Biochimica et Biophysica Acta, 1151 (1993) 193–200

BBAMEM 76082

Solubilization and reconstitution of high- and low-affinity Na^+-dependent neutral L-α-amino acid transporters from rabbit small intestine

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(Received 3 June 1993)

Key words: Amino acid transporter; Small intestine; Reconstitution; Proteoliposome; (Rabbit)

High- and low-affinity Na^+-dependent neutral L-α-amino acid transporters were solubilized with 0.25% octaethylene glycol dodecyl ether ($C_{12}E_8$) after removal of the proteins from the brush-border membrane vesicles with 2% CHAPS and 4 M urea. When the CHAPS-insoluble protein was treated with papain before its solubilization with $C_{12}E_8$, a substantial amount of protein was removed without any decrease of the transport activities. The solubilized transporters were reconstituted into proteoliposomes after removal of $C_{12}E_8$ with Bio-Beads SM2. Several parameters proved to be important for optimal reconstitution efficiency: (a) the type of detergent, and (b) the phospholipid/protein and detergent/protein ratio during reconstitution, and (c) the salt concentration during reconstitution. Reconstituted proteoliposomes showed rapid uptake of neutral L-α-amino acids but not imino acid, basic or acidic amino acids driven by an electrochemical potential of Na^+ (out > in). The uptakes under low- and high-substrate condition were further augmented by an artificial membrane potential introduced by K^+ diffusion via valinomycin (negative interior). Kinetic analysis revealed that both the brush-border membranes and the solubilized fraction involved two carrier-mediated pathways for alanine transport. The kinetic parameters were determined by curve fitting with a computer to be $K_{t1} = 0.28$ mM (0.21 mM) and $K_{t2} = 43.2$ mM (28.4 mM), respectively (those with brush-border membrane vesicles in parentheses). Studies on the specific activities for transport of individual amino acids under low or high substrate concentration and the cross-inhibitory effects of various amino acids on alanine uptake (low concentration) revealed that these transporters possess broad specificity for neutral L-α-amino acids.

Introduction

Several transport systems for amino acids have been found in the small intestine and renal proximal tubules by studies using brush-border membrane vesicles [1–5]. Most of these transport systems are driven by an electrochemical gradient of Na^+ ($\Delta\tilde{\mu}_{Na^+}$) [1–3,5].

Neutral amino acid transport in the small intestine is mainly mediated by an NBB system (Na^+-dependent), which is clearly distinct from either an A system or an ASC system (Na^+-dependent) observed in the plasma membranes of most non-polar cells, such as Ehrlich cells [6] and mouse fibroblasts [7]. The mechanisms of these transport systems have been studied extensively [8–11], but, the actual number of transport systems involved is still controversial, and the molecular basis of amino acid transport and of co-transport in general is not known. The isolation of the transporter in an active form is obviously required to understand these problems. The only active amino acid transporter so far purified from mammalian cells is the sodium- and chloride-coupled γ-aminobutyric acid transporter [12] and sodium-coupled glutamate transporter [13] from rat brain. Very recently these two transporters have been cloned and sequenced [14,15]. There are several reports of the solubilization and reconstitution of Na^+-dependent amino acid transport activity from Ehrlich ascites cells [16], hepatocytes [17], renal proximal tubules [18,19] and Chang liver cells [20]. But the

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Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; E-64, L-*trans*-epoxysuccinylleucylamidyl-(4-guanidinobutane); APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone; FCCP, carbonylcyanide *p*-trifluorophenylhydrazone.

methods used in these studies have not yet been applied successfully to brush-border membranes of the small intestine. Previously, we reported that proteoliposomes reconstituted with the 0.4% deoxycholate-insoluble fraction from rabbit small intestine showed Na^+-dependent alanine transport activity [21]. In this report, we describe conditions for selective solubilization and functional reconstitution of high- and low-affinity Na^+-dependent neutral L-α-amino acid transporters from rabbit small intestine. This preparation will be helpful in order to monitor the transporter activities during the isolation and purification of the transporter, now in progress in our laboratory, providing new insights into the molecular mechanisms of the process.

Materials and Methods

Materials

^{14}C-labelled amino acids were purchased from New England Nuclear, Boston, MA. Soybean phospholipids (Asolectin), purchased from Associated Concentrates, Woodside, NY, were partially purified by the methods of Kagawa and Racker [22]. Crude rabbit small intestine lipids were extracted by the method of Folch et al. [23]. CHAPS and EDTA were from Dojindo, Kumamoto. Octaethylene glycol dodecyl ether ($C_{12}E_8$) was from Nikko, Tokyo. Bio-Beads SM2 was from Bio-Rad, Richmond, CA. Papain, trypsin, and chymotrypsin were from Sigma. All other reagents were of the highest purity available.

Isolation and NaI treatment of brush-border membrane vesicles

Membrane vesicles from rabbit small intestine were prepared as described previously [21]. All subsequent procedures were carried out at 0–4°C. Isolated membrane vesicles were suspended in 20 mM Hepes-Tris (pH 7.4), 1 mM DTT, and 2 mM EDTA (EDTA buffer) containing 0.6 M NaI at a final protein concentration of 4 mg/ml and sonicated for 10 min at 4°C in a bath sonicator (Elma Transsonic T 460). Then the mixture was centrifuged at $105\,000 \times g$ for 30 min, and the precipitate was suspended in EDTA buffer and centrifuged at $105\,000 \times g$ for 30 min. The resulting pellet was resuspended in the same EDTA buffer at a protein concentration of 10 mg/ml. The NaI-treated membranes were mixed sequentially with CHAPS (2%), NaCl (1 M), and urea (4 M) (final concentrations in parentheses). The mixture at a protein concentration of 2 mg/ml was sonicated for 10 min in a bath sonicator, and then centrifuged at $105\,000 \times g$ for 60 min. The pellet was washed, resuspended in the EDTA buffer at a protein concentration of 4 mg/ml, and stored at −80°C until use.

Proteinase treatment

In some experiments, the CHAPS-treated membranes were treated with papain, trypsin, and chymotrypsin (40:1 by mass) at 35°C. The proteinase reactions were stopped by the addition of E-64 to papain, APMSF to trypsin, and TPCK to chymotrypsin (1:10 by mass). The proteinase-treated membranes were centrifuged at $500\,000 \times g$ for 20 min and the pellets were washed twice and suspended in the EDTA buffer.

Solubilization and reconstitution of proteoliposomes

CHAPS- and papain-treated membranes were solubilized with 0.25% $C_{12}E_8$ in the presence of 4 mM alanine at a protein concentration of 2 mg/ml by sonication for 10 min in a bath sonicator. The mixture was centrifuged at $500\,000 \times g$ for 60 min and the resulting supernatant was used as solubilized transporter.

Reconstitution of proteoliposomes with the preparations described above was carried out by two different methods. Brush-border membranes, CHAPS-treated membranes, or CHAPS- and papain-treated membranes were reconstituted into proteoliposomes by the freeze-thaw-sonication method as follows: Partially purified Asolectin at a concentration of 20 mg dry weight of lipid per ml in potassium buffer was sonicated three times for 3 min periods with a probe sonicator (Branson Sonifier Cell Disruptor 200) (sonicated liposomes). Samples of 1 mg of brush-border membrane vesicles, CHAPS-treated membranes, or CHAPS- and papain-treated membranes were mixed with the sonicated liposomes at a ratio of phospholipids to protein of 20:1 by weight (total volume, 1–2 ml). The mixture was sonicated for 3 min on ice at a 0.5 s pulse mode. Sonicated samples were quickly frozen in a solid CO_2/methanol bath. The mixture was thawed at room temperature, subjected to brief pulse sonication for 30 s and centrifuged at $500\,000 \times g$ for 20 min, and the resulting pellet was suspended in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.4), and 1 mM DTT and used for measurements of amino acid transport.

The solubilized proteins were reconstituted into proteoliposomes by treatment with Bio-Beads SM2. For this, the proteins (0.5–2 mg protein) were mixed with an appropriate amount of Asolectin (200 mg/ml in the presence of 1.5% $C_{12}E_8$) to give a final lipid/protein ratio of 20:1 in the presence of 1 M NaCl and 10% glycerol. Then the mixture was treated with Bio-Beads SM2 (0.6 g wet weight of beads per 2 ml) for 6 h on ice with stirring, and filtered to remove the Bio-Beads. The filtrate was centrifuged at $500\,000 \times g$ for 60 min, and the pellet was washed with 0.15 M potassium phosphate buffer (pH 7.4), containing 1 mM DTT and 1 mM $MgSO_4$ (potassium buffer) and resuspended in the potassium buffer. The suspension was then sub-

jected to the freeze-thaw-sonication procedure described above.

Measurements of amino acid transport

The transport of amino acids was measured essentially as described previously [21]. Reconstituted proteoliposomes, obtained as described above, were pre-incubated for 5 min at 30°C. Then the proteoliposome suspension was mixed with 1 μl of valinomycin solution (1 mg/ml of methanol) and uptake experiments were initiated by mixing 300-μl aliquots of proteoliposome suspension with 1 ml of pre-warmed incubation medium (0.26 mM or 13 mM L-[^{14}C]alanine (37 kBq/ml) in 0.15 M sodium or potassium phosphate buffer, pH 7.4). Aliquots (100 μl) of the suspension were withdrawn at appropriate times and filtered through membrane filters (Sartorius, pore size 0.45 μm) that had been soaked in the stopping solution (10 mM Hepes-Tris buffer (pH 7.4), containing 0.15 M NaCl). The filters were washed with two 4 ml volumes of the stopping solution, and their radioactivities were then measured in a liquid scintillation counter (Aloka LSC-3500). The Na^+-dependent uptake rate was calculated by subtracting the uptake rate in potassium phosphate buffer (K^+ control).

Other methods

Protein concentrations were determined by the method of Lowry et al. [24] with the slight modifications described by Wessel and Flügge [25] with bovine serum albumin as a standard. Proteins reconstituted into proteoliposomes were recovered by the method of Wessel and Flügge [25].

Results and Discussion

Solubilization of the Na^+-dependent neutral L-α-amino acid transporter

Several procedures for reconstitution of amino acid transporters have recently been reported [16–20,26,27], but there are only a few reports of chromatographic purification procedures. We found that none of these procedures was effective for either solubilization or functional reconstitution of the high- and low-affinity Na^+-dependent neutral L-α-amino acid transporters from rabbit small intestine. Various detergents were tested for ability to solubilize the Na^+-dependent neutral L-α-amino acid transporters. Triton X-100, octyl glucoside, sucrose monocaprate, and Mega 10 caused great loss of high- and low-affinity transport activities. Only $C_{12}E_8$, which has been used for solubilization of Ca^{2+}-ATPase [28] and Na^+/K^+-ATPase [29], was effective for solubilization and reconstitution of these Na^+-dependent neutral L-α-amino acid transporters from rabbit small intestine. Bile acid derivatives such as cholic acid, deoxycholic acid, and CHAPS solubilized substantial amounts of proteins from the brush-border membranes, leaving the transporting activity under low alanine concentration in the residual insoluble fraction (Fig. 1). The similar result was observed under high-substrate condition (10 mM alanine) (data not shown). Simple liposomes without proteins showed passive uptakes of L-[^{14}C]alanine at almost the same rates as those in the K^+ control. For further removal of proteins other than the transporter, the CHAPS-treated membranes were subjected to proteolytic digestion. Trypsin and chymotrypsin caused great loss of both activities (data not shown), but papain removed a substantial amount of protein without affecting these activities. As shown in Fig. 2, specific activity (0.2 mM alanine) was increased as a function of the incubation period which reached a plateau at 60 min or more, while the total activity remained almost constant. These results are somewhat contradictory since the latter two enzymes have rather more strict substrate specificities than papain. However, the structural integrity of these transporters in membranes may result in different sensitivities toward these proteinases. In fact, after solubilization with $C_{12}E_8$ these transporters were rapidly digested by papain (data not shown). The kinetic parameters, substrate specificities, and solubility in $C_{12}E_8$ were essentially unaltered by papain treatment, indicating that papain has no proteolytic action on these transporter proteins in the membrane. Thus, we used the CHAPS-insoluble and papain-treated fraction as starting material for solubilization of the neutral L-α-

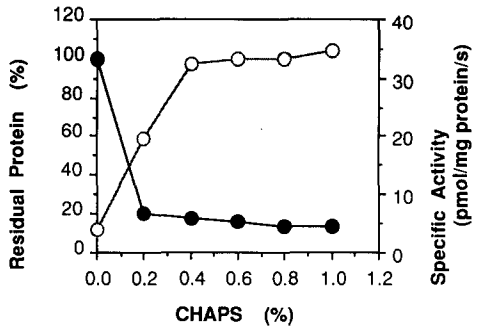

Fig. 1. Effect of CHAPS on solubilization of brush-border membranes and alanine transport activity. The NaI-treated membranes in EDTA buffer were incubated for 60 min in the presence of various concentrations of CHAPS, 4 M urea, and 1 M NaCl. The insoluble proteins were reconstituted into proteoliposomes and alanine transport activity (○) as described under Materials and Methods. Reconstituted proteoliposomes were incubated with incubation medium (0.26 mM L-[^{14}C]alanine in 0.15 M sodium or potassium phosphate buffer, pH 7.4). The alanine transport activities were assayed with the duration of 15 s. In the presence of potassium buffer the alanine uptake rates were almost constant (about 1000 cpm/100-μl aliquots). The specific activities were calculated by subtracting the uptake rate in potassium phosphate buffer. The residual proteins (insoluble proteins) (●) were given as percentage of total proteins in the original membrane vesicles.

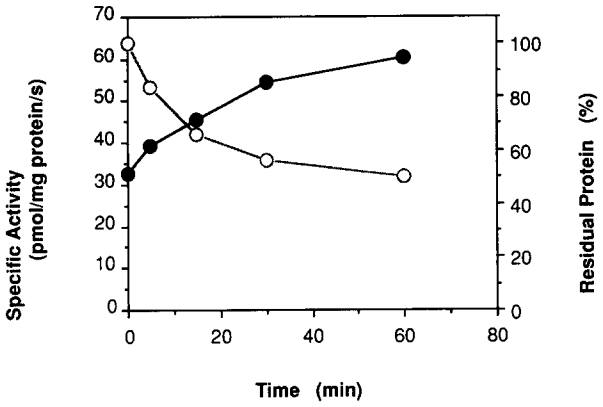

Fig. 2. Effect of papain digestion on alanine transport. CHAPS-treated membranes (2 mg proteins) in EDTA buffer were treated with 50 μg of papain at 35°C. Proteinase reactions were stopped by the addition of 5 μg of E-64. Papain-treated membranes were reconstituted into proteoliposomes and alanine transport activities were assayed as described under Materials and Methods. Symbols: ○, residual proteins were shown as percentage of total proteins in the original CHAPS-treated membranes; ●, specific activity.

amino acid transporters with $C_{12}E_8$ as described below.

For determination of the optimal $C_{12}E_8$ concentration for solubilization of the transporters, CHAPS- and papain-treated membrane proteins were treated with various concentrations of $C_{12}E_8$ (Fig. 3). The amount of proteins solubilized reached a plateau with 0.25% $C_{12}E_8$ or more. However, the specific activity of the high-affinity Na^+-dependent alanine transport changed markedly as a function of the detergent concentration, being maximal with 0.25% $C_{12}E_8$ and decreasing at

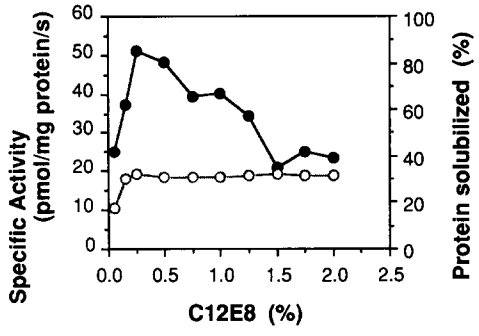

Fig. 3. Solubilization with $C_{12}E_8$ of the neutral L-α-amino acid transporter from CHAPS and papain-treated brush-border membranes. CHAPS- and papain-treated membranes (3 mg proteins) were solubilized with $C_{12}E_8$ at the indicated concentrations (0.05–2.0%) as described under Materials and Methods. The supernatants were centrifuged at 500000 × g for 60 min, mixed with 1 M NaCl and Asolectin in the presence of 1.5% $C_{12}E_8$ and treated with Bio-Beads SM2. The resulting protein fractions were used for reconstitution of proteoliposomes and assay of alanine transport activity (●) as described under Materials and Methods. After reconstitution, protein concentration was determined and the percentage of CHAPS-treated membrane proteins solubilized (○) was calculated.

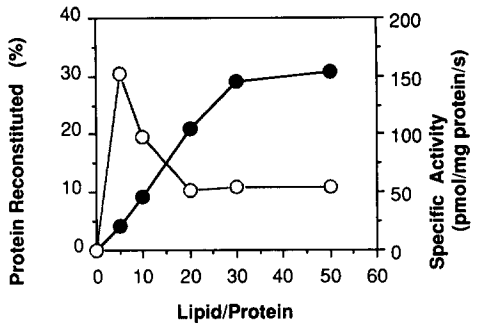

Fig. 4. Effect of lipid/protein ratio on reconstitution of alanine transport. The 0.25% $C_{12}E_8$ extract from CHAPS-treated membranes was reconstituted into proteoliposomes with Asolectin at the indicated lipid/protein ratios. Alanine transport activity (○) was assayed as described under Materials and Methods. The amounts of protein reconstituted are shown as percentages of that in the original $C_{12}E_8$ extract (●).

higher concentrations. The specific activity of low-affinity transport was also maximal with 0.25% $C_{12}E_8$. Similar results have been reported for the solubilization of the sodium- and chloride-coupled glycine transporter from rat spinal cord with either cholate or CHAPS [30]. With 0.25% $C_{12}E_8$, most of the activity was recovered in the soluble fraction and essentially no activity was found in the insoluble fraction.

Factors influencing the reconstituted transport activity

To optimize the experimental conditions and to characterize the reconstitution system, we investigated the effects of several factors on the activity of the reconstituted transporters. First, the phospholipid/protein ratio was found to be important in reconstitution experiments. As shown in Fig. 4, when the $C_{12}E_8$ solubilized proteins were reconstituted at various lipid/protein ratios, the amount of protein recovered in the proteoliposomes increased almost linearly with up to a ratio of 30:1. On the other hand, the specific activity of high-affinity alanine transport was maximal at a ratio of 5:1 and decreased at higher ratios. Similar result was obtained with the low-affinity system (10 mM alanine). These results indicate that these transporters are rather selectively reconstituted from the $C_{12}E_8$-solubilized proteins into proteoliposomes at a lipid/protein ratio of 5 : 1. However, these data do not necessarily reflect the real effect of the lipid/transporter protein ratio, because the results may be due to more efficient reconstitution of these transporter proteins than of other proteins. Furthermore, since the total activity increased as a function of the lipid/protein ratio, reaching a plateau at a ratio of 20 : 1 or more, the ratio of 20 : 1 was chosen for routine experiments for characterization of the amino acid transport. The biological activity of transporters [26,27,30,31] or channels [32,33] is enhanced by the addition of lipids

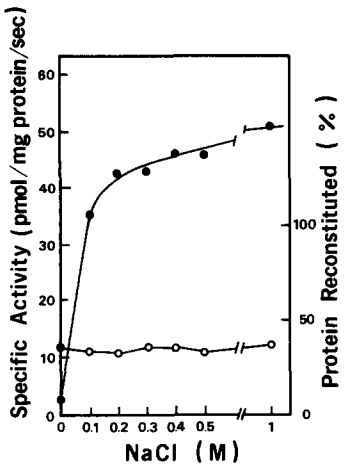

Fig. 5. Effect of NaCl concentration on reconstitution of the alanine transport activity. The 0.25% $C_{12}E_8$ extract was mixed with asolectin in the presence of 1.5% $C_{12}E_8$ and the indicated concentrations (0–1 M) of NaCl. The mixtures were used for reconstitution, and alanine transport activity (●) was assayed as described under Materials and Methods. The amounts of protein reconstituted are shown as percentages of that in the original $C_{12}E_8$ extract (○).

derived from the original membranes, but in our experiments, the supplemental addition of native lipids extracted from the small intestine or cholesterol had no effect on both high- and low-affinity transporter activities (data not shown).

Second, the salt concentration was found to be important for optimal reconstitution. Fig. 5 shows the effect of the NaCl concentration on the specific activity of high-affinity Na^+-dependent alanine transport during treatment with Bio-Beads SM2. The amount of protein reconstituted into proteoliposomes did not vary appreciably, but the specific activity increased markedly as a function of the NaCl concentration, reaching a plateau with 1 M NaCl or more of more than 20 times that observed in the absence of NaCl. Other monovalent cations (K^+, Li^+, NH_4^+) were found to have the similar effect as Na^+ on both high- and low-affinity transport activities (data not shown).

Characterization of the reconstituted alanine transport activity

Fig. 6 shows the effects of various ionophores on alanine transport (0.2 mM alanine). The reconstituted proteoliposomes showed rapid uptake of alanine driven by an electrochemical potential of Na^+ plus an artificial membrane potential induced by K^+ diffusion mediated by valinomycin. The uptake reached a maximum in 2.5 min, showing an apparent overshoot. In the presence of FCCP, the initial rate of alanine uptake was reduced 32%, indicating that the process was electrogenic. On the contrary, an Na^+–H^+ exchanger, monensin, caused 85% inhibition of alanine uptake in either the presence or absence of valinomycin. These results clearly indicate that an electrochemical potential of Na^+ is the driving force for alanine uptake. The uptake of D-alanine into the reconstituted proteoliposomes was less than 10% of that of L-alanine, indicating that the transporter had specificity for the L-stereoisomer.

The specificity of Na^+ in stimulating the rate of L-alanine uptakes under low (0.2 mM) and high (10 mM) substrate concentration by reconstituted proteoliposomes is shown in Table I (Experiment 1). The uptake rates in the K^+ control were 14.5 ± 1.2 pmol/mg protein per s and 0.25 ± 0.04 nmol/mg protein per s, respectively. Simple liposomes without proteins showed passive uptakes of L-[^{14}C]alanine at almost the same rates as those in the K^+ control, indicating that the reconstituted proteoliposomes did not possess an Na^+-independent carrier mediated pathway (data not shown). Rb^+, Li^+, NH_4^+ and choline ions did not influence these uptakes either in the presence or absence of valinomycin. However, Na^+ increased these uptake rates about 2-fold in the absence of valinomycin. In this character, it differs from the ASC system of rat hepatocytes for which Na^+ can be replaced by Li^+ [34]. Both high- and low-affinity Na^+-dependent uptake rates were further stimulated by an artificial membrane potential introduced by K^+ diffusion via valinomycin. Subtraction of the value for the K^+ control showed that the presence of a membrane potential increased these uptakes more than 2-fold. These results indicated that these alanine transporters are specific for Na^+ as a coupling ion. Furthermore, Na^+-

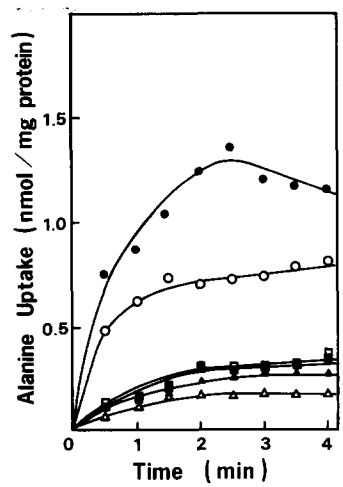

Fig. 6. Effects of ionophores on alanine transport by reconstituted proteoliposomes. The 0.25% $C_{12}E_8$ extract was reconstituted into proteoliposomes and alanine transport activity was assayed as described under Materials and Methods. The external medium contained 0.2 mM L-[^{14}C]alanine and 116 mM sodium phosphate buffer (pH 7.4) with: ●, 1 μg of valinomycin; ▲, 1 μg of valinomycin plus 1 μg of monensin; ■, 1 μg of monensin; ○, 1 μg of valinomycin plus 1 μg of FCCP; (□), D-[^{14}C]alanine (0.2 mM) in place of L-[^{14}C]alanine with 1 μg of valinomycin; and △, potassium phosphate buffer in place of 116 mM sodium phosphate buffer (pH 7.4).

TABLE I

Sodium dependency of alanine transport

Reconstituted proteoliposomes were incubated with 0.2 mM or 10 mM L-alanine for 15 s at 30°C as described under Materials and Methods. Means ± S.D. of three separate experiments are shown.

Experiment 1

Out (150 mM)	In (150 mM)	Val (1 μg)	Uptake (0.2 mM) (pmol/mg protein/s)	Uptake (10 mM) (nmol/mg protein/s)
Na^+	K^+	+	68.3 ± 8.1	1.69 ± 0.21
Na^+	K^+	−	37.1 ± 2.7	0.91 ± 0.19
K^+	K^+	+	14.5 ± 1.2	0.25 ± 0.04
Rb^+	K^+	+	15.8 ± 0.6	0.28 ± 0.02
Rb^+	K^+	−	15.0 ± 1.5	0.18 ± 0.03
Li^+	K^+	+	12.0 ± 3.2	0.23 ± 0.01
$NH4^+$	K^+	+	14.1 ± 2.8	0.31 ± 0.04
$Choline^+$	K^+	+	13.6 ± 1.8	0.40 ± 0.02

Experiment 2

Out (150 mM)	In (150 mM)	Val (1 μg)	Uptake (0.2 mM) (pmol/mg protein/s)	Uptake (10 mM) (nmol/mg protein/s)
Na^+	K^+	+	68.9 ± 4.9	1.66 ± 0.34
K^+	K^+	−	11.7 ± 2.3	0.15 ± 0.04
Na^+	Na^+	−	29.3 ± 2.1	0.58 ± 0.21
K^+	Na^+	−	14.0 ± 0.9	0.24 ± 0.11

loaded proteoliposomes (both out and in were 150 mM NaCl) showed significant amount of the uptake (Table I, Experiment 2). These uptake rates was increased 24-fold for high affinity and 19-fold for low affinity than those of brush-border membrane vesicles (1.2 pmol/mg protein per s and 31 pmol/mg protein per s in brush-border membrane vesicles, respectively). These increases of the uptake may mean the enrichment of the transporter protein(s) during solubilization and reconstitution procedure.

Kinetic analysis and substrate specificity of reconstituted neutral L-α-amino acid transporter

An Eadie-Hofstee plot of activity with alanine as substrate gave a concave curve, indicating the presence of two carrier-mediated pathways (Fig. 7). The kinetic parameters were determined by curve fitting with a computer and were summarized in Table II. The K_t values of reconstituted proteoliposomes coincided with those observed with brush-border membrane vesicles. On the other hand, the J_{max} values were significantly increased in reconstituted proteoliposomes, suggesting that the enrichment of the transporters occurred. Furthermore, these findings could be explained by supposing that the solubilized fraction contained two independent transporters; one with high affinity and the other with low affinity, or a single transporter with two

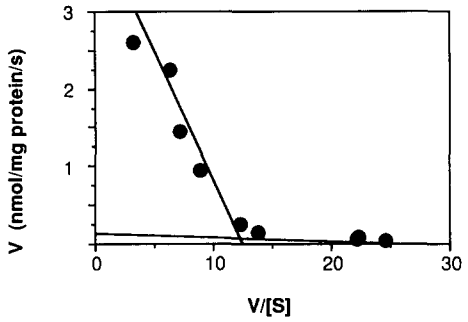

Fig. 7. Kinetic analysis of the Na^+-dependent alanine transport. Eadie-Hofstee plot of the Na^+-dependent alanine uptake. Abscissa unit is 10^{-6} l/mg per s. Means of three separate experiments are shown.

different affinity sites. Our results, that is, several conditions of solubilization and reconstitution, Na^+ dependency, and substrate specificity, indicate that these transport proteins had similar properties except for their affinity constants. From these findings, the latter possibility is more probable. However, complete purification of the active transporter protein is obviously required to distinguish between these possibilities.

As shown in Table III, brush-border membrane vesicles have Na^+-dependent transport activities for all neutral and acidic amino acids, including proline under low (0.2 mM) or high (10 mM) substrate concentration. Using the reconstituted proteoliposomes, we examined the substrate specificity of the neutral L-α-amino acid transporter (Table III). Its specific activities were the highest with valine and methionine. All the neutral amino acids tested except glycine and proline were also effectively transported into the proteoliposomes. Furthermore, the relative activities of the reconstituted proteoliposomes with papain treatment coincided well with those without papain treatment (data not shown). Under low-substrate condition, the inhibitory effects of several unlabelled amino acids (10 mM) on alanine transport were also studied. Amino acids that were transported effectively were strong inhibitors of alanine transport, whereas others were not inhibitory. Thus there was a good correlation between the specific activities for amino acids and the inhibitory effects of

TABLE II

Kinetic parameters of brush-border membrane vesicles and reconstituted proteoliposomes

Sample	K_t (mM)	J_{max} (pmol/mg protein per s)
Brush-border membranes	$K_{t1} = 0.12$	$J_{max1} = 2.1$
	$K_{t2} = 13.0$	$J_{max2} = 163$
Reconstituted proteoliposomes	$K_{t1} = 0.28$	$J_{max1} = 63.4$
	$K_{t2} = 43.2$	$J_{max2} = 4280$

TABLE III

The substrate specificity of the neutral L-α-amino acid transporter

The proteoliposomes were reconstituted and the Na^+-dependent transport activities for each amino acid (0.2 mM or 10 mM) were assayed as described under Materials and Methods. The inhibitory effects (low substrate concentration) of concentrations of 5 mM of each amino acid on [^{14}C]alanine transport are shown as percentage inhibitions of the control activity (no additions). Means ± S.D. of three separate experiments are shown.

Amino acid	Brush-border membranes	Reconstituted proteoliposomes		
	Specific activity (0.2 mM)	Specific activity		Inhibition of alanine uptake (%)
		(0.2 mM) (pmol/mg protein per s)	(10 mM) (nmol/mg protein)	
Ala	3.2	52 ± 12	1.63 ± 0.40	58 ± 11
Val	4.3	77 ± 4	1.47 ± 0.42	76 ± 5
Met	N.D.	75 ± 22	2.81 ± 0.21	90 ± 5
Ile	N.D.	64 ± 6	2.01 ± 0.34	80 ± 7
Phe	22.7	37 ± 14	2.90 ± 0.72	83 ± 10
Ser	N.D.	39 ± 6	2.23 ± 0.38	48 ± 13
Gly	8.0	3 ± 7	0.04 ± 0.22	15 ± 10
Pro	5.3	12 ± 4	0.25 ± 0.14	15 ± 10
Lys	0.7	3 ± 5	0.12 ± 0.12	3 ± 2
Glu	8.0	3 ± 5	0.08 ± 0.12	5 ± 3

N.D. was not determined.

these amino acids on alanine uptake, suggesting that the Na^+-dependent neutral L-α-amino acid transporter recovered in the reconstituted proteoliposomes has broad specificity for neutral L-α-amino acids. These results are well consistent with those reported previously [21]. Phenylalanine was transported into brush-border membrane vesicles much more effectively than the other neutral L-α-amino acids, but, it was not a specific substrate for the reconstituted high- and low-affinity neutral L-α-amino acid transporters. A specific phenylalanine transporter, the Phe system, has been described [5]. The Na^+-dependent transporters for acidic amino acids, imino acids, glycine, and phenylalanine present in the brush-border membranes seemed to be eliminated during the solubilization and reconstitution procedure.

Acknowledgments

We thank Drs. Toshiro Hamamoto and Eiro Muneyuki for helpful discussion. We also thank Yasuko Narita and Mayumi Masubuchi for their excellent assistance.

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