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Development of transporter-control technology for fermentation

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Research aims

In some strains of the lactic acid bacterium *Tetragenococcus halophilus*, a proton-motive force (PMF) is generated by the combined action of an intracellular L-aspartate decarboxylation reaction, catalyzed by an L-aspartate-4-decarboxylase (AspD, EC 4.1.1.12), and an electrogenic aspartate^{1-} : alanine^{0} exchange reaction, catalyzed by an aspartate : alanine antiporter (AspT, TC# 2.A.81.1.1). The PMF generated is sufficiently high to drive ATP synthesis via the bacterial F_0F_1-ATPase. This combination of PMF and ATP synthesis has been proposed as a proton-motive metabolic cycle (Fig. 1). Such decarboxylation reactions are thought to be advantageous for cells because they generate metabolic energy and regulate intracellular pH[1,2]. In previous work with proteoliposomes, we found that the aspartate:alanine exchange catalyzed by AspT is electrogenic[1–3]. AspT is classified as a conventional secondary transport protein and belongs to the newly classified aspartate:alanine exchanger (AAEx) family (TC# 2.A.81) of transporters (http://www.tcdb.org/index.php). AspT is a membrane protein containing 543 amino acids (57.2 kDa). AspT has a unique topology (Fig. 2)[3] and the transmembrane domain (TM) 3 participates in the formation of a hydrophilic cleft in the membrane, implicating the TM3 in the ligand-induced conformational changes[4]. Previously, we developed a solubilization and purification scheme of AspT by using *n*-dodecyl-β-D-maltoside (DDM)[4]. Here, we characterized, in detail, the kinetic properties of AspT by analyzing the transport kinetics of reconstituted AspT under various conditions. In addition, to reveal the substrate recognition mechanism of AspT, we performed competition analysis with a series of amino acids and the aspartate analogs. The competition analysis suggested that the putative L-aspratate and L-alanine binding sites are independently located in the substrate translocation pathway of AspT.

Methods

Reconstitution and Transport of Purified AspT-His6

Purified AspT-His6 was reconstituted in a final volume of 1 ml with 800 μl of detergent DDM extracts (10 to 20 μg of purified AspT protein) (or control lipid extract), 130 μl of bath-sonicated liposomes (5.9 mg of *E. coli* phospholipid), and 18 μl of 15% 1-*O*-*n*-Octyl-β-D-glucopyranoside, with

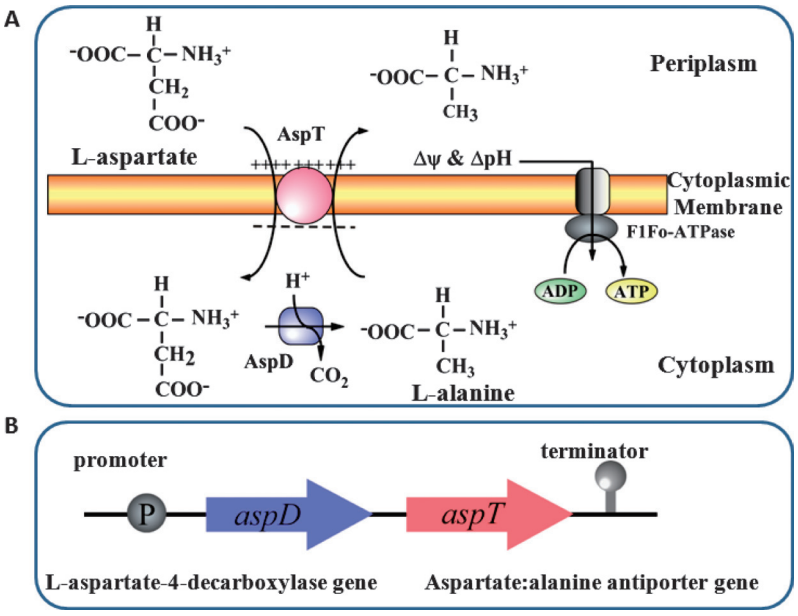

Fig. 1. (A) Energy generation coupled with L-aspartate decarboxylation, and (B) the structure of the *asp* operon encoding L-aspartate-4-decarboxylase and L-asparate : L-alanine antiporter.

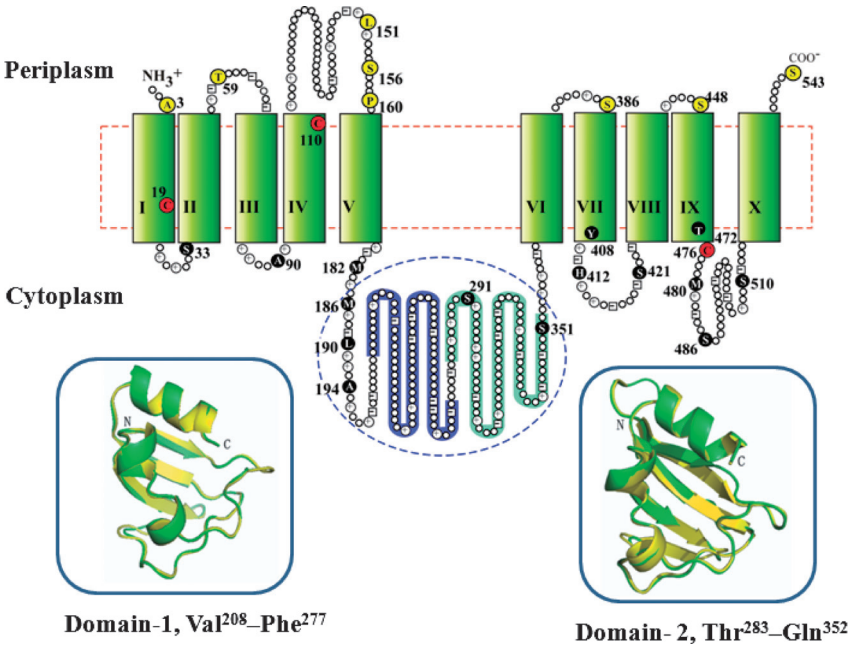

Fig. 2. Membrane topology of AspT and 3D-model of the large cytoplasmic loop region of AspT. AspT possesses 10 transmembrane helices, a large cytoplasmic loop between TM5 and TM6, and N- and C-termini facing periplasm.

the balance made up by 50 mM potassium phosphate (pH 7). After incubation for 20 min on ice, proteoliposomes (or control liposomes) were formed at room temperature (RT) by rapid injection of the mixtures into 20 ml of loading buffer containing buffer solution and suitable counter substrate such as 100 mM L-aspartate or L-alanine as the potassium salt for L-aspartate or L-alanine exchange reaction. Buffer components were changed in each experiment. The substrate-loaded proteoliposomes (or liposomes) were kept at RT for 20 min. To assess L-aspartate transport by L-[^{3}H] labeled aspartate or alanine-loaded particles, proteoliposomes were diluted 20-fold from the concentrated stock into an appropriate volume of assay buffer (50 mM potassium phosphate [pH 7], 100 mM K_2SO_4). After 1 to 3 min of pre-incubation at 25°C, L-[^{3}H]aspartate or L-[^{3}H]alanine was added to a final concentration of 100 μM; at various times, 50- to 100-μl aliquots were removed for membrane filtration with 0.22-μm-pore-size GSTF Millipore filter (Millipore Co., Billerica, MA). The membrane filters were washed twice with 5 ml of assay buffer[5].

Filtration Assay of Substrate Transport

Initial rates of L-[^{3}H]aspartate or L-[^{3}H]alanine entry were measured in duplicate at 25°C by means of a filtration assay[5]. Proteoliposomes were applied directly to the center of a 0.22-μm pore-size GSTF Millipore filter and washed twice with 5 ml of chilled assay buffer (50 mM potassium phosphate [pH 7], 100 mM K_2SO_4). Upon release of the vacuum, the proteoliposomes were covered with assay buffer that was pre-incubated at 25°C with 0.1 mM L-[^{3}H]aspartate or L-[^{3}H]alanine, and the reaction was terminated after 1 min by use of filtration and washing.

Results

Kinetic Analysis of AspT Transport

A kinetic study was undertaken, relying on samples filtered after a 10 s reaction, to estimate the initial velocities of the L-aspartate and L-alanine self-exchange reactions. A Hanes-Woolf plot was used to determine the Michaelis constant (K_m) and the maximum reaction (V_{max}) (Table 1). We found that the L-aspartate self-exchange reaction had a Michaelis constant (K_m) of 0.35±0.03 mM and a maximum velocity of 175±21 μmol min^{-1} mg^{-1} of protein and that the L-alanine self-exchange reaction had a K_m of 26±2 mM and a maximum velocity of 155±9 μmol min^{-1} mg^{-1} of protein (mean±S.E.). L-Aspartate had a higher affinity than L-alanine for AspT, but the L-aspartate and L-alanine self-exchange reactions had almost the same maximal velocities (V_{max}). Thus, the L-alanine self-exchange reaction showed almost the same transport rate as that of the L-aspartate self-exchange reaction in the presence of sufficiently high concentrations of L-alanine. A kinetic study of D-aspartate and D-alanine self-exchange reactions was also undertaken; the results are summarized in Table 1. D-Asparate and D-alanine showed a higher affinity than L-aspartate and L-alanine, respectively, for AspT. On the other hand, the L-isomers had higher V_{max} values than those of the D-isomers. These results suggest that the D-isomers form a tight and stable complex with AspT, with slow dissociation rates and low V_{max} values. Thus, AspT recognizes not only L-aspartate and L-alanine, but also their D-isomers, as substrates.

Table 1. Kinetic parameters of AspT

	K_m [mM]	V_{max} [μmol/min/mg protein]	V_{max}/K_m
L-Asp	0.35±0.03	175±21	500
L-Ala	26±2	155±9	6.0
D-Asp	0.098±0.000	3.8±0.3	39
D-Ala	3.3±0.2	3.3±0.4	1.0

Substrate Screening of AspT

To examine the substrate specificity of AspT, substrate-competition experiments were performed by measuring the uptake of L-[^{3}H]aspartate and L-[^{3}H]alanine in self-exchange reactions in the presence of 15 mM of various unlabeled amino acids. L-Asparagine and L-cysteine strongly inhibited L-[^{3}H]aspartate uptake (relative accumulation of L-[^{3}H]aspartate: 10% to 30%) whereas L-glutamate and L-tryptophan moderately inhibited L-[^{3}H]aspartate uptake (relative accumulation of L-[^{3}H]aspartate: 70% to 80%). None of the amino acids completely inhibited L-[^{3}H]alanine uptake. L-Asparagine and L-serine considerably inhibited L-[^{3}H]alanine uptake (relative accumulation of L-[^{3}H]alanine: 40% to 60%). L-Phenylalanine, L-arginine, L-leucine, L-threonine, D-serine, L-methionine, β-alanine, and D-cycloserine moderately inhibited L-[^{3}H]alanine uptake (relative accumulation of L-[^{3}H]alanine: 60% to 80%). These results suggest that AspT recognizes as substrates not only L-aspartate, L-alanine, D-aspartate, and D-alanine, but also many other D- and L-amino acids, such as L-serine and L-cysteine. L-cysteine and L-serine had unique inhibitory effects. L-[^{3}H]aspartate uptake was strongly inhibited by L-cysteine; however, L-[^{3}H]alanine uptake was not inhibited. On the other hand, L-serine apparently inhibited L-[^{3}H]alanine uptake and barely inhibited L-[^{3}H]aspartate uptake.

Inhibitory effect of L-cysteine, L-serine, and aspartate analogs

To evaluate the inhibitory effects of L-cysteine and L-serine, we performed a Dixon–Webb plot analysis of data from samples that were filtered after a 10-s reaction to estimate initial velocities. External substrate concentrations were adjusted to 0.07 mM, 0.35 mM (= K_m value), and 0.7 mM for L-aspartate self-exchange, and 5.8 mM, 26 mM (=K_m value), and 58 mM for L-alanine self-exchange. The three lines in the Dixon plot crossed at one point, demonstrating competitive inhibition of L-aspartate and L-alanine by L-cysteine and L-serine, respectively. The calculated inhibition constant values (K_i) were 2.0±0.2 mM (L-aspartate exchange inhibited by cysteine), 20±2.7 mM (L-alanine exchange inhibited by cysteine), and 5.3±1.7 mM (L-alanine exchange inhibited by serine) (Table 2). The K_i of L-serine for L-aspartate self-exchange was too large to measure, suggesting that serine is a specific inhibitor of alanine self-exchange but not of aspartate self-exchange. These analyses reveal that the mechanism of inhibition of L-aspartate self-exchange and L-alanine self-exchange by L-cysteine and L-serine is competitive inhibition. Because the K_m of L-serine to AspT was larger than the K_m of L-aspartate to AspT but smaller than the K_m of L-alanine to AspT, the apparent selective inhibitory effect of L-serine on L-alanine self-exchange can be explained by the difference in the K_m values of L-serine and L-alanine. However, the K_m of L-cysteine was larger than that of L-aspartate; this cannot explain the apparent selective inhibition of L-aspartate self-exchange with L-cysteine.

To further examine the selective inhibition of L-aspartate self-exchange with L-cysteine, we used substrate analogs for both cysteine and aspartate, namely L-CSA, L-CA, and D-CA. We measured L-aspartate and L-alanine self-exchange in their presence. L-CSA, D-CA, and L-CA inhibited both the L-aspartate and the L-alanine self-exchange reaction via AspT (Table 2). Dixon plot analyses revealed the competitive inhibition of L-aspartate and L-alanine by the analogs. D-CA was the strongest inhibitor of both the L-aspartate and the L-alanine self-exchange reactions. All three analogs markedly inhibited L-aspartate self-exchange but only weakly inhibited L-alanine self-exchange.

Table 2. K_i values for compounds competitive for L-Asp or L-Ala uptake

	L-Asp self-exchange		L-Ala self-exchange	
	K_i [mM]	IC_{50} [mM]	K_i [mM]	IC_{50} [mM]
L-cysteine	2.0±0.2	8.9	20±2.7	65
L-serine	N.D.*	N.D.*	5.3±1.7	28
L-CSA	0.59±0.08	0.83	28±0	28
L-CA	0.29±0.05	0.47	14±0	16
D-CA	0.14±0.02	0.19	5.0±0.2	9.0

* N.D., not determined. The K_i value of L-Ser for L-Asp self-exchange was too large.

Conclusion

T. halophilus AspT catalyzes the electrogenic exchange of L-asparate^{1-} with L-alanine^0. Here we show that the binding sites of L-aspartate and L-alanine are independently present in AspT by means of the kinetic analyses. We purified AspT-His6 and characterized its kinetic properties when reconstituted in liposomes (K_m=0.35±0.03 mM for L-aspartate, K_m=0.098±0 mM for D-aspartate, K_m=26±2 mM for L-alanine, Km=3.3±0.2 mM for D-alanine). Competitive inhibition by various amino acids of L-aspartate or L-alanine in self-exchange reactions revealed that L-cysteine selectively inhibited L-aspartate self-exchange but only weakly inhibited L-alanine self-exchange. Additionally, L-serine selectively inhibited L-alanine self-exchange but barely inhibited L-aspartate self-exchange. The aspartate analogs L-CSA, L-CA, and D-CA competitively and strongly inhibited L-aspartate self-exchange compared with L-alanine self-exchange. Taken together, these kinetic data suggest that the putative binding sites of L-aspartate and L-alanine are independently located in the substrate translocation pathway of AspT.

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