Role of Transmembrane Domain 8 in Substrate Selectivity and Translocation of SteT, a Member of the l-Amino Acid Transporter (LAT) Family* §

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System l-amino acid transporters (LAT) belong to the amino acid, polyamine, and organic cation superfamily of transporters and include the light subunits of heteromeric amino acid transporters and prokaryotic homologues. Cysteine reactivity of SteT (serine/threonine antipporter) has been used here to study the substrate-binding site of LAT transporters. Residue Cys-291, in transmembrane domain 8 (TM8), is inactivated by thiol reagents in a substrate protectable manner. Surprisingly, DTT activated the transporter by reducing residue Cys-291. Cysteine-scanning mutagenesis of TM8 showed DTT activation in the single-cysteine mutants S287C, G294C, and S298C, lining the same α-helical face. S-Thiolation in Escherichia coli cells resulted in complete inactivation of the single-cysteine mutant G294C. l-Serine blocked DTT activation with an EC50 similar to the apparent Km of this mutant. Thus, S-thiolation abolished substrate translocation but not substrate binding. Mutation of Lys-295, to Cys (K295C) broadened the profile of inhibitors and the spectrum of substrates with the exception of imino acids. A structural model of SteT based on the structural homologue AdiC (arginine/agmatine antipporter) positions residues Cys-291 and Lys-295 in the putative substrate binding pocket. All this suggests that Lys-295 is a main determinant in the recognition of the side chain of SteT substrates. In contrast, Gly-294 is not facing the surface, suggesting conformational changes involving TM8 during the transport cycle. Our results suggest that TM8 sculpts the substrate-binding site and undergoes conformational changes during the transport cycle of SteT.

The l-amino acid transporters (LAT) correspond to 1 of the 13 families within the large APC superfamily of transporters (20). The LAT family has prokaryotic and eukaryotic members. The eukaryotic members are the light subunits of the heteromeric amino acid transporters (HAT) (2, 3). SteT (serine/threonine antipporter) from Bacillus subtilis is the first characterized prokaryotic member of the LAT family and shares ~30% amino acid sequence identity with the human light subunits of HAT (30).

HAT are antiporers composed of two subunits, a polypeptide membrane protein (the light subunit; SLC7 family) and a disulfide-linked N-glycosylated type II membrane glycoprotein (the heavy subunit; SLC3 family) (1–3). The light subunit is the catalytic component of the transporter (4), whereas the heavy subunit appears to be essential only for trafficking to the plasma membrane (5–7). Two heavy subunits (4F2hc and rBAT) and 10 light subunits have been identified in mammals (see Ref. 8 for the last member identified). Several human pathologies highlight the physiological roles of HATs. Two transporters of this family are responsible for inherited amino acidurias; mutations in either of the two genes coding for the subunits of system b0,+ (rBAT and b0,+AT) lead to cystinuria (MIM 220100) (9, 10), whereas mutations in system y is LAT1 (a 4F2hc-associated system y L) result in lysinuric protein intolerance (MIM222700) (11, 12). In addition, xCT (the 4F2hc-associated system xCT), which mediates cystine uptake and glutamate efflux (13, 14), is essential for Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) infection (15, 16). This transport system is also involved in cocaine relapse through the control of the basal levels of extrasynaptic glutamate (17), and it contributes to the maintenance of the cell redox balance (18). Finally, LAT1 (a 4F2hc-associated system L) is overexpressed in many human tumors and transports essential neutral amino acids through the

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plasma membrane for mammalian target of rapamycin signaling (19).

Recently, the atomic structures of two prokaryotic APC amino acid transporters, AdiC and ApcT, solved in the apo (i.e. substrate unbound)-state (21–23), showed the prototypical “5 + 5 inverted repeat” fold first identified in LeuT (Na+–dependent amino acid transporter) from *Aquifex aeolicus* (24) and also shared by other unrelated prokaryotic transporters (25–27). Very recently, the crystal structure of the functional double mutant N22A/L123W of AdiC from *Escherichia coli* bound to L-arginine showed an occluded conformation of the transporter where the substrate interacts with residues in the unwound segments of the transmembrane domains (TM) 1 and TM6 and in TM3, TM8, and TM10 (28). This binding is similar to L-leucine binding to LeuT (24), as expected from previous mutational analysis of APC transporters (29). However, it remains to be established whether LAT transporters, which are distant homologues of AdiC (<20% amino acid sequence identity) (supplemental Fig. S1), share the same substrate binding design.

To gain insight into the substrate-binding site of LAT transporters, we performed a structure-blind search for residues sensitive to thiol modification and protected by substrate in SteT, and Cys-291 in TM8 was identified as such a residue. Then a cysteine scanning mutagenesis study of TM8 provided evidence that two residues neighboring Cys-291 (Gly-294 and Lys-295) are closely linked to the substrate-binding site. A derivative of SteT bearing the mutation G292C in an otherwise Cys-less background is inactivated by S-thiolation in *E. coli* but retains substrate binding, although introduction of cysteine at position 295 (mutant K295C) into Cys-less SteT broadens substrate specificity. The locations of these residues in a structural model of SteT based on the AdiC structure support the concept that Lys-295 is a main determinant of the substrate interaction and that TM8 undergoes conformational changes during the transport cycle.

**EXPERIMENTAL PROCEDURES**

Wild-type and mutants of SteT and AdiC-SteT (*B. subtilis*) and AdiC (*E. coli*) cloned into the EcoRI and PstI sites of a modified version of the vector pTTQ18 (31) were used (30, 32). SteT and AdiC mutants were obtained by site-directed mutagenesis (QuickChange site-directed mutagenesis, Stratagene). All mutants were verified by sequencing.

**Expression of Transporters in *E. coli***—Expression experiments were carried out with freshly transformed cultures of *E. coli* strain BL21(DE3) harboring pTTQ18-His6-SteT or pTTQ18-His6-AdiC (wild-type and mutants). Either 6×0.8 liters of medium in flasks or 10 liters of medium in a fermentor were inoculated with *E. coli* cultures harboring the desired vector. Cells were induced with 0.5 mM isopropyl β-D-thiogalactoside at an *A*₅₆₂₈ of 0.5 and harvested after 3 h of growth at 30 °C for SteT and 37 °C for AdiC. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8, 0.5 mM EDTA) and stored frozen at −20 °C.

**Preparation of *E. coli* Membranes**—Cell pellets were thawed and disrupted by passage through a French pressure cell (20,000 p.s.i., three times). Unbroken cells were removed by centrifugation (10 min at 10,000 × g and 4 °C). The supernatant was ultracentrifuged (1 h at 100,000 × g and 4 °C), and the pellet was resuspended and homogenized (30-ml glass homogenizer for 2 min) in lysis buffer and ultracentrifuged again. Peripheral membrane proteins were removed by homogenization in 20 mM Tris-HCl, pH 8, 300 mM NaCl, and ultracentrifugation. Finally, the membrane pellet was resuspended in 20 mM Tris-HCl, pH 8, 150 mM NaCl at a protein concentration between 13 and 25 mg/ml. Aliquots were frozen in liquid nitrogen and stored at −80 °C until use. Expression of the different SteT variants was tested by Western blot analysis using HRP (Thermoscientific, Rockford, IL).

**Purification of Transporters**—Frozen membranes were thawed and solubilized in 0.5% n-dodecyl β-D-maltospyranoside (DDM; Anatrace, Maumee, OH), 20 mM Tris-HCl, pH 8, 20% glycerol, 50 mM NaCl on a roller shaker (1 h, 4 °C) at a protein concentration of 2–3 mg/ml. The supernatant after ultracentrifugation (1 h at 100,000 × g, 4 °C) was incubated overnight at 4 °C with Ni²⁺–nitrilotriacetic Superflow beads (Qiagen; Hilden, Germany) equilibrated with washing buffer (20 mM Tris-HCl, pH 8, 20% glycerol, 200 mM NaCl, 0.05% DDM, 10 mM imidazole). The supernatant fraction was removed by centrifugation (1 min at ~160 × g). Protein-bound beads were washed three times with 10 ml of washing buffer and centrifuged as before. The columns were then packed with 5 ml of protein-bound beads each. Two more washes were performed with 15 ml of washing buffer before elution with 10 ml of elution buffer (washing buffer supplemented with 500 mM imidazole). The purified protein was concentrated by centrifugation in an Amicon Ultra (10,000 molecular weight cut off; Millipore) at 3220 × g down to a volume of 1 ml. Imidazole was removed by the addition of 10 ml of 20 mM Tris-HCl, pH 8, 20% glycerol, 200 mM NaCl, 0.05% DDM and re-concentration to the desired final volume. A similar protocol, with some differences, was used to purify wild-type and G292C AdiC, as described elsewhere (32).

**Transport Measurements in Proteoliposomes**—For functional studies, purified SteT or AdiC proteins were reconstituted into proteoliposomes as described previously (30). Proteoliposomes contained no amino acids or the desired amino acid at a concentration of 4 mM, unless otherwise indicated. Influx measurements in proteoliposomes were made as described previously (4) with minor changes. 10 μl of cold proteoliposomes in dialysis buffer (120 mM KPi, pH 7.4, 0.5 mM EDTA, 1 mM MgSO₄, 5 mM Tris-SO₄, 1% glycerol) were mixed with 180 μl of transport buffer (150 mM choline chloride, 10 mM Tris-HEPES, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5–1.0 μCi of radiolabeled L-amino acid, and unlabeled amino acid to the desired final concentration) and incubated at room temperature for different periods of time. Reactions were stopped by the addition of 850 μl of ice-cold stop buffer (150 mM choline chloride, 10 mM Tris-HEPES, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5–1.0 μCi of radiolabeled L-amino acid, and unlabeled amino acid to the desired final concentration) and incubated at room temperature for 10 min. Filters were then washed three times with 2 ml of ice-cold stop buffer and dried, and the trapped radioactivity was counted. All experimental values were corrected by subtracting zero time values obtained by adding the stop solu-
tion into the transport buffer before the proteoliposomes. The protein concentration in the proteoliposomes was determined using the Amido Black protein assay (33), and the transport was expressed as picomoles/µg of protein per unit of time and reported as the mean ± S.E. Radiolabeled L-[3H]serine was from GE Healthcare; L-[3H]arginine was from American Radiolabeled Chemicals (St. Louis, MO), and amino acids were from Sigma.

**Treatment with Thiols Reagents**—Proteoliposomes were treated with the indicated final concentration of thiol reagents at room temperature for 5 min. As a negative control, proteoliposomes were treated with the equivalent volume of the solvent (dimethyl sulfoxide, DMSO). To stop reactions, proteoliposomes were diluted in dialysis buffer and ultracentrifuged (100,000 × g, 1 h at 4 °C), and the pellet was resuspended in the original volume of dialysis buffer prior to transport measurements. For the protection assays, the indicated concentration of amino acids was added simultaneously with the thiol reagents.

The MTS reagents (MTSET, MTSEA, MTSES, and pCMBS) were purchased from Toronto Research Chemicals, Inc. pCMB, N-ethylmaleimide, DTt (dithiothreitol), and DMSO were purchased from Sigma.

**Generation of E. coli QC458—**E. coli YG228 (λ- rph-1 ΔaroP ΔbrnQ Δ(liv-ylhk-livKHMGF) ΔpheP) (34) (kindly provided by Takashi Koyanagi, Kyoto University) served as recipient for the P1-mediated knock-out (35) of the chromosomal genes of the native serine/threonine transport systems sstT and tdcC. Chromosomal fragments carrying the kanamycin resistance gene in place of sstT or tdcC were obtained by collecting P1 lysates of previously infected E. coli JW3060-1 (F Δ(arad-araB)567 ΔlacZ4787::rrnB-3 λ- rph-1 Δ(araD-araB)568 hsdR514 ΔstT7779::kan) or JW3087-2 (F Δ(arad-araB)567 ΔlacZ4787::rrnB-3 λ- ΔtdcC732::kan rph-1 Δ(araD-araB)568 hsdR514) (36), obtained via The Coli Genetic Stock Center (CGSC) at Yale University, respectively. After infecting YG228 with the ΔsstT7779::kan-containing P1 phages, cells were selected on LB/kanamycin plates. Subsequently, the kanamycin resistance gene was eliminated by transforming the cells with pCP20, a helper plasmid expressing the flippase recombinase, which eliminates the repeated flippase recognition target sites flanking the kanamycin resistance gene in the resulting cells, followed by temperature-induced removal of the helper plasmid (37). pCP20 was obtained as part of the CGSC gene disruption kit. The cured cells were subjected to a second P1 transduction cycle, this time with the ΔtdcC732::kan-containing DNA fragment of JW3087-2, followed by selection of positive clones in the presence of kanamycin and curing of the cells as described above, yielding E. coli QC458 (λ- rph-1 ΔaroP ΔbrnQ Δ(liv-ylhk-livKHMGF) ΔpheP ΔsstT7779 ΔtdcC732). Thus, E. coli QC458 is defective in the L-serine transporters Liv1, SteT, and TdcC.

**Influx Measurements in E. coli QC458—**Cells expressing single-Cys G294C SteT (vector pTTQ18-His6) were washed once with 100 mM potassium phosphate (KP), pH 7.5, 10 mM MgSO4, and adjusted to an absorbance of 10.0 at 420 nm (0.7 mg of protein/ml). Immediately before the experiment, cells were incubated for 10 min on ice with 50 µM carbonyl cyanide m-chlorophenylhydrazone (Sigma) to dissipate both the pH gradient and the membrane potential. Transport was initiated by the addition of 10 µl of L-[3H]serine (0.5 µCi, final concentration 10 µM) to 50 µl of these cells. Samples were quenched at given times with 2 ml of ice-cold 100 mM KP, pH 5.5, 100 mM LiCl, 10 mM L-serine and assayed by rapid filtration. Control experiments were performed in parallel using E. coli QC458 cells transformed with nonrecombinant vector.

**Sulfhydryl Modification of Single-Cys SteT Mutant G294C—**A biochemical assay followed by the quantification of free cysteines in the protein was used to determine single-Cys SteT mutant G294C oxidation by either S-nitrosylation, S-thiolation, or cysteine sulfenic acid formation. In these experiments, 1–2 µM of detergent-purified protein in 20 mM Tris buffer, pH 7.6, 150 mM NaCl, 0.02% DDM was treated with either 20 mM Na+-ascorbate (Sigma), 20 mM Na+-arsenate (Sigma), or 1 mM of 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (Sigma), respectively. Also, control samples were treated with 1 mM or no DTT, respectively. All samples were rotated at room temperature for 1 h in the dark. After the reaction, samples were subjected to a 50-fold dilution in the protein buffer followed by concentration up to the original volume using Vivaspin 6 30-kDa cutoff concentrators (Sartorious Stedim Biotech S.A., France). Before dilution, the sample containing dimedone was treated with 1 mM DTT. At this stage, protein concentration was measured using the BCA protein assay (Thermoscientific, Rockford, IL). Protein-reduced thiols were assayed by two methods as follows: cysteine modification with biotin (38) and cysteine modification with the fluorescent probe BODIPY® 499/508 maleimide (Invitrogen). Both types of modification were carried out by treating the samples (1.3 µM protein) with 50 µM of either N-(6-(biotinamido)hexyl)-3′-(2′-pyrididyldithio)-propionamide (Thermoscientific, Rockford, IL) or BODIPY® 499/508 maleimide. For the reaction, samples were rotated at room temperature for 30 min in the dark. Finally, an equal amount of protein from each sample was resolved using 12% SDS-PAGE. Biotinylation was analyzed by immunoblotting with avidin horseradish peroxidase conjugate (avidin-HRP) (Thermoscientific, Rockford, IL), whereas the extent of BODIPY labeling was measured by “in gel” fluorescence using a G-box gel analysis instrument (Syngene, Cambridge, UK).

**Model of SteT Structure—**SteT from B. subtilis was modeled on the crystal structure of AdiC from Salmonella typhi-
murium (Protein Data Bank accession 3HQK; chain D) (22) using the program Modeler version 8.2 (39). Alignment of the SteT (Swiss-Prot O34739) and AdiC (Swiss-Prot P60066) sequences (supplemental Fig. S1), which exhibit only ~22% sequence identity, was aided by comparison of the patterns of residue conservation in closer homologues of each of these proteins (175 and 75 representatives, respectively, obtained from the UniRef90 data base), using the program ConSeq (40) One hundred models were made, and the five of lowest energy were further analyzed using MolProbity (41). Of these, the model chosen for further analysis had only six residues in the disallowed region of the Ramachandran plot.
RESULTS

Covalent Modification of Cysteine 291 by MTSET Inactivates SteT in a Substrate-protectable Manner—To search for SteT-inactivating cysteine reagents, purified SteT reconstituted in proteoliposomes was incubated with MTSET, MTSEA, MTSES, pCMB, pCMBS, or N-ethylmaleimide. The transport of 10 μM 1-[3H]serine was then measured in proteoliposomes containing 4 mM 1-serine (1-serine/1-serine antiport activity) or L-arginine (negative control of antiport; i.e. 1-arginine is not a SteT substrate (30)). All the cysteine reagents tested inactivated the serine antiport activity of SteT proteoliposomes (supplemental Fig. 2a). A SteT mutant in which all five Cys residues were substituted with Ser (designated Cys-less SteT) was still active upon reconstitution and, as expected, was not inactivated by the cysteine reagents used (supplemental Fig. S2b). Mutation of each Cys residue individually to Ser in all cases resulted in retention of transport activity, and all the mutants were inactivated by MTSET with the exception of mutant C291S (Fig. 1a). Replacement of Cys-291 in the Cys-less background (single Cys-291) rescued inactivation by MTSET (Fig. 1a) and by all the other cysteine reagents tested (supplemental Fig. S2c). Thus, residue Cys-291, located in TM8 (42) (supplemental Fig. S1), is the target of MTSET inactivation of SteT. It is worth mentioning that Cys-291 is accessible to nonpermeant reagents of large (e.g. pCMBS) and small (e.g. MTSET, MTSES, and MTSEA) size, independently of their net charge (supplemental Fig. S2c).

Next, we analyzed whether inactivation of SteT by MTSET was protectable by substrate. L-Serine protected against the inactivation of wild-type and single Cys-291 SteT at all the concentrations of MTSET studied (Fig. 1b). Protection was ~90% with a saturating concentration of L-serine (30 mM) (Fig. 1b). Consistent with an apparent K_M of 1.2 mM for L-serine uptake (30) at 4 mM, L-serine was able to protect ~60% against MTSET-mediated inactivation of wild-type SteT (data not shown). L-Arginine, which is not a SteT substrate, was unable to protect against the inactivation of wild-type and single Cys-291 SteT by MTSET (Fig. 1b). These results suggest that L-serine protection is due to the occupancy of the substrate-binding site. All these results are consistent either with Cys-291 being close to the substrate-binding site or with conformational changes in SteT, induced by substrate binding, blocking accessibility to MTSET.

Activation of SteT by DTT—Reversal of MTSET inactivation by DTT was studied to confirm the covalent modification of residue Cys-291 in wild-type SteT (Fig. 2a). Surprisingly, DTT treatment after MTSET inactivation not only restored SteT activity but resulted in higher L-serine/L-serine antiport activity. Similarly, DTT treatment of non-MTSET-modified wild-type SteT also increased its activity. In contrast, Cys-less SteT was not activated by DTT (Fig. 2b). These findings suggest that cysteine oxidation, either in host E. coli cells or upon solubilization, purification, and reconstitution, inactivates SteT. A closer analysis to identify the responsible cysteine residue(s) showed that only mutation of Cys-291 to serine (C291S) abolished DTT activation (Fig. 2b). Moreover, restitution of Cys-291 in a Cys-less SteT background (single Cys-291) rescued DTT activation (Fig. 2b). Thus, Cys-291 appears to be oxidized when assayed for transport activity in proteoliposomes and could be reduced back to full activity by DTT.

Cysteine Scanning Mutagenesis in TM8 of SteT—To search for accessibility and substrate interaction along TM8 residues, several single-cysteine mutants in this segment were prepared in the Cys-less background. These mutants were assayed for 10 μM L-serine transport activity in proteoliposomes containing 4
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Three single-Cys mutants (I288C, K295C, and F299C) showed mutant being constructed in a Cys-less SteT background. In all cases, the mutant proteins were expressed in higher transport activity than Cys-less SteT (see under “Discussion”). In the negative control conditions (4 mM l-arginine inside the proteoliposomes, all but one version of SteT (the wild-type, Cys-less, and most of the single-cysteine mutants) showed only a level of l-serine incorporation into proteoliposomes consistent with simple diffusion, as demonstrated earlier for wild-type SteT (30). In contrast, the single-Cys SteT mutant K295C showed significant transport of l-serine in proteoliposomes containing 4 mM l-arginine (Fig. 3a). Two possible explanations can be made for these data as follows: (i) uncoupling of l-serine transport or (ii) l-serine/l-arginine exchange activity by the mutant (see below).

All the TM8 single-cysteine mutants were then assayed for transport activity after MTSET and DTT treatment (Fig. 3b). Substantial inactivation of SteT by MTSET (≥70%) occurred in wild-type and single Cys-291 (as expected; see Fig. 1, a and b), whereas moderate inactivation (≤30%) occurred in the single-Cys SteT mutants L292C, K295C, and F299C. Activation by DTT was moderate in single Cys-291 (as expected; see Fig. 2b); however, a dramatic increase in activity was observed in the single-Cys SteT mutants S287C, G294C, and S298C after DTT treatment (Fig. 3b). This finding suggests that oxidation of the cysteine residue in these positions causes an almost complete inactivation of SteT. It is worth mentioning that residues Ser-287, Cys-291, Gly-294, and Ser-298 are spaced with α-helical periodicity and are probably aligned on the same face of TM8. Mutant L297C had a completely different behavior; it was moderately activated by MTSET and substantially inhibited by DTT, suggesting that increasing the size of the side chain at this position, either by covalent modification with MTSET or through cysteine oxidation, is beneficial for the transport activity.

#### S-Thiolation of Cys-294 in the Single-Cys SteT Mutant G294C Occurs in E. coli

The nature of oxidation of the single-Cys SteT mutant G294C was further investigated because this mutant showed the largest activation after DTT treatment (36-fold; see Fig. 3b) and has a homologous location in the vicinity of the substrate-binding site of AdiC (supplemental Fig. S1) (28). The mutant was similarly activated by treatment with β-mercaptoethanol (data not shown). Three types of oxidation products of the sulfhydryl group, which can be reversed by DTT and β-mercaptoethanol, have been described in proteins: S-nitrosylation (yielding Cys-SNO), S-sulfenation (yielding Cys-SOH), and S-thiolation (yielding disulfide derivatives, i.e. Cys-SS-R) (43–45). To find out which type of oxidation was occurring with the single-Cys SteT mutant G294C, the effects of reagents known specifically to reduce or further modify each type of oxidation product were next explored. As reported elsewhere (46), ascorbate is able to specifically reduce protein-SNO groups to SH via a nitroso transfer mechanism. Similarly, arsenite has the ability to reduce sulfenic acid to a free thiol (47). Protein sulfenic acids can also be covalently modified by dimerdone, forming stable thioether groups that cannot be converted back to the original free thiol group by DTT (48). Modification of a free thiol (i.e. labeling) with biotin—HPDP of purified single-Cys SteT mutant G294C was more effective only after reduction with 1 mM DTT, but not when treated with 20 mM ascorbate or 20 mM arsenite (Fig. 4a), excluding the possibility of S-nitrosylation and S-sulfenation of Cys-294, respectively. Moreover, sequential treatment with 1 mM dimerdone and 1 mM DTT also increased biotin labeling (Fig. 4a). This indicates that

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![FIGURE 2. Residue Cys-291 is the target of activation of SteT by DTT. Proteoliposomes containing wild-type SteT (a) or the indicated SteT mutants (b) were treated with 1 mM MTSET (a) or 10 mM DTT (a and b) for 5 min as indicated. Exchange of 10 μM [3H]serine, 4 mM l-serine was subsequently measured and calculated as indicated in the legend of Fig. 1. In no case was transport into proteoliposomes containing l-arginine affected by thiol reagent treatment (data not shown). Data (mean ± S.E.) correspond to representative experiments run in triplicate. α, DTT reverses inactivation of wild-type SteT by MTSET. α-serine/α-serine exchange inactivated by MTSET (p < 0.001) was reversed by DTT and reached an exchange activity higher than controls (proteoliposomes treated with DMSO) (p ≤ 0.01). DTT treatment alone also activated exchange (p ≤ 0.01). b, l-serine/l-serine exchange in proteoliposomes, containing the indicated mutated versions of SteT, treated (closed bars) or not (control; open bars) with DTT. Exchange activity was activated by DTT in all the mutants (at least p ≤ 0.01), except for Cys-less SteT and C291S. When not visible, error bars are smaller than lines. prot, protein.](image-url)
dithiobenzaldehyde was not able to block DTT reduction of Cys-294, further discounting S-sulfenylation of Cys-294. Similar results were obtained using fluorescence labeling with BODIPY maleimide after the indicated treatments (data not shown). The same analysis with biotin-HPDP labeling of purified single-Cys SteT mutant S298C rendered similar results (supplemental Fig. S3). These findings suggest that residues Cys-294 and Cys-298, which are the only Cys residues in the corresponding single-Cys SteT mutants, had formed a disulfide bridge with another molecule. Although the identity of the latter is unknown, the mobility of the G294C and S298C mutants on SDS-PAGE corresponded to that of the SteT monomer (Fig. 4a), suggesting that S-thiolation of Cys-294 and S298C has been accomplished by a low molecular weight molecule.

It was next investigated whether S-thiolation of residue Cys-294 occurred during expression of the mutant in E. coli or during subsequent biochemical manipulations (detoxification, purification, and reconstitution into liposomes). An E. coli strain devoid of LIV1, SteT, and TdcC l-serine transporters (see “Experimental Procedures”) was transformed with a vector expressing the single-Cys SteT mutant G294C or with nonrecombinant vector, and uptake of 10 μM l-[3H]serine was subsequently measured for 10 min in the absence or presence of 5 mM DTT (Fig. 4b). Only cells expressing the mutant and then treated with DTT showed l-serine uptake in amounts greater than background (i.e. that in cells transformed with nonrecombinant vector and then treated with or without DTT). This activation of transport by DTT treatment of intact cells expressing the single-Cys SteT mutant G294C demonstrated that S-thiolation of Cys-294 occurs during cell culture. In any case, these results provide us with the opportunity to study the functional impact of Cys-294 S-thiolation.

**Blockage of DTT Activation of the Single-Cys SteT Mutant G294C by Substrate**—Among the functional important residues of SteT identified so far, three stand out because they are located in a cluster in TM8 (Gly-291, Gly-294, and Lys-925). As shown before (Fig. 1b), the substrate protects against the inactivation of the single Cys-291 mutant by MTSET. The proximity of these residues prompted investigation of whether the substrate also blocks the activation of the single-Cys SteT mutant G294C by DTT. A concentration of DTT of 100 μM was chosen for these studies because DTT activation of this mutant showed a half-maximal dose of ≈200 μM (data not shown). Increasing...
concentrations of L-serine blocked DTT activation (up to ~70%), with an EC$_{50}$ value of ~1 mM (Fig. 5a). The single-Cys SteT mutant G294C, following activation by DTT, exhibited an apparent $K_M$ value for L-serine/L-serine exchange of 0.7 ± 0.1 mM, in close agreement with the EC$_{50}$ value for blockage of
Single-Cys SteT Mutant K295C Mediates L-Serine/L-Arginine Exchange—The fact that the single-Cys SteT mutant K295C mediated substantial L-serine uptake in proteoliposomes loaded with L-arginine (Fig. 3a) prompted investigation of whether this mutant conserves the mechanism for the coupled exchange of substrates. This coupled mechanism of exchange of 10 μM L-[3H]serine into proteoliposomes containing 4 mM unlabeled L-serine results in an overshoot in the case of wild-type SteT, which reaches a maximum around 5 h and equilibrium after 20 h (30). The time courses of transport of 10 μM L-[3H]serine into single-Cys SteT mutant K295C proteoliposomes containing 4 mM L-serine or L-arginine are shown in Fig. 6a. L-Serine/L-serine exchange gave a typical overshoot with a maximum uptake of label at 30 min, and the system returned to near equilibrium after 22 h. These results demonstrate that L-serine/L-serine exchange catalyzed by the K295C mutant is not only coupled but is faster than by wild-type SteT. In contrast, L-[3H]serine transport into L-arginine-loaded proteoliposomes did not show an overshoot, but nonetheless transport was faster than the passive diffusion of L-arginine into these proteoliposomes (Fig. 6a).

To distinguish between uncoupled transport of L-serine and L-serine/L-arginine exchange, L-serine transport was measured in linear conditions of velocity into proteoliposomes containing no amino acid, L-arginine, or L-serine (Fig. 6b). As expected, wild-type and Cys-less SteT showed significant transport in L-serine/L-serine conditions, but transport into proteoliposomes containing L-arginine or lacking amino acids was minimal and identical. Thus, wild-type and Cys-less SteT only mediate L-serine/L-serine exchange, and transport into proteoliposomes containing L-arginine is only due to passive diffusion, which is not mediated by SteT. In contrast, the single-Cys SteT mutant K295C showed a significantly greater rate of L-serine/L-arginine exchange than of L-serine diffusion (i.e., L-serine transport into proteoliposomes containing no amino acids). To ensure that the observed functional changes in the single-Cys SteT mutant K295C were due solely to mutation of Lys-295, the same mutation was introduced in wild-type SteT. This single mutation (K295C) yielded a form of SteT with transport properties very similar to that of the mutation made in the Cys-less background; it exhibited not only a very active L-serine/L-serine exchange but also L-serine/L-arginine exchange (Fig. 6b). To further confirm the observation that the mutant catalyzes L-serine/L-arginine exchange, transport of 10 μM L-[3H]arginine was measured into proteoliposomes containing unlabeled L-serine (supplemental Fig. S4). Again, in contrast to wild-type SteT, the rate of transport mediated by the mutant protein was significantly greater than that for passive diffusion (i.e., seen for proteoliposomes containing no amino acids). However, L-arginine/L-arginine exchange was only barely detectable after 30 min of uptake in K295C SteT proteoliposomes (supplemental Fig. S4).

FIGURE 6. SteT K295C mutants showed coupled exchange of L-serine and L-arginine. a, time course of 10 μM L-[3H]serine transport into proteoliposomes harboring the single-Cys SteT mutant K295C and containing 4 mM unlabeled L-serine (black squares) or L-arginine (gray squares). Diffusion of 10 μM L-[3H]serine into liposomes containing no protein is indicated by the dotted line. This was calculated using a diffusion coefficient of 2.5 × 10⁻⁶ cm²·s⁻¹ and a liposome volume of 62 nl/μg protein, as measured previously (30). Data (mean ± S.E.) correspond to a representative experiment with three replicates. Error bars when not visible are smaller than the symbols. b, transport of L-serine into proteoliposomes harboring K295C mutants. Proteoliposomes containing wild-type (SteT), its Cys-less variant, the single-Cys SteT mutant K295C, and the K295C mutant generated in a wild-type background were used. Transport of 10 μM L-[3H]serine into proteoliposomes containing 4 mM L-serine (black bars), L-arginine (gray bars), or no amino acid (white bars) was measured over a period of 10 min, except for the two K295C mutants, for which transport was measured for 30 s (during the linear portion of the uptake time course). Only for the two K295C mutants transport was greater in proteoliposomes containing L-arginine than in those containing no amino acids (at least p < 0.01) (inverted triangles). Transport is expressed as picomoles/μg of protein in 10 min. Data (mean ± S.E.) correspond to a representative experiment with three replicates. prot, protein.

Mutant K295C Broadens the Substrate Specificity of SteT—The ability of the K295C mutant of SteT to transport L-arginine prompted a search for additional amino acids that might act as novel substrates or interactors with the transporter. The cis-inhibition of 10 μM L-[3H]serine, 4 mM unlabeled L-serine exchange by a 500-fold excess of a panel of amino acids showed the following pattern for wild-type SteT: Ser, Thr (transport
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FIGURE 7. Amino acid cis-inhibition pattern of wild-type (open bars) and K295C (closed bars) SteT transport activity. Proteoliposomes harboring wild-type SteT or its K295C mutant were used, as indicated. Transport of 10 μM L-[3H]serine was measured for 10 min (SteT) and 30 s (K295C) (linear conditions) in proteoliposomes containing or lacking 4 mM L-serine. Transport was measured in the absence or presence of the indicated amino acids at a concentration of 5 mM in the external medium (2 mM in the case of L-tyrosine). L-Serine/L-serine exchange was calculated by subtracting transport in proteoliposomes containing no amino acid from that in those containing L-serine. Protein-mediated transport corrected for diffusion in this manner is expressed as the percentage of L-serine/L-serine exchange in the absence of cis-inhibitors (3.5 ± 0.8 pmol/μg protein in 10 min for wild-type SteT and 5.0 ± 0.2 pmol/μg protein in 30 s for the K295C mutant of SteT). Common amino acids and d-stereoisomers are indicated with the three-letter code. Homoser, L-homoserine; P-L-Ser, phospho-L-serine; OH-Pro, hydroxyproline. Data (mean ± S.E.) correspond to a representative experiment with three replicates.

Therefore, all these experiments suggest that the side chain of residue Lys-295 is one of the main determinants of the substrate side chain recognition in SteT.

All the previous results indicate that shortening the side chain of Lys residue 295 by mutation to cysteine or glycine increases the capacity of the substrate-binding site of SteT to interact with almost any amino acid, with the exception of imino acids (i.e., L-proline). To test whether the additional amino acids shown to interact efficiently with the K295C mutant were also acting as substrates, proteoliposomes containing this mutant or wild-type SteT were filled with 4 mM selected amino acids, and transport of 10 μM L-[3H]serine was measured (Fig. 8). In wild-type SteT proteoliposomes, L-serine exchange was highly efficient only with L-serine, whereas d-serine, L-γ, and L-leucine were less efficient substrates. L-Proline and d-alanine were very poor substrates, and L-arginine (as expected) did not exchange with L-serine. In contrast, all the tested amino acids, with the exception of L-proline, exchanged with L-serine in K295C SteT proteoliposomes. It is worth mentioning that L-alanine and the original substrate L-serine (also L-threonine; data not shown) showed a similar efficiency in the exchange with L-[3H]serine. L-Leucine showed less efficient exchange, and L-arginine (as expected) exchanged with L-serine but with very low efficiency. Interestingly, d-serine and d-alanine proved to be very poor substrates for K295C SteT. Thus, mutation of Lys-295 to Cys broadened the substrate selectivity of SteT but maintained stereoisomer specificity, while retaining, and indeed enhancing, the severe restriction of imino acid transport. In summary, these data strongly suggest that Lys-295 plays a key role in the binding site of SteT by recognizing the side chains of substrates.

DISCUSSION

The very recently solved structure of the functional AdiC mutant N22A bound to its substrate L-arginine (AdiC-Arg) (28) and previous mutational analysis (22, 32) support the central role of residue Trp-293 in TM8 in the interaction with the side chain of the substrate by this APC transporter. It has been proposed, based on this structure, that upon substrate binding to Trp-293 a conformational change in TM8 opens the so-called Trp-293 gate allowing the transporter to continue further conformational changes to reach the inward open conformation and finally releasing the substrate into the cytosol. Here, we offer functional evidence for the involvement of different TM8 residues in substrate binding and translocation in the LAT transporter SteT as follows: Cys-291 (structurally equivalent to Ser-289 in AdiC) is close to the substrate binding. Lys-295
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FIGURE 8. Exchange of \( \text{l-serine} \) and different amino acids by wild-type SteT and its K295C mutant. Transport of \( 10 \mu M \cdot L^{[3]}\text{H}\text{serine} \) was measured in wild-type (a) and K295C (b) SteT proteoliposomes containing no amino acids or the indicated amino acids at a concentration of \( 4 \text{ mM} \) concentration. Transport was measured over periods during which uptake was a linear function of time, namely 10 min (wild-type) or 30 s, 2 or 5 min, depending on the substrate (K295C). \( \text{l-serine} \) exchange was calculated by subtracting transport in proteoliposomes containing no amino acid from that in those containing the assayed amino acid. Exchange is expressed as picomoles of \( \text{l-serine} / \mu \text{g protein} \cdot 10 \text{min} \).

FIGURE 9. Putative substrate binding pocket of wild-type and K295C SteT. Upper view of the putative substrate binding pocket of wild-type SteT (a) and its K295C mutant (b), seen from the periplasmic space. This structural model is based on the open-to-out structure conformation of AdiC (22). The TM8 residues Cys-291 and Lys-295 are located at the surface of the bottom of the substrate binding pocket. In contrast, the TM8 residue Gly-294 (spheres) is not accessible to the solvent. Mutation K295C enlarges the substrate binding pocket in \( \sim 90 \text{ Å}^3 \) and residue Tyr-102, in TM3, became accessible at the bottom of the cavity.

Several cysteine mutants introduced into Cys-less SteT (S287C, Cys-291, G294C, and S298C), with \( \alpha \)-helical periodicity, showed increased transport activity upon treatment of proteoliposomes with DTT (Figs. 2b and 3b). This activation was particularly dramatic for the S287C, G294C, and S298C mutants. The effect of reduction with DTT, coupled with the lack of effect of ascorbate or arsenite, supports the concept that Cys at position 294 is forming a disulfide bridge with a low molecular weight molecule (Fig. 4a). Interestingly, activation by DTT of the single-Cys SteT mutant G294C was blocked by \( \text{l-serine} \) with an \( EC_{50} \) of \( 0.7 \text{ mM} \) (Fig. 4b). The apparent \( K_M \) for \( \text{l-serine} \) transport by wild-type SteT is similar (1.2 mM) (30), suggesting that mutation of Gly-294 to Cys and its subsequent oxidation does not alter significantly the binding of \( \text{l-serine} \). Consequently, our data suggest that Cys-294 \( S \)-thiolation blocks substrate translocation.

Interestingly, Gly-294 is not accessible to the solvent in the SteT structural model (Fig. 9) based on AdiC or ApdC apostructures (22, 23). Indeed, the homologous residues of AdiC (Gly-292; supplemental Fig. S6a) and ApdC (Ala-290; data not shown) are packed against TM1 and TM5 residues or facing the lipid bilayer in the apo-structures of AdiC and ApdC, respectively. Similarly, residue Gly-292 is packed against TM1 and TM5 in the AdiC structure with \( \text{l-arginine} \) bound (28). In contrast, \( S \)-thiolation of the SteT residue Cys-294 suggests accessibility to the solvent. To test whether the structure of AdiC represents a good model for SteT Gly-294, we checked the solvent accessibility of the residue equivalent in AdiC, Gly-292. To this end, G292C AdiC was generated. Interestingly, AdiC wild-type was not inactivated by MTSET (5 min of treatment at 1...
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mm), whereas G292C AdiC was fully active and was almost completely inactivated by MTSET, demonstrating full accessibility to this nonpermeant reagent (supplemental Fig. S6b). In these experiments, proteoliposomes loaded with 4 mM L-arginine were treated with MTSET in the absence of substrate in the external medium. AdiC is an obligatory exchanger (32, 51). Thus, after interacting with the internal L-arginine, the transporter translocates the substrate to the external medium and remains in the apo-state unable to continue the transport cycle because there is no substrate outside. Thus, in these conditions only the following two possible conformations are present depending on the orientation of the transporter after reconstitution: (i) substrate-free outward open for transporters in the right side out orientation, and (ii) substrate-free inward open for transporters in the inside out orientation. The almost complete inactivation of G292C AdiC by MTSET suggests that in both substrate-free conformations Cys-292 is accessible to MTSET. Alternatively, the transporter incorporates in the proteoliposomes only with an inside out orientation, and Cys-292 is accessible to MTSET in the substrate-free inward open conformation. In any case, these results support that the accessibility of the SteT residue Cys-294 to a small S-thiolation molecule occurs through the substrate translocation pathway. A similar phenotype could be envisaged for the other two single-cysteine mutants that showed a dramatic activation by DTT (single-Cys SteT mutants S287C and S298C). Ser-287 is packed against TM1 and TM5, whereas Ser-298 is packed against TM1 and TM6. Indeed, these three residues (Ser-287, Gly-294, and Ser-298) lie in the same face of TM8 according to the model of SteT mutants S287C and S298C. Ser-287 is packed against the substrate binding pocket of the SteT model based on AdiC structure (supplemental Fig. S6). All this suggests that S-thiolation of Cys residues at positions 287, 294, and 298 blocks a conformational change of TM8 versus TM1, TM5, and/or TM6 during the transport cycle. This is in agreement to the two proposed mechanisms of transition from the outward to the inward facing conformation of other transporters with the 5 + 5 inverted repeat fold as follows: 1) rotation of the hash segment of TM1-TM2 and TM6-TM7, described in the sodium-hydantoin transporter Mhp1 (51), or 2) the rocking bundle mechanism of LeuT and related transporters, where the bundle with respect to TM3 and TM8, respectively (52, 53).

Interestingly, S-thiolation of residue Cys-294 by a low molecular weight compound occurs during culture of E. coli expressing the single-Cys G294C mutant of SteT (Fig. 4b). S-Thiolation is crucial for protection and regulation of thiol-containing proteins during oxidative stress in bacteria (54–57). It is considered that S-thiolation is frequently achieved by the formation of mixed disulfides with glutathione in Gram-negative bacteria (e.g. E. coli) (54, 55). However, many Gram-positive bacteria lack genes for glutathione biosynthesis. Thus, these bacteria (e.g. Bacillus, Staphylococcus, and Actinomyces) rely on different thiol buffers based on cysteine, coenzyme A, bacillithiol, or mycophenolate (56–60). It is difficult to envisage how glutathione is able to reach Cys-294 and the other Cys residues that show activation by DTT in SteT, because they are located deep within the putative translocation pathway (Fig. 9). It seems more likely that a lower molecular weight compound such as cystine (236 Da) might have access to these residues. In contrast, mutation to Cys of the AdiC residue, Gly-292, equivalent to Gly-294 of SteT, did not result in either transport inactivation or DTT activation of transport by the arginine/agnatine exchanger (supplemental Fig. S6b). Even though S-thiolation of SteT from B. subtilis occurs during heterologous expression in E. coli cells, S-cysteinylglutathione is a general mechanism for thiol protection in B. subtilis (56). Thus, our results suggest that Cys-291 in SteT might be a target for S-cysteinylglutathione. Indeed, under oxidative stress, cysteine biosynthesis from serine and methionine is fortified in B. subtilis (57). Similarly, biosynthesis of branched neutral amino acids (leucine, isoleucine, and valine) from threonine is inhibited under oxidative stress, conserving the use of NADPH for oxidoreductases involved in maintaining the thiol redox buffer in the cell (57). SteT exchanges serine and threonine, and therefore, the transporter might be a target of S-thiolation during adaptation to oxidative stress. In any case, further research is needed to identify the compound that participates in the S-thiolation of Cys-291 and the cysteine residues of the single-cysteine mutants of SteT activated by DTT.

Mutation of residue Lys-295 to Cys (or Gly) broadens dramatically the amino acid profile of inhibitors and the range of efficient substrates of SteT (Figs. 7 and 8 and supplemental Fig. S5). Lys-295 is located at the surface of the putative substrate binding pocket of the SteT model based on AdiC (Fig. 9) (22) and ApcT (data not shown) (23) structures. The atomic structures of two 5 + 5 inverted repeat fold amino acid transporters with substrate bound, LeuT (24) and AdiC (28), are available. In these structures, the α-amino and carboxyl moieties of the substrates interact mainly with residues in the unwound α-helical segments of TM1 and TM6, whereas the side chain of the amino acid substrate interacts with residues in TM3, TM6, and TM8 (Ser3–55 and Ile-359 for LeuT; Trp-293 in AdiC). SteT residues Lys-295 and Cys-291 (see above) correspond to the substrate-interacting residues Ile-359 and Ser-355, respectively, in LeuT (alignment reported in Ref. 23). The corresponding residues in AdiC are Trp-293 and Ser-289 (supplemental Fig. S1). Thus, the increased spectrum of substrates transported by the K295C mutant of SteT (e.g. L-alanine and L-arginine) suggests a similar interaction between the side-chain moiety of the substrate and TM8 residues in SteT, AdiC, and LeuT. It is reasonable to think that the positive charge of the side chain of Lys-295 decreases interaction of SteT with L-arginine. Moreover, substitution of Lys-295 by smaller residues (Cys or Gly) would enlarge the substrate binding pocket by ~90 Å³ (Fig. 9b), potentially facilitating the interaction of additional amino acid substrates (e.g. L-arginine). Mutation of the homologous residue in AdiC to leucine (W293L) completely blocks substrate binding and transport (22, 32) in agreement with the proposed cation–π interaction between the guanidinium group of L-arginine and Trp-293 (28). In contrast, mutation K295C in SteT retains the transport activity of the original substrates (L-serine and L-threonine) (Figs. 6 and 8). In addition, the steric restriction imposed by the ring structure of imino acids is not accepted by the SteT Lys-295 mutants (mutants K295G and K295C, constructed in a Cys-less background). Furthermore, D-stereoisomers (D-serine and D-alanine) are not better substrates in the K295C mutant than for...
wild-type SteT (Fig. 8). These findings are consistent with the participation of residues in other TM segments, probably 1 and 6 as for AdiC and LeuT, in the recognition of the α-amino and -carboxyl moieties of the substrate. Indeed, it has been proposed that residues in TM6, which are specific for the APC proline transporters PmB and Put4p, determine imino acid specificity (29). In conclusion, Lys-295 is a key residue implicated in the recognition of the side chain of amino acid substrates; however, it does not have an essential role in the interaction or translocation of the main substrates of SteT.

The SteT mutant K295C also shows a very high transport activity (10–20-fold higher than wild-type SteT; Figs. 6 and 8 and supplemental Fig. S4). The reason for this is not clear at present. On the one hand, l-serine apparent affinity is 4–5-fold increased in the SteT mutant K295C. Thus, the apparent \( K_M \) values for l-serine/l-serine exchange in K295C-proteoliposomes was 255 ± 29 \( \mu M \) (supplemental Fig. S7), whereas in wild-type SteT it has been estimated in 1.2 mM (30). On the other hand, the SteT mutant K295C showed an increased maximal transport rate compared with wild-type SteT (1110 ± 42 pmol/μg of protein/min, respectively). In this scenario, two possibilities could be considered. First, mutation K295C rendered a more stable transporter, and thus a higher proportion of active transporters is reconstituted in the proteoliposomes. Indeed, size exclusion chromatography analysis and concentration-dependent precipitation studies indicated that K295C is more stable in detergent micelles than wild-type SteT (data not shown). Second, K295C mutant might have an intrinsically higher transporter turnover rate than wild-type SteT. In the absence of an experimental approach to measure the proportion of functional SteT molecules in the preparation, it cannot be distinguished between an increase of SteT turnover rate or an increase of function protein.

LAT family members range from transporters with very broad substrate specificity (e.g. LAT2, which exchanges any neutral amino acid with the exception of imino acids (61)) to very specific transporters (e.g. xCT, which is almost restricted to the exchange of glutamate and cystine (13)). Similar extreme examples are also found in members of other APC families, whereas ApoC1 mediates \( \Delta^{2} \)-coupled transport of almost any amino acid (23), AdiC is almost restricted to the exchange of l-arginine and agmatine (32, 50). The large change in the amino acid substrate specificity of SteT as a result of single mutations in residue Lys-295 highlights the plasticity of the substrate-binding site of LAT transporters (and APC transporters in general) and the role of TM8 residues such as SteT Lys-295 in sculpting the substrate-binding site.

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