Functional and Structural Characterization of the First Prokaryotic Member of the L-Amino Acid Transporter (LAT) Family

A MODEL FOR APC TRANSPORTERS

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We have identified YkbA from Bacillus subtilis as a novel member of the L-amino acid transporter (LAT) family of amino acid transporters. The protein is ∼30% identical in amino acid sequence to the light subunits of human heteromeric amino acid transporters. Purified His-tagged YkbA from Escherichia coli membranes reconstituted in proteoliposomes exhibited sodi- um-independent, obligatory exchange activity for L-serine and L-threonine and also for aromatic amino acids, albeit with less activity. Thus, we propose that YkbA be renamed SteT (Ser/Thr exchanger transporter). Kinetic analysis supports a sequential mechanism of exchange for SteT. Freeze-fracture analysis of purified, functionally active SteT in proteoliposomes, together with blue native polyacrylamide gel electrophoresis and transmission electron microscopy of detergent-solubilized purified SteT, suggest that the transporter exists in a monomeric form.

Freeze-fracture analysis showed spherical particles with a diameter of 7.4 nm. Transmission electron microscopy revealed elliptical particles (diameters 6 × 7 nm) with a distinct central depression. To our knowledge, this is the first functional characterization of a prokaryotic member of the LAT family and the first structural data on an APC (amino acids, polyamines, and choline for organocations) transporter. SteT represents an excellent model to study the molecular architecture of the light subunits of heteromeric amino acid transporters and other APC transporters.

The APC (amino acids, polyamines, and choline for organocations) superfamily of transport proteins includes nearly 250 members that function as solute-cation symporters and solute-solute antiporters (1). They occur in all phyla from prokaryotes to higher eukaryotes and vary in length between 350 and 850 amino acid residues. The smaller proteins are generally of prokaryotic origin, whereas the larger ones are of eukaryotic origin and have N- and C-terminal hydrophilic extensions. Most APC members are predicted to possess 12 transmembrane (TM) α-helical domains.

The L-amino acid transporter (LAT) family belongs to the APC superfamily. LAT family members correspond to the light subunits of the heteromeric amino acid transporters (HATs), also called glycoprotein-associated amino acid transporters (2–4). HATs are composed of two subunits, a polytopic membrane protein (the light subunit) and a disulfide-linked N-glycosylated type II membrane glycoprotein (the heavy subunit). The light subunit is the catalytic component of the transporter, whereas the heavy subunit appears to be essential only for trafficking to the plasma membrane. Two types of heavy subunit (4F2hc and rBAT) and 10 types of light subunit have so far been

The abbreviations used are: TM, transmembrane; BN-PAGE, blue native polyacrylamide gel electrophoresis; DDM, N-dodecyl-β-D-maltopyranoside; DM, N-decyl-β-D-maltopyranoside; PL, proteoliposome; TEM, transmission electron microscopy; LAT, L-amino acid transporter; HAT, hetero-meric amino acid transporter; NTA, nitritriacetic acid.
identified. A number of human pathologies have highlighted the physiological roles of HATs. For example, two transporters of this family are responsible for inherited aminoacidurias; mutations in either of the two genes coding for the subunits of system b0,+ (b0,+AT and rBAT) lead to cystinuria (MIM 220100) (5, 6), whereas mutations in y+LAT1 (a 4F2hc-associated system y+L) result in lysinuric protein intolerance (LPI) (MIM222700) (7, 8). In addition, xCT, a LAT transporter that in association with 4F2hc mediates cystine uptake and glutamate efflux (9, 10), has been recently identified as the receptor of Kaposi’s sarcoma-associated Herpesvirus (human herpesvirus 8) (11). In vivo, this transport system is involved in cocaine relapse through the control of the basal levels of extrasympathetic glutamate (12), and it contributes to the maintenance of the plasma redox balance (13).

Despite the important roles attributed to HATs, only a few studies have addressed the structure-function relationships of these transporters: (i) most HATs are obligate antiporters with a 1:1 stoichiometry (14), and a sequential mode of exchange has been proposed for system b0,+ (15); (ii) light subunits appear to be sufficient for transport activity, as demonstrated for b0,+AT (16); (iii) using xCT as a model for the light subunits, a membrane topology with 12 transmembrane segments and with a re-entrant loop between transmembrane segments 2 and 3 has been reported (17); and (iv) the xCT residues His110 and Cys327 have been shown to be crucial for function (17, 18), whereas the cystinuric-specific mutation A354T inactivates b0,+AT (16). Similarly, structure-function studies on the APC superfamily as a whole have been very limited and primarily related to membrane topology studies and the identification of relevant residues for substrate interaction (19–21).

The recent elucidation of atomic structures for several prokaryotic transporters has given key insights into the molecular dynamics of fundamental transport processes. Toward a similar increase in our understanding of amino acid transport, we describe here the identification and characterization, at the functional and structural levels, of the first example of a prokaryotic transporters (23), we first constructed a multiple protein sequence alignment of all known APC members (listed on the World Wide Web at www.tcdb.org/tcdb/superfamily.php) with test sequences using ProbCons (24) in combination with manual refinements. All APC members that appeared to be too distant to provide information (i.e. that could not be properly aligned) were not further considered. The resulting alignment was further evaluated with G-BLOCKS (25) in order to select the most conserved and hence most informative regions of the multiple alignment. The latter were subsequently processed with Clustal (26) to obtain a neighbor-joining phylogenetic tree (1000 bootstraps with 111 random seeds).

**Cloning of Prokaryotic APC Transporters—Genomic DNA from Escherichia coli strain DH5α and from B. subtilis strain 168t+ was prepared from cells collected after an overnight liquid culture. Cell pellets were incubated for 1 h at 50 °C in 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.5% SDS with 0.1 mg/ml Proteinase K. Three extractions with the same volume of phenol were performed with centrifugations at 5,000 × g. Next, a chloroform/ethanol precipitation gave final pellets that were resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The following primers (5'-3') were used to amplify open reading frames encoding the indicated proteins from genomic DNA by PCR: ATCTGAATTTCTGATGCCAGAAAACCCCTGTGTC and CATTCTCCAGAGAACGTCATCACT for PotE and ATCTGAATTTCTCCTCCATACCATACATGCA and CATTCTCCAGAGATCCAGTGTCTTATATCAAT for YfhM from E. coli and ATCGAGATTCTGAGTCCACATCAAATCATCA from B. subtilis. The PCR products were digested with EcoRI and Xhol and ligated into a pBluescript vector (Stratagene). Another PCR with the following primers (5'-3') was performed to subclone each transporter open reading frame into the EcoRI and PstI sites of a modified version of the vector pTTQ18 (27), thereby placing its expression under the control of the tac promoter: TGATGAATTTGATGAGGAGACGCTAAGGCTTATCAGT for PotE, TGATGAATTTGATGAGCACCCCTCCCCCTAATCCGCCCTGAGAACGCTTAATCCGC and ATCCGCTCGAGCACCAAGAGCTTATTGAGATGACGCTGAAGTGGGGAAAAGGGCCATCTGCATGTC and TCTG for SteT and ATGGTAATCTCAAATATATACAAAAAGAACTGC and TCTACTCGAGTATCGCTTCATCTGTGTTGG for YfnA from B. subtilis. PCR products were digested with EcoRI and Xhol and ligated into a pBlueScript vector. Another PCR with the following primers (5'-3') was performed to express the four APC transporters (PotE, YhfM, SteT, and YfnA) was investigated in 50-ml samples cultured in LB medium containing 50 µg/ml ampicillin. When the A600 had reached 0.5, protein expression was induced by the addition of 0.5 mM isopropyl-β-d-thiogalactoside (Roche Applied Science). To estimate protein expression, cells were harvested after incubation for different time periods (1, 2, 3, and 4 h and overnight) at 30 or 37 °C. For biochemical or functional analy-
ses of SteT and PotE, either 6 × 0.8 liters of medium in flasks or 10 liters of medium in a fermentor were inoculated with *E. coli* cultures harboring pTTQ18-His$_6$-YkbA or pTTQ18-His$_6$-PotE. Cells were induced with 0.5 mM isopropyl-$\beta$-d-thiogalactoside at an $A_{560}$ of 0.5 and harvested after 3 h growth at 30 and 37 °C, respectively. In all cases, the cell pellet was 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**—Cells 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.

**Purification of APC Transporters**—To estimate protein expression and functional reconstitution of SteT and PotE, frozen membranes were thawed and solubilized in 0.5% n-dodecyl-$\beta$-d-maltosyanoside (DDM; Anatrace), 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 mg/ml. The supernatant after ultracentrifugation (1 h at 100,000 × g and 4 °C) was incubated for 2 h at 4 °C with equilibrated Ni$_2^+$-NTA Superflow beads (Qiagen) with washing buffer (20 mM Tris-HCl, pH 8, 20% glycerol, 200 mM NaCl, 0.05% DDM, 10 mM imidazole). The supernatant after ultracentrifugation (1 min at ~160 × g). Protein-bound beads were washed three times with 10 ml of washing buffer and centrifuged as before. Then columns were 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 3,220 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 reconcentration to the desired final volume.

For negative stain transmission electron microscopy (TEM) studies, frozen SteT-containing membranes were thawed and solubilized for 1 h at 4 °C in 1% n-dodecyl-$\beta$-d-maltosyanoside (DDM; Anatrace), 20 mM Tris-HCl, pH 8, 300 mM NaCl, 10% glycerol, 0.01% NaN$_3$. The protein concentration during solubilization was between 2 and 3 mg/ml. After ultracentrifugation (100,000 × g, 45 min at 4 °C), the supernatant was diluted 2-fold with 20 mM Tris-HCl, pH 8, 300 mM NaCl, 0.3% DM, 3 mM histidine, 10% glycerol, 0.01% NaN$_3$ (washing buffer) and bound for 2 h at 4 °C to Ni$_2^+$-NTA Superflow beads (Qiagen). The beads were then loaded onto a spin column (Promega), washed with washing buffer, and eluted with the same buffer containing 200 mM histidine.

**Reconstitution into Proteoliposomes**—*E. coli* polar lipid extract (Avanti Polar Lipids) solubilized in chloroform (50 mg/ml) was dried under a stream of nitrogen to remove the solvent and to obtain a thin layer of dry lipids in a glass tube. The dried lipids were resuspended in dialysis buffer (120 mM K$_2$SO$_4$, pH 7.4, 0.5 mM EDTA, 1 mM MgSO$_4$, 5 mM TrisSO$_4$, 1% glycerol, and a 4 mM concentration of the desired amino acid, unless otherwise indicated) to yield a final lipid concentration of 40 mg/ml. After four 30-s sonication and vortexing cycles, the liposomes were extruded in a LiposFast-Pneumatic Aevator (Avestin) through a 400-nm polycarbonate filter (Avestin) to obtain unilamellar vesicles of homogeneous size. Liposomes were mixed with purified protein at a 1:100 (occasionally 1:40) protein/lipid ratio (w/w). To destabilize the liposomes, 1.25% TBB-decyl glucoside (Roche Applied Science) was added and incubated in ice with occasional agitation for 5 min. DDM and TBB-decyl glucoside were removed by dialysis for 40 h at 4 °C against 100 volumes of dialysis buffer. Finally, proteoliposomes were ultracentrifuged (100,000 × g, 1 h at 4 °C), and the pellet was resuspended in one-third of the initial volume of dialysis buffer without amino acids.

**Transport Measurements**—Influx measurements in proteoliposomes were made as described (16) with minor changes. Cold proteoliposomes (10 μl) were mixed with 180 μl of transport buffer (150 mM choline chloride, 10 mM Tris-HEPES, pH 7.4, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 0.5 μM of radioisotopically labeled amino acid, and unlabeled amino acid to the desired final concentration) and incubated at room temperature for different periods of time. To test the effect of an imposed membrane potential, 2.8 μM valinomycin (Sigma) was added to the transport buffer. Reactions were stopped by the addition of 850 μl of ice-cold stop buffer (150 mM choline chloride, 10 mM Tris-HEPES, and 5 mM L-serine or putrescine for SteT- and PotE-containing proteoliposomes, respectively) and filtration through membrane filters (Sartorius; 0.45-μm pore size). Filters were then washed three times with 2 ml of 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 solution before the proteoliposomes into the transport buffer. The following radioisotopically labeled compounds (American Radiolabeled Chemicals) were used in this study: L-[H$^3$]serine, L-[H$^3$]putrescine, L-[H$^3$]ornithine, L-[H$^3$]arginine, L-[H$^3$]lysine, L-[H$^3$]glutamate, and L-[H$^3$]alanine.

For efflux measurements, proteoliposomes (400 μl) were mixed with 200 μl of 3-fold concentrated transport buffer (450 mM choline chloride, 30 mM Tris-HEPES, pH 7.4, 3 mM MgCl$_2$, 3 mM CaCl$_2$, 35 μg of L-[H$^3$]serine at a final concentration of 10 μM) and incubated at room temperature for 2 h. The proteoliposome suspensions were divided into two halves (295 μl each), and these were diluted 13-fold with transport buffer (without L-[H$^3$]serine) with or without 7 mM L-serine. At the indicated times, aliquots (195 μl) were mixed with 200 μl of ice-cold stop buffer (850 μl), vortexed, and filtered (0.45-μm pore size; Sartorius). Filters were then washed three times with 2 ml of stop buffer and dried, and the trapped radioactivity was counted.

**Simulation of SteT Exchange Activity**—Simulation of the time course of transport of radioisotopically labeled L-serine into proteoliposomes containing purified SteT (SteT-PLs) was performed using a model based on the following premises: (i) the induced
amino acid transport activity is an obligatory exchange process with a 1:1 stoichiometry; (ii) an additional diffusive, protein-independent flux is necessary to explain transport observed in the absence of transporter (i.e. in proteoliposomes containing no SteT) or in the absence of exchangeable amino acids (SteT-PLs containing l-arginine instead of l-serine). The computer program, previously designed to simulate transport in oocytes (28) and membrane vesicles (15), was adapted to model amino acid exchange and simulated the experimental influx and efflux rates governed by a concerted (i.e. sequential) mechanism of exchange, as described in a previous study for system b0 + (15). Different types of mechanisms (e.g. ping-pong), however, would produce similar results (data not shown). The computer program is available upon request. The simulations were set up using the following rules. (i) Characteristics of the simulated system were as follows. The experimental setup was reproduced as two separate compartments. The outside compartment volume was set to 86 μl (taking 1 μg of protein as a reference). The inside volume, 62 nl, together with the Fick parameter, was deduced from extrapolation to infinity of non-specific influx measurements (SteT-PLs containing l-arginine), fitted to the Fick law by standard nonlinear regression. (ii) Kinetic parameters were as follows. Experimental parameters were used when available. Best values for the unknown parameters and transporter concentration were determined after a systematic search of the appropriate range of values. (iii) The simulation procedure was as follows. Transport rates were evaluated from the relative concentrations of transporter complexes. Transport rates were estimated on the assumption that the limiting step is the translocation of the transporter bound to the substrate, as described for system y+L (29), and therefore all binding steps were considered as being at equilibrium. Possible inactivation of the transporter was included as an exponential first order law with the appropriate parameters. During simulation, amino acid concentrations were calculated by numerical integration of the transport rates. Integration was performed with a constant time step of 0.02 min during the desired period.

**Results**

**Freeze-Fracture and Electron Microscopy of Proteoliposomes**—A freeze-fracture electron microscopy study was performed as described (30). The suspension was sandwiched between two copper platelets using a 400-mesh gold grid as spacer. The samples were frozen by liquid propane immersion, at –189 °C and fractured at –150 °C and 10–5 millibars in a BAL-TEC BAF 060 freeze-etching system (BAL-TEC). The replicas were obtained by unidirectional shadowing at 45° with 2 nm of Pt/C and at 90° with 20 nm of C and subsequently floated on distilled water for 5 min. Electron micrographs (at ×50,000) were recorded in a Jeol 1010 electron microscope operated at 80 kV.

**Measurement of Freeze-Fracture Particles**—Particle diameters were measured from scanned electron micrographs using AnalySIS software. The diameter was obtained by measuring the width of the particle edge-to-edge in a direction perpendicular to the direction of the shadow. Duplicate measurements of individual particles of SteT and PotE revealed a ±0.2-nm error of measurement. The accuracy of the diameter measurements was tested by measuring the diameter of 10-nm gold particles (Chemicon) placed directly on Formvar-coated copper grids (9.94 ± 0.04 nm; n = 125). All values are reported as means ± S.E. of the mean.

**Negative Stain TEM**—DM-solubilized SteT protein as eluted from the Ni2+ -NTA column was adsorbed for 10 s to parlodion carbon-coated copper grids rendered hydrophilic by glow discharge at low pressure in air. Grids were washed with four drops of double-distilled water and stained with 2 drops of 0.75% uranyl formate. This washing step is to effectively remove the buffer solution and not adsorbed protein. The former, if not removed, can lead to precipitation of the uranyl salts with buffer components. Electron micrographs were recorded at a magnification of ×50,000 and an underfocus of –400 nm on Eastman Kodak Co. 50-163 sheet films with a Hitachi H-7000 electron microscope operated at 100 kV.

**Blue Native Gel Electrophoresis**—Linear 5–12% gradient gels for blue native polyacrylamide gel electrophoresis (BN-PAGE) were prepared and run as previously described (31). Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) were used as standard proteins.

**Results**

**Screening for an Appropriate Prokaryotic Homolog Candidate of LAT Transporters**—In order to identify appropriate prokaryotic homolog candidates for studying the structure-function relationships of the light subunits of HATs, an exhaustive search was made on several available protein sequence data bases. Among the most significant matches, four candidates were selected for further investigation: PotE and YhfM from *E. coli* and YkbA (SteT) and YfnA from *B. subtilis*. These proteins had amino acid sequence identities to the light subunits of HATs ranging from 17 to 29%. All of these candidates were hypothetical proteins (32, 33) with the exception of PotE, the putrescine/ornithine exchanger (34), from *E. coli*. In preliminary trials, each of these APC members was C-terminally tagged with His6 and successfully expressed in *E. coli* as described under “Experimental Procedures.” However, detailed investigation was subsequently confined to PotE and SteT because of the high yield of purified protein: up to 3 and 1 mg/liter of bacterial culture, respectively. In contrast, purified protein yield was lower than 0.5 mg/liter for the other APC members (data not shown).

In a wide phylogenetic study of the APC superfamily of transporters, the PotE and SteT proteins were initially classified as basic amino acid/polyamine antiporters within one of the 11 independently defined APC subfamilies (1). To our surprise, and in disagreement with this existing classification, our BLAST comparisons of the SteT protein sequence with those of all other APC members clearly showed a preferred match with members of the LAT subfamily (with identities ranging between 31 and 29%) rather than with any other APC transporters, including PotE (with 22% identity). To solve this discrepancy, we performed a new phylogenetic analysis of SteT and PotE using a restricted collection of closely related APC subfamilies that, in contrast to wide APC studies, allowed not only the usage of a more accurate protein alignment algorithms (ProbCons), but also a manual examination and verification of the alignment results, which is therefore expected to provide a
FIGURE 1. a, phylogenetic relationship of SteT with other prokaryotic and eukaryotic members of the APC transporter superfamily. The neighbor-joining tree illustrates the phylogenetic relationships of SteT (YkbA from *B. subtilis*) with all alignable members of the APC superfamily that are either functionally characterized or putative amino acid transporters. The abbreviation of each of the subfamilies is in boldface type (see Ref. 23 and, on the World Wide Web, www.tcdb.org/tcdb/superfamily.php for a description of the subfamilies). The tree was rooted using yeast VAL1 permease (YAT family). Bootstrap values (as a percentage of 1000 replicates) are indicated for each of the nodes. Thicker strokes denote branches supported with bootstrap values larger than 70%. In order to simplify the tree, clades with terminal branches that include clear orthologs were collapsed and labeled with the general name of the transporter followed by an asterisk. We have identified other bacterial protein sequences that were annotated during the course of our studies; the sequences were closely related to SteT (probably orthologs) and clustered with LAT members as well (data not shown; GenInfo Identifiers, gi: 37521919, 77543889, 67931606, 29898613, and 41582385). b, membrane topology model of SteT. This model is based on the alignment of the amino acid sequence of SteT with the light subunits of mammalian HATs that interact with the heavy subunits rBAT or 4F2hc (see supplemental Fig. 1), the experimentally probed membrane topology of human xCT (17), and the predicted membrane topology of SteT (supplemental Fig. 1). The membrane topology model of SteT contains 12 transmembrane domains (TM1 to -12) with intracellular N and C termini. Due to the high amino acid sequence similarity to human xCT (see supplemental Fig. 1), the intracellular loop connecting TM2 and TM3 is depicted as a re-entrant loop, as demonstrated for human xCT (17). Conserved residues (circled) in SteT and the mammalian light subunits that interact functionally with rBAT or 4F2hc are distributed along the protein but are more frequent in the first part of the protein (TM1, TM2, and the re-entrant loop) (see also supplemental Fig. 1). The conserved Cys residue in the external loop between TM3 and TM4 involved in the disulfide bridge connecting the light and the heavy subunits of eukaryotic HATs is not present in SteT (see also supplemental Fig. 1).
more consistent phylogenetic classification. The sequence comparisons and the constructed phylogenetic tree indicated that SteT clearly clusters with all of the members of the LAT family (Fig. 1a). Furthermore, these results are also in agreement with a revised analysis recently performed by Saier and co-workers. Thus, SteT and the other related bacterial sequences described in the legend to Fig. 1a can be considered as the first identified prokaryotic members of the LAT family. SteT is predicted to contain 438 amino acid residues (estimated molecular mass 48,879 Da), and its sequence can be aligned in its entirety with almost the full lengths of the sequences of all of the eukaryotic LAT family members (supplemental Fig. 1). The estimated membrane topology of SteT (supplemental Fig. 1) is similar to that of these transporters and fits the experimental topology model of xCT, which contains 12 transmembrane (TM) domains (17). Totally conserved residues in SteT and the mammalian rBAT- or 4F2hc-associated LAT family members (see selected transporters in supplemental Fig. 1) are indicated in Fig. 1b. The regions most highly conserved between SteT and the eukaryotic LAT family members correspond to TM1, TM2, and the re-entrant loop between TM2 and TM3. The main differences are shorter N and C termini in SteT and the absence of the cysteine residue, in the loop between TM3 and TM4, that is involved in the disulfide bridge between the light and the heavy subunits in the eukaryotic LAT family members.

Functional Characterization of SteT—In order to characterize the function of SteT, C-terminally His6-tagged SteT was overexpressed in E. coli (used as a negative control) (Fig. 4a). SteT showed accumulation of L-serine over the equilibrium levels, as indicated by the overshoot observed in L-serine-containing SteT-PLs, which was not observed in L-arginine-containing SteT-PLs. SteT capacity for active accumulation is characteristic of an exchanger and is not compatible with an allosteric trans-activation mechanism. Moreover, the transport activity of SteT was

kDa (17, 35, 36). The faster migration of these proteins in SDS-PAGE than would be expected from their predicted molecular masses probably reflects their hydrophobic nature and is commonly observed for membrane transport proteins. A higher molecular mass band (~115 kDa; Fig. 2, lane 5), visible in some but not all protein preparations, corresponded to a low abundance contaminant, since it was not detected with HisProbe-horseradish peroxidase (Qiagen) (i.e. His tag detection) (data not shown). From scanning densitometry of multiple Coomassie Blue-stained gels, the purity of a typical SteT preparation was estimated to be >86%. Purified SteT was reconstituted in the presence of E. coli lipids to form proteoliposomes (SteT-PLs) at a protein/lipid ratio of 1:100. Again, the ~40-kDa band is visible in SteT-PLs (Fig. 2, lanes 6 and 7).

SteT was a hypothetical protein identified in the B. subtilis genome sequence (32). Based on its phylogenetic relationship with eukaryotic LAT family members, we reasoned that SteT could be an amino acid exchanger. To identify the SteT transport activity, a fast screening approach for amino acid exchanger activity was set up. The first step consisted in preparing SteT-PLs with or without a mixture of 10 representative amino acids (L-Arg, L-Orn, Gly, L-Pro, L-Ala, L-Leu, L-Met, L-Phe, L-Tyr, and L-Glu at 1 mM each) contained inside the proteoliposomes. Then influx of a group of radiolabeled, structurally related amino acids was measured. In agreement with the proposed transport function determined in whole bacteria expressing PotE and in membrane vesicles derived from these cells (19, 34), PotE reconstituted in liposomes (PotE-PLs) showed putrescine/ornithine and ornithine/ornithine exchange (supplemental Fig. 2). As expected, PotE-PLs showed higher transport in the amino acid-filled than in the empty proteoliposomes (i.e. trans-stimulation) with the group of radiolabeled L-arginine, L-lysine, L-ornithine, and putrescine (Fig. 3a). In contrast, SteT-PLs showed trans-stimulated uptake only with the group of L-serine, glycine, and L-proline (Fig. 3a). Next, trans-stimulation of transport of these amino acids, including L-alanine, was measured individually with SteT-PLs (Fig. 3b). Only L-serine transport was trans-stimulated in amino acid-filled SteT-PLs. Similarly, substitution of the intraproteoliposome mixture of amino acids by L-serine as the unique internal substrate also showed trans-stimulation of L-serine transport in SteT-PLs (Fig. 3b). Indeed, the initial velocity of transport of 10 μM L-serine was 11-fold higher in L-serine-filled than in L-arginine-filled SteT-PLs (2.12 ± 0.15 and 0.19 ± 0.05 pmol/μg protein per 10 min, respectively; n = 5 independent experiments). As expected, transport of the amino acids from this group was not trans-stimulated in PotE-PLs (Fig. 3b).

Trans-stimulation can result either from allosteric trans-activation or exchange activity. In order to distinguish between these two possible mechanisms, L-serine transport was examined over time in SteT-PLs containing L-serine or L-arginine (used as a negative control) (Fig. 4a). SteT showed accumulation of L-serine over the equilibrium levels, as indicated by the overshoot observed in L-serine-containing SteT-PLs, which was not observed in L-arginine-containing SteT-PLs. SteT capacity for active accumulation is characteristic of an exchanger and is not compatible with an allosteric trans-activation mechanism. Moreover, the transport activity of SteT was
bidirectional, as shown by L-serine efflux measurements from SteT-PLs (Fig. 4b). Efflux of radiolabeled L-serine from pre-loaded SteT-PLs was dramatically trans-stimulated by L-serine. In contrast, L-serine was apparently transported by simple diffusion in SteT-PLs containing L-arginine. (i) L-Serine transport in PotE-PLs was indistinguishable from that in SteT-PLs when only L-arginine was present inside the proteoliposomes (data not shown). (ii) Moreover, pretreatment with the cysteine-reactive reagent 2-(trimethylammonium) ethylmethanethiosulfonate bromide (1 mM for 5 min) inactivated L-serine transport in L-serine-containing (65% inhibition) but not in L-arginine-containing SteT-PLs (data not shown). SteT contains five cysteine residues, which have been mutated to serine to construct a cysteineless version of the transporter (SteT-Cysless). This mutated protein retains 25% of the transport activity of the wild-type SteT (data not shown). Pretreatment with 2-(trimethylammonium) ethylmethanethiosulfonate bromide (1 mM for 5 min) did not affect L-serine transport in SteT-Cysless-PLs containing L-serine (data not shown). These findings indicate that L-serine transport in SteT-PLs containing L-serine is SteT-mediated, whereas that in SteT-PLs containing L-arginine is not SteT-mediated. Taken together, all of these results demonstrate that SteT is an obligate exchanger.

The kinetics of L-serine/L-serine exchange in SteT-PLs were examined by varying L-serine concentrations inside and outside proteoliposomes (Fig. 5a). Lineweaver-Burk plots showed straight lines with different slopes where internal and external apparent K_m values were independent of the substrate concentration in trans. This kinetic behavior supports a sequential
mechanism and rules out a ping-pong mechanism, which is characterized by a nonintersecting pattern (37). This initial kinetic analysis estimated the apparent $K_m$ values from inside and outside the proteoliposomes to lie in the range of 1–5 and 1–3 mM, respectively. Subsequently, a second kinetic analysis was performed with 15 mM L-serine inside SteT-PLs and varying the external L-serine concentration (Fig. 5b). In agreement with these previous results, the external apparent $K_m$ was 1.2 ± 0.2 mM. In these two experiments (with up to 15 mM L-serine inside SteT-PLs), the $V_{\text{max}}$ of L-serine/L-serine exchange was 67 pmol/µg protein-min. Thus, the turnover rate of SteT L-serine/L-serine exchange was 0.06 s$^{-1}$.

In order to test whether ions were cotransported via SteT, we studied the effect of membrane potential on L-serine/L-serine exchange (supplemental Fig. 3). A membrane potential (negative inside) was generated by inclusion of 120 mM K$_i$ in the internal medium of the proteoliposomes followed by the addition of valinomycin to the external medium. PotE-PLs were used as a positive control. PotE exchanges putrescine and L-ornithine with 1:1 substrate stoichiometry (34). At neutral pH, putrescine/L-ornithine exchange would be electrogenic (two charges (putrescine) will be exchanged for one charge (L-ornithine)). Consistent with this stoichiometry, imposing a membrane potential increased the putrescine (influx)/L-ornithine (efflux) exchange in PotE-PLs by ~80%. In contrast, L-serine/L-serine exchange in SteT-PLs was independent of the membrane potential. Moreover, SteT exchange activity was independent of Na$^+$ and Cl$^-$ in the external medium (i.e. substitution by choline and acetate, respectively, had no effect) and was not affected by variation of the external medium pH (from 5.5 to 7.5; internal proteoliposome, pH 7.4) (data not shown). Similarly, SteT exchange activity was independent of K$^+$ gradient (i.e. equilibration of the K$^+$ gradient by the addition of 120 mM K$^+$ to the external medium; data not shown).

To further confirm that SteT mediates obligate exchange, the time course of L-serine/L-serine exchange via SteT was simulated (see “Experimental Procedures”). Transport of 10 µM radiolabeled L-serine into SteT-PLs containing 4 mM L-arginine fitted well to a model of simple diffusion with a diffusion coefficient of 2.5 × 10$^{-6}$ m$^2$s$^{-1}$ and an internal SteT-PLs volume estimated at equilibrium of 62 nl/µg of purified protein (Fig. 4a). Transport of 10 µM radiolabeled L-serine into SteT-PLs containing 4 mM L-serine was simulated using two transport components: (i) electroneutral Ser/Ser exchange with 1:1 exchange stoichiometry and apparent internal and external substrate affinities of 2.0 and 1.2 mM, respectively, plus (ii) the simple diffusion component (Fig. 4a). The internal SteT-PLs volume estimated at equilibrium was 62 nl/µg of protein. Interestingly, the best agreement with the experimental data was obtained when a decrease in transport activity over time was included in the simulation ($V_{\text{max}}$ from 70 to 22 pmol/µg protein-min within the first 2 h with a $t_{1/2}$ of 21 min). The nature of this inactivation during time course transport measurements is unknown. However, these simulations support an electroneutral and obligate exchange of L-serine by SteT and indicate that this exchange is balanced (probably with 1:1 stoichiometry).

Finally, the amino acid specificity of SteT transport activity was characterized by cis-inhibition experiments (Fig. 6a). A 500-fold excess of the L-stereoisomers of serine and threonine almost completely abolished L-serine/L-serine exchange. Amino acids structurally related to L-serine (D-serine and L-homoserine) also inhibited transport, but to a much lower extent. Similarly, the phosphorylated form of L-serine (L-phosphoserine) was a much poorer inhibitor. Many other amino acids, including glycine, proline, arginine, cysteine, lysine, and glutamate, inhibited transport very weakly or not at all. Surprisingly, L-aromatic amino acids (phenylalanine, tyrosine, and tryptophan) signifi-
clearly trans-stimulated by L-serine and L-threonine and to a lesser extent by the L-aromatic amino acids. Because serine and threonine were the more efficient transported, we propose that YkbA be redesignated as a SteT (serine/threonine exchanger transporter).

The results presented demonstrate that SteT exhibits an obligate exchange activity for serine, threonine, and aromatic amino acids. Because serine and threonine were the more efficiently transported, we propose that YkbA be redesignated as a SteT (serine/threonine exchanger transporter).

Structure and Oligomeric State of SteT and PotE—To ascertain whether SteT and PotE exist in a monomeric or oligomeric form, three independent methods were used: BN-PAGE, freeze-fracture TEM, and negative stain TEM. BN-PAGE results of SteT are summarized in supplemental Fig. 4. DM-solubilized and purified His-tagged SteT migrated as a strong protein band at an apparent molecular mass (M_m) of ~80 kDa. In addition, two faint bands at ~155 and ~205 kDa as well as a stronger one at ~40 kDa were detected. The latter band had a bright blue color and corresponds to detergent/Coomassie Brilliant Blue G-250 micelles as demonstrated previously (38). In Western blot analysis, the ~80-, ~155-, and ~205-kDa bands reacted with HisProbe-horseradish peroxidase and therefore corresponded to SteT (data not shown). BN-PAGE with purified PotE was not successful because of its strong tendency to precipitate during gel electrophoresis.

In a second approach, freeze-fracture TEM analysis was applied to estimate the size of functional His-tagged SteT and PotE reconstituted in proteoliposomes (SteT-PLs and PotE-PLs). Aliquots (5 μl) of proteoliposomes, as used for the transport assays, at a protein/lipid ratio of 1:100 and for these studies also at 1:40, were prepared for freeze-fracture TEM. SteT- and PotE-PLs at protein/lipid ratios of 1:100 and 1:40 showed identical transport activity when corrected by μg of protein (data not shown). In the replicas after freeze-fracture, proteoliposomes appeared as concave or convex surfaces with a diameter of 150–200 nm (Fig. 7). The majority (90%) of the proteoliposome fracture faces contained 0–2 and 0–6 intramembrane vesicles, indicating a random insertion of the protein in the bilayer. d, PotE proteoliposomes prepared for transport assays at a protein/lipid ratio of 1:100. Intramembrane particles (arrows) are visible. Scale bar, 100 nm. Insets, high magnification view of one SteT (c) and one PotE (d) particle. Scale bar in insets, 10 nm.
single populations of particles with diameters of 7.4 ± 0.1 nm (n = 119) and 8.0 ± 0.1 nm (n = 109) for SteT and PotE, respectively. As a control for our measurements, we used lactose permease from E. coli, and we found a similar particle size. Thus, freeze-fracture TEM analysis applied to the C154G lactose permease mutant (i.e. the same mutant protein used for the crystal structure resolution) (39) reconstituted in liposomes (1:100 protein/lipid ratio) showed a particle diameter of 6.0 ± 0.1 nm (n = 72) (data not shown).

Finally, DM-solubilized His-tagged SteT protein was negatively stained and examined by TEM. The homogeneity of purified SteT is documented in Fig. 8a. Single SteT proteins were distinguished and displayed an elliptic “donut-like” shape with diameters of ~6 nm by ~7 nm and a central stain-filled indentation (see gallery of well preserved SteT top views in Fig. 8b). As already indicated from BN-PAGE, PotE had a tendency to aggregate when solubilized in detergent. This behavior was further supported by negative stain TEM, which showed a heterogeneous population of particles (data not shown).

**DISCUSSION**

In this work, we have provided, for the first time, information on the functional and structural features of SteT from *B. subtilis*. The first part of our extensive analysis shows that SteT is an excellent prokaryotic model for structural and functional studies of eukaryotic amino acid exchangers, in particular members of the LAT family. (i) Phylogenetic analysis clustered the SteT amino acid sequence together with LAT family members within the APC superfamily (Fig. 1a). (ii) The putative membrane topology of SteT (Fig. 1b and supplemental Fig. 1) is compatible with that experimentally determined for eukaryotic LAT transporters (i.e. the catalytic subunit of HATs) (17). (iii) SteT showed obligate amino acid exchange activity (Figs. 3b, 4, and 6b), which is characteristic of the catalytic subunits of HATs (14). (iv) Furthermore, kinetic analysis supports a sequential mechanism of exchange for SteT (Fig. 5), which has also been described for the HAT system b^0^,+ in chicken small intestine (15). Thus, SteT is the first identified and characterized prokaryotic member of the LAT family of amino acid transporters.

In agreement with SteT amino acid exchange activity and in support of our functional characterization protocol, the SteT ortholog in *Bacillus cereus* (GenBank™ accession number AAP11885, gi 29898613, annotated as a hypothetical protein in Ref. 40) showed the same L-serine/L-threonine exchange activity when purified and reconstituted in proteoliposomes (data not shown). Other than the clear amino acid exchange activity detected here for both orthologues, to our knowledge, a SteT-related transport activity still remains to be described in cells or membrane vesicles of *Bacillus* sp.

Analysis of SteT in PLs by freeze-fracture TEM indicated that the protein is integrated in the lipid bilayer of unilamellar vesicles. Reconstituted SteT resides in two orientations in the bilayer, as suggested by freeze-fracture TEM images of SteT-PLs showing particle retention on concave as well as convex faces (Fig. 7, b and c). Distinct substrate affinities may be expected for the two orientations (up to 3 orders of higher apparent affinity for the external than the cytoplasmic side), as has been demonstrated for the reconstituted catalytic subunit (b^0^, AT) of system b^0^,-AT (16) and for other HATs expressed in oocytes (41). In contrast, curve fitting of kinetic data to Michaelis-Menten equations for two distinct affinities for L-serine outside SteT-PLs contraindicates a second K^m^ for L-serine and gives a unique external apparent K^m^ of 1.2 mM for L-serine (Fig. 5). This suggests that SteT has similar apparent affinities at both sides of the membrane.

The blue native gel of purified SteT indicates a strong and major band with an M^obs^ of ~80 kDa. This finding suggests that the majority of SteT molecules exist in monomeric form, given that the polypeptide has a molecular mass of ~49 kDa (based on its amino acid sequence) and migrates in BN-PAGE in association with a detergent/Coomassie Brilliant Blue G-250 micelle. The use of the conversion factor (1.8) determined by Heuberger et al. (42) to estimate the mass of membrane proteins from the M^obs^ also supports the existence of SteT in monomeric form. In addition, experiments performed with a wide range of amino-specific (dithiobis(succinimidyl)propionate, disuccinimidyl tartarate, and dimethyl sulfoximide) and sulfhydryl-specific (bis-maleimidotetraethyleneglycol) reagents did not yield cross-linking products (data not shown), in line with the results from BN-PAGE.

Freeze-fracture TEM of functional SteT and PotE in PLs showed homogeneous round-shaped particles with diameters of ~7.4 and ~8.0 nm, respectively. These values are only slightly higher than the diameter estimated for the lactose permease monomer (~6 nm; see “Results”) and in agreement with previous studies (43). Lactose permease has 12 transmembrane segments and similar molecular mass to SteT and PotE, and its monomeric state is well established (39). This suggests that functional SteT and PotE are monomers. The thickness of the platinum-carbon film deposited to produce our replicas may have led to overestimation of the true particle diameter by ~2 nm, as described for freeze-fracture TEM analysis of other transporters inserted in proteoliposomes (44). Thus, the diameter of the SteT and PotE particles can be assumed to be in the range of 5–6 nm. TEM of negatively stained SteT proteins
revealed an elliptical donut-like structure with diameters of ~7 nm by ~6 nm. Similar to freeze-fracture TEM, the measured dimensions are overestimated. With detergent-solubilized membrane proteins, the additional mass arising from the detergent belt and endogenous lipid bound to the protein has to be considered. According to Dekker et al. (45), the true protein volume is about 20% smaller than the volume estimated from the negatively stained detergent-solubilized membrane protein. Thus, the corrected dimensions of SteT from negative stain TEM are in agreement with those from freeze-fracture TEM. The “red permease” is a lactose permease fusion protein that consists of 12 transmembrane helices, has a molecular mass comparable with that of SteT, and forms monomers and trimers (46). The reported dimensions for the “red permease” monomer reconstituted into lipid membranes, as determined by negative stain TEM, are about 5 × 4 nm (46) and therefore similar to the dimensions of SteT after correction for the detergent bound to the protein (see above and Ref. (45)). Besides the size, both proteins possess a similar overall structure and a pronounced central indentation. As a control experiment and additional support for the monomeric nature of negatively stained detergent-solubilized SteT (Fig. 8), its dimensions were compared with those of negatively stained detergent-solubilized glutamate transporter (GltP). GltP forms trimers, and the molecular mass of the monomer is similar to that of SteT. The measured dimensions of the GltP monomer within the trimer are comparable with those of SteT, reinforcing the conclusion that SteT is monomeric (data not shown). In summary, our data suggest SteT and PotE to be functional in the monomeric form.

Little is known about the structure of the light subunits of HATs (LAT family). The elucidation of its structure is mainly limited by the low HAT expression in native cells and tissues and the difficulty of overexpressing these proteins in heterologous systems. An excellent alternative to understand the molecular mechanism of HATs represents the elucidation of the atomic structure of a prokaryotic transporter with high sequence identity to eukaryotic light subunits of HATs. To this end and for the first time, we have identified, cloned, overexpressed, and purified the SteT protein, a prokaryotic member of the LAT family. Functional and structural characterization of SteT has provided insights into the transport mechanism, kinetics, structure, and oligomeric state of this important family of amino acid transporters. However, a high resolution structure is needed to fully understand the molecular mechanism of SteT and to relate the previous functional data. The first step toward the growth of two- and three-dimensional protein crystals suitable for high resolution structure analysis has been achieved here by the successful overexpression and purification of high amounts of functional SteT.

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