisiae* high affinity sulphate transporter (P38359); pendrin, human sodium-independent chloride/iodide transporter (O43511); CLD, human chloride anion exchanger (P40879); and DTDST, human diastrophic dysplasia sulphate transporter (P50443).

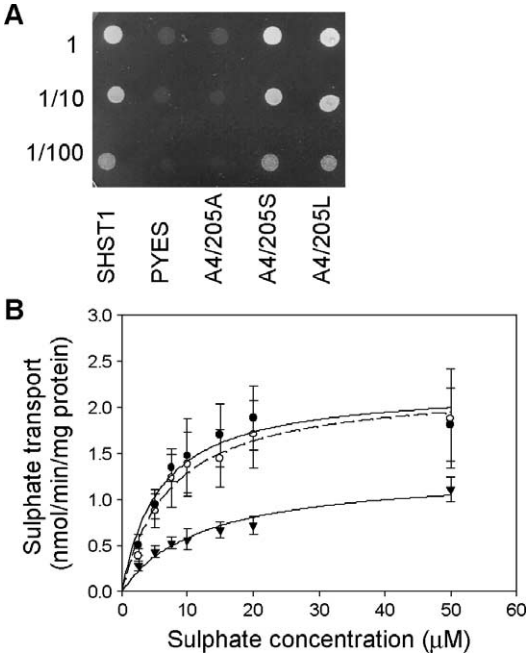

Fig. 4. A. Complementation of YSD1 on 100 μM sulphate media by cysless SHST1 variants. Cells were resuspended in water and diluted as shown. B. Kinetics of sulphate transport activity of cys-less variants of SHST1. Uptake of radioactive sulphate was measured at different concentrations of sulphate and 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. SHST1 (○), dashed line; A4/205L (●), solid line; A4/205S (▼), solid line.

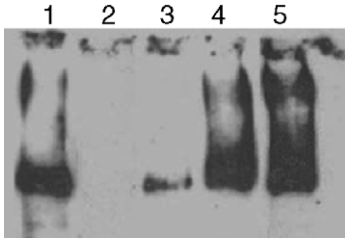

Fig. 5. Western blot of plasma membranes from cys-less variants of SHST1. Yeast membranes were separated on a sucrose gradient as described in Materials and methods. Plasma membrane proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with polyclonal antisera to SHST1. Lane 1, SHST1; Lane 2, pYES; Lane 3, A4/205A mutant; Lane 4, A4/205S mutant; Lane 5, A4/205L mutant.

3.2. Construction and characterization of a cysteine-less SHST1

Repeated rounds of mutagenesis resulted in the production of three cys-less SHST1 variants in which C205 was replaced by alanine, serine or leucine and the other four cysteine residues were replaced by alanine. As expected, the cys-less transporter with alanine at position 205 (A4/205A) was non-functional as indicated by complementation (Fig. 4A). The cys-less transporters with serine or leucine at position 205 (A4/205S and A4/205L) were both able to complement YSD1 and had significant levels of sulphate transport activity (Fig. 4). In the case of A4/205L, the V_{max} for sulphate transport was not significantly different from wild type SHST1. This mutant also had a K_m value of 5.7±1.3 μM, similar to the wild type value of 7.3±1.4 μM (Fig. 4B). The A4/205S mutant had a somewhat lower V_{max} of 1.3±0.1 nmol/min/mg protein, compared to 2.2±0.1 nmol/min/mg protein for wild type SHST1 and a slightly increased K_m of 12.2±2.4 μM.

The fact that residue size at position 205 made such a difference to activity suggests that the conformation of this region of the transporter may be important for structure or function of the transporter. If the altered conformation in the mutants prevented correct folding, this would result in reduced trafficking to the plasma membrane as the unfolded mutant proteins would be degraded. To examine this possibility, plasma membranes from yeast cells expressing each of the cys-less transporters were isolated on sucrose gradients and used for Western blotting as described in Materials and methods. Fig. 5 shows the results of this experiment. The A4/205A mutant shows significantly reduced cell surface expression while the A4/205S and A4/205L mutants are present on the plasma membrane in amounts similar to or greater than wild type SHST1.

4. Discussion

The aim of this work was to determine if it was possible to construct a cys-less variant of SHST1 that retained significant function so that it could be used for cysteine scanning mutagenesis. We have successfully constructed a functional cys-less variant of SHST1, which has transport properties indistinguishable from the wild type protein. Cysteine residues in proteins may function in the active site or in formation of disulphide bonds but our results indicate that no cysteine residue is essential for function of SHST1. There are numerous examples of transporters where individual cysteine residues are not essential [18–23]; however, in some cases, mutation of multiple cysteine residues results in a progressive decrease in activity which is likely due to an effect on protein folding and trafficking [22,23]. In contrast, the cys-less SHST1 mutant, A4/205L, retained wild type activity but this resulted from a decrease in activity due to the C421A mutation, which was compensated by an increase in activity resulting from the C205L mutation. Of the five cysteine residues present in SHST1, three (C403, C421 and C484) are very highly conserved among the plant members of the SulP family, with C403 and C421 also being present in some mammalian homologs (see alignment in Ref [10]). However, of these three, only the C421A mutation had a significant effect on activity (Fig. 2). C118 and C205 are unique to SHST1. Transport activity was not affected when C118 was replaced with alanine, although other mutations in helix 1 have had significant effects [10–12].

The size of the residue at position 205 had a dramatic effect on the transport activity of SHST1. In single mutants (Fig. 2), there was no activity when C205 was replaced by a smaller residue (alanine) but a 3-fold increase in the sulphate transport rate was observed when C205 is replaced by a larger residue (leucine). The function of the cys-less variants of SHST1 (Fig. 4) showed similar results, ranging from no activity for A4/205A to the A4/205L mutant with transport properties indistinguishable from wild type SHST1. The A4/205S mutant was intermediate in character, with a slightly increased K_m and a V_{max} around half the wild type level. The A4/205L mutant will be a valuable tool for a variety of cysteine labelling experiments, e.g. cys-scanning mutagenesis to identify the transport pathway. Cysteine labelling techniques have played a major role in elucidating transport mechanisms in some transporters [24–28] and should lead to a greater understanding of the transport of sulphate by SHST1.

The effects of the different substitutions at position 205 can be rationalised by a consideration of related transporters, all of which have a large, hydrophobic residue such as leucine in the equivalent position (Fig. 3). It seems that the native SHST1 has suffered a mutation that renders it less active, at least when expressed in yeast. SHST1 is one of a pair of very closely related sulphate transporters in *S. hamata* [6] and both contain cysteine in this position indicating that this mutation occurred before duplication of the ancestral gene. SHST3, the third transporter in this family in *S. hamata*, has leucine at this position but it is much less closely related and also has a 10-fold lower affinity for sulphate [6].

C205 is located in a predicted intracellular loop between helices 4 and 5 (Fig. 1). The effects of different substitutions

at this position suggest that conformation of this loop is sensitive to size changes. The A4/205A mutant protein was present in the plasma membrane at substantially reduced levels compared to the wild type and the other cys-less mutants (Fig. 5). This suggests that the C205A mutation resulted in misfolding of SHST1 since misfolded membrane proteins are rejected by the quality control system of the endoplasmic reticulum and are not trafficked to the plasma membrane [29]. However, there is still some protein present in the plasma membrane and this mutant completely lacked measurable sulphate transport activity. This is consistent with an effect on activity as well as folding. The A4/205S and A4/205L mutants are present in the membrane at similar levels, appearing to be trafficked to a greater level than wild type, but have different V_{max} values, again consistent with an effect on activity as well as folding. It is unusual for replacement by a smaller residue to have such a significant effect, with alanine scanning mutagenesis being a common technique to identify functionally important regions of transporters. The fact that the A4/205A mutant is misfolded suggests that packing of this region is important. Substitution by a smaller residue may leave a 'hole' which disrupts folding of this loop. Similar results have been obtained in the human organic anion transporter, where substitution of a leucine residue by smaller residues reduced activity due to an effect on cell surface expression [30]. Previous studies have identified amino acid residues in SHST1 with functional roles [10–12,17] but none has been in helices 4 and 5. There are, however, mutations resulting in diastrophic dysplasia [31], Pendred Syndrome [32] or congenital chloride diarrhoea [33] that are found in the helix 4/5 region of the homologous proteins. Interestingly, there are a number of absolutely conserved residues in putative helix 4 and the following loop in SHST1, including three glycine residues (Fig. 3). This helix and loop may be quite flexible. In most homologous transporters from plants and fungi, the loop also contains two oppositely charged residues that may form a charge pair. Our results raise the possibility that this loop plays a role in the transport pathway, either directly or by positioning other residues in helices 4 and 5. Further mutagenesis will be required to test this idea.

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