Purification and Reconstitution of an Intestinal Na\textsuperscript{+}-dependent Neutral L-\(\alpha\)-Amino Acid Transporter*

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Using an improved reconstitution method, we have purified an Na\textsuperscript{+}-dependent neutral L-\(\alpha\)-amino acid transporter from rabbit small intestine to apparent homogeneity. The preparation solubilized with octaethylene glycol dodecyl ether (C\textsubscript{12}E\textsubscript{8}) was purified by successive chromatographies on DEAE-Toyopearl and lentil lectin-Sepharose 4B columns. The transport activity was assayed by reconstitution of the protein into liposomes. The specific activity of the final preparation was 1364-fold that of brush border membrane vesicles. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the active fractions gave one band of 90 kDa. Kinetic analysis using proteoliposomes reconstituted with the purified fraction showed that alanine transport was mediated by high affinity system with \(K_a\) value of 0.19 mM and \(V_{max}\) value of 2.8 nmol/mg protein/s. Analysis of the amino acid composition of the purified transporter revealed that the transporter is very hydrophobic protein. From its specific activities for transport of individual amino acids this transporter was concluded to possess broad specificity for neutral L-\(\alpha\)-amino acids. Furthermore, inhibition study of other amino acids allowed us to identify this transport pathway as the intestinal system B.

A metabolically important function of the intestine and kidney is the uptake of amino acids and sugars by active transport systems. Several transport systems for amino acids have been found in the intestine by studies using brush border membrane vesicles (1–5). Most of these transport systems are driven by an electrochemical gradient of Na\textsuperscript{+} (ApNa\textsuperscript{+}) (1–3, 16). However, to determine the transport activity using other amino acids, we conclude that this transporter protein has been involved in single transport systems without interference from other potentially more active transport systems.

The transport of neutral amino acids in the small intestine is mainly mediated by the NBB system (16), which was recently renamed system B (17). System B is distinct from either system A or the ASC system observed in the plasma membranes of most non-polar cells, such as Ehrlich cells (18) and mouse fibroblasts (19). Furthermore, this neutral amino acid transport appears to be mediated by two systems with different affinities (20, 21). The presence of two affinity sites for neutral amino acid transport prompted us to determine whether the binding sites are located in the same transporter or two separate ones. Recently, Daniel et al. (22) suggested that dipeptide transporters with high and low affinity are localized on two separate carriers because they differ in sensitivities to a pH gradient and membrane potential. In addition, Mønzen et al. reported that the low affinity system B is identical with the low affinity system of acidic amino acid transport (23) and threonine is a model substrate for system B (24). However, to determine whether system B transport with high and low affinities is due to one or two transporters, the transporter(s) must be purified in an active form.

Mammalian transport proteins constitute only a small fraction of the total membrane proteins (<0.2%) and are easily denatured, making their isolation in an active form difficult. In fact, little information is available on the solubilization, reconstitution, and purification of these transporters. The reconstitutions of the Na\textsuperscript{+}-dependent glucose transport system and several amino acid transport systems from renal and intestinal brush border membranes have recently been reviewed (25). Other reconstituted co-transport systems are the Na\textsuperscript{+}/Pi symport system of the brush border membrane of the renal proximal tubules (26), the Na\textsuperscript{+}-coupled amino acid transport system from liver tissue (27, 28), and the 4-aminobutyric acid transporter from rat brain (29, 30).

Recently, we have developed a solubilization and reconstitution procedure for the Na\textsuperscript{+}-dependent neutral L-\(\alpha\)-amino acid transporter from rabbit small intestine (22). Here we report purification of the Na\textsuperscript{+}-dependent neutral L-\(\alpha\)-amino acid transporter monitored by reconstitution of the transporter into liposomes. The activity of the purified transporter retained the same properties as those of the "native" transporter in the brush border membrane. Furthermore, from the inhibition study of the transport activity using other amino acids, we conclude that this transporter protein has been involved in intestinal system B.

EXPERIMENTAL PROCEDURES

Materials

\(^{13}C\)-Labeled amino acids were purchased from DuPont NEN. Soybean phospholipids (Asolectin), purchased from Associated Concentrates (Woolside, NY), were partially purified by the methods of Ka-

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Na+-dependent Neutral l-α-Amino Acid Transporter

Polyacrylamide Gel Electrophoresis and Electrolabelling—Slab-gel electrophoresis was carried out on 8.5% polyacrylamide gels in 25 mM Tris, 192 mM glycine, pH 8.3, containing 0.1% SDS by the method of Laemmli (32). Proteins in the gels were stained with silver essentially as described (33). In some cases, after SDS-polyacrylamide gel electrophoresis, proteins were electroblotted with a Bio-Rad Trans-Blot system onto a Problott membrane for 2 h for 0.5-mm-thick gel. The blotting buffer was the same buffer as that for SDS-polyacrylamide gel electrophoresis. After transfer, proteins were stained with Coomassie Brilliant Blue R-250. In order to determine a pl value of the transporter protein, the commercial model (Rotofer) from Bio-Rad was used. 3 ml of lentil lectin fraction was diluted 1:10 in 4 x urea, 1% C₂₃E₈, 10% glycerol (w/v), and 1% (3:10) carrier ampholyte (Bio-Lyte 3/10) was loaded into the Rotofer. The limits on the power supply were 1500 V, 25 mA, and 12 watt. After 4 h, fractions were collected and their pH values measured.

Analysis of Amino Acid Composition—Bands of the neutral l-α-amino acid transporter on the Problott membrane were cut into small pieces (2 x 5 mm) and pooled in a single Eppendorf tube. The pieces were washed twice with 20% methanol and 0.25 mg/ml potassium EDTA, washed once with H₂O, and applied to an Applied Biosystems model 420A derivatizer system.

RESULTS AND DISCUSSION

Purification of the Na+-dependent Neutral l-α-Amino Acid Transporter—Recently, several ion-coupled amino acid transporters have been cloned from mammalian cells and expressed in a Xenopus oocyte system (6, 8-12, 14), and these have been identified with monoclonal antibodies (36, 37). However, to understand the transport mechanism of single transporters without interference from other potentially more active transporters and to characterize the transporter proteins, these transporters must be isolated in an active form.

In the small intestine there are many specific amino acid transporters that differ from those of non-polar cells (38). In particular, the Na+-dependent neutral l-α-amino acid transport system, named system B, is unique, having broad substrate specificity and appearing to be mediated by two different affinity transport mechanisms (21, 22).

Purification of system B from the small intestine is difficult because its content in the enteroctye is low and enteroctye brush border membrane also contains many other Na+-coupled organic solute transporters. For the purification of the transporter, the development of a reliable reconstitution system is important because no molecular tools such as affinity probes, irreversible inhibitors, or sensitive binding assays are available. At present, functional assay of the solubilized transporter protein is the only way to monitor purification and assay its activity in a complex protein mixture. Recently, we reported the improved solubilization and reconstitution method of Na+-dependent neutral l-α-amino acid transport system, named system B, is unique, having broad substrate specificity and appearing to be mediated by two different affinity transport mechanisms (21, 22).

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were pooled and applied to a lentil lectin column equilibrated with the lectin buffer as described under "Experimental Procedures." The activity was eluted with 150 mM α-methylmannopyranoside in the lectin buffer. The protein and activity profiles of the eluate are illustrated in Fig. 2. Under these conditions, most of the protein and about 80% of the alanine transport activity was not retained on the column, and only a small portion of protein (0.4%) with about 10% of the activity applied was eluted with 150 mM α-methylmannopyranoside. At higher sugar concentration (300 mM α-methylmannopyranoside), almost no protein was eluted (data not shown). Affinity columns for glycoproteins have been used successfully for the purifications of several Na+-dependent transporters (39–41). In present work, this step resulted in about 20-fold increase in specific activity over that of the DEAE fraction and an actual overall purification of 1364-fold. These purification steps were summarized in Table I.

Analysis of the polypeptide composition at each purification step by SDS-polyacrylamide gel electrophoresis with silver staining revealed the presence of one polypeptide with a molecular mass of about 90 kDa in the lentil lectin 150 mM α-methylmannopyranoside fraction (Fig. 3, lane B). By isoelectrofocusing (Bio-Rad, Rotofor), this lentil lectin 150 mM α-methylmannopyranoside fraction was composed by one 90-kDa protein, showing the pl value of 6.8 (data not shown). The alanine transport activities at these purification steps corresponded well with the amount of 90-kDa material. From these results, we conclude that this 90-kDa protein is a neutral l-α-amino acid transporter. Alanine transport activity was also detected in the flow-through fraction from the lentil lectin column, as described above. The SDS-polyacrylamide gel electrophoretic pattern of this flow-through fraction was almost the same as that of the DEAE-Toyopearl fraction (data not shown).  

Uptake of Alanine by the Na+-dependent Neutral l-α-Amino Acid Transporter Reconstituted into Proteoliposomes—Fig. 4 shows the [14C]alanine uptake by the purified transporter reconstituted into proteoliposomes. Closed circles show the uptake in the presence of Na+ and valinomycin, and open circles that in the presence of K+ in the outer solution. These results demonstrate that the purified transporter reconstituted into proteoliposomes mediated rapid uptake of alanine driven by an electrochemical potential of Na+.

The kinetics of Na+-dependent alanine uptake were also examined. An Eadie-Hofstee plot (Fig. 5) of the alanine transport activities using the purified transporter reconstituted into proteoliposomes revealed one saturated system. These results clearly indicate that this transport protein shows high affinity transport system (K0.5 = 0.19 mM, Jmax = 2.8 nmol/mg protein/s). The K0.5 value determined with the purified fraction was very similar to that of brush border membrane vesicles (Table II), indicating that the transport system remained functionally normal during solubilization and purification of the transporter. Recently, Daniel et al. (23) reported that the high and low affinity transport systems for dipeptides in the kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. Because of these differences, they suggested that two distinct transporters were responsible for the high and low affinity transports. Our present result also indicates that two distinct transporters were responsible for the high and low affinity transports because this transporter shows only high affinity K0.5 for alanine. In addition, Maenz et al. (24) also reported that the low affinity system of acidic amino acid transport in rabbit jejunal brush border membrane is identical with the low affinity one of system B by inhibition studies.

Substrate Specificity of Purified Na+-dependent Neutral l-α-Amino Acid Transporter—As shown in Table II, the purified transporter has Na+-dependent transport activities for most neutral amino acids. Acidic amino acids, basic amino acids, glycine, and proline were not transported into proteoliposomes (data not shown). The specific activity was highest with valine (4.58 ± 0.39 nmol/mg protein/s). In order to get a better appraisal as to the nature of the transport system, we also have studied the effect of different amino acids on the initial uptake rates of alanine. Table II shows the result of such an experiment using 15 mM concentrations of these agents in the uptake media in comparison to a control run. It appears that the neutral amino acids, with the possible exception of glycine and proline, were all potent inhibitors, reducing uptake rates to the level of diffusion obtained with 15 mM alanine in external potassium media (K+ control). None of the other compounds tested, however, could demonstrate any capacity to inhibit alanine uptake. This inhibition profile was almost same as that described previously for system B (16). Furthermore, Maenz et al. (25) also suggested that threonine is a definitive substrate for system B. In our inhibition study, threonine was a potent inhibitor of alanine uptake, indicating that this 90-kDa protein is a transporter responsible for system B.

![Fig. 1. DEAE-Toyopearl column chromatography of Na+-dependent neutral l-α-amino acid transporter.](image-url)
Na⁺-dependent Neutral L-α-Amino Acid Transporter

**Purification of the Na⁺-dependent neutral l-α-amino acid transporter from rabbit small intestine**

Aliquots of fractions at each step were reconstituted into proteoliposomes, and their alanine transport activity was determined as described under "Experimental Procedures."

| Step | Activity (pmol/s) | Protein (mg) | Specific activity (pmol/mg proteins/s) | Purification | Yield % |
|------|------------------|--------------|---------------------------------------|--------------|---------|
| Brush-border membranes | 1948 | 696 | 2.8 | 1 | 100 |
| CHAPS pellet | 2494 | 126 | 19.8 | 7 | 128 |
| Papain pellet | 1768 | 42 | 42.1 | 15 | 91 |
| C₁₅E₆ extract | 1232 | 16.8 | 73.3 | 26 | 63 |
| DEAE-Toyopearl | 1174 | 5.5 | 213.5 | 76 | 60 |
| Lentil lectin | | | | | |
| Sepharose 4B | 122 | 0.032 | 3820 | 1364 | 6.3 |

**Table I**

Amino Acid Composition of the Na⁺-dependent Neutral L-α-Amino Acid Transporter—Amino acid analysis of the Na⁺-dependent neutral l-α-amino acid transporter on a Problott membrane was carried out as described under "Experimental Procedures." Tryptophan and cysteine were not determined by the present method. Leu, Val, and Ile were the most abundant amino acid residues (each more than 10% of the total amino acid residues), while the contents of Gin and Asx were low (each are about 5% of the total amino acids) (Table III). These results indicate that this transporter is hydrophobic like many other transporters. All known transporters have been found to have 10–12 transmembrane domains, except the Na⁺-independent
neutral amino acid transporter, which has four transmembrane domains, low amounts of Val and Ile, and high amounts of Glx and Asx (12). In preliminary experiments on the amino acid sequence by Edman degradation, the N terminus appeared to be blocked (data not shown).

In the present study we developed a procedure for purification of the Na⁺-dependent neutral l-α-amino acid transporter from the small intestine. Current studies in our laboratory are aimed at cloning and expression of the intestinal Na⁺-dependent neutral l-α-amino acid transporter.

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