Solubilization and Reconstitution of Sodium-dependent Transport System for Branched-chain Amino Acids from *Pseudomonas aeruginosa*

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The sodium-dependent transport system for branched-chain amino acids of *Pseudomonas aeruginosa* was solubilized with n-octyl-β-D-glucopyranoside and reconstituted into liposomes by a detergent-Sephadex G-50 gel filtration procedure. The reconstituted proteoliposomes exhibited Na⁺-dependent counterflow and Na⁺-gradient-driven transport of l-leucine, l-isoleucine, and l-valine. The leucine counterflow was specifically inhibited by only branched-chain amino acids and the uphill transport of two species of amino acids among the three was induced by counterflow of the other substrate. These results show that the transport system for branched-chain amino acids was reconstituted into liposomes from *P. aeruginosa* cells and strongly suggest that three branched-chain amino acids are transported by a common carrier system.

*Pseudomonas aeruginosa* has the transport system of branched-chain amino acids, l-leucine, l-isoleucine, and l-valine, which is mediated by two kinetically distinguishable systems, LIV-I and LIV-II (1). These two transport systems have been identified by genetic study (2). The LIV-I system, with a high affinity, is binding protein-dependent and specific for alanine and threonine in addition to branched-chain amino acids. The LIV-II system, with a low affinity, is membrane-bound and specific for branched-chain amino acids and is operative only when sodium is present (1). The LIV-II transport system is one of the bacterial transport systems which require the presence of Na⁺ or are driven by a Na⁺-concentration gradient (3-10). Among them, the Na⁺-dependent meliobiose transport system of Salmonella typhimurium and *Escherichia coli* utilizes either Na⁺ or Li⁺ as the coupling cation for co-transport (5, 6, 11), while the LIV-II transport system of *P. aeruginosa* depends specifically on Na⁺ as the coupling cation (3).

The study of the molecular mechanism of bacterial active transport driven by specific coupling of a cation such as proton or sodium ion has been progressing for several years. Some important progresses in biochemical research have been made as follows: solubilization and reconstitution of transport systems followed by purification and characterization of carrier proteins (12-20).

This communication describes the reconstitution of the LIV-II transport system from *P. aeruginosa* having an enhanced transport activity which was isolated by Hoshino and Kageyama (2). The reconstituted proteoliposomes exhibited Na⁺-dependent counterflow and Na⁺ gradient-driven transport of branched-chain amino acids.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Two strains of *P. aeruginosa* were used. One strain is PML1455, a derivative of PML14, having an enhanced transport activity of LIV-II and lacking a LIV-I transport activity (2). The enhanced LIV-II activity in PML1455 is characterized by a 6-fold increased Vₘₐₓ and an unchanged apparent Kₘ, compared to those in wild type strain PML14. The other is PML1463, a mutant strain devoid of both LIV-I and LIV-II transport activites (2).

**Materials**—Crude phosphatidylethanolamine (Type IX) extracted from *E. coli* cells was obtained from Sigma and designated as *E. coli* lipid. The lipid was partially purified by the procedure of Kugawa and Racker (21). n-Octyl-β-D-glucopyranoside and DL-dithiothreitol were purchased from Sigma. Sephadex G-50 was from Pharmacia Fine Chemicals. Radioactive L-U⁻¹⁴C-amino acids were purchased from Amersham. All other reagents were of analytical grade.

**Preparation of Membranes**—PML1455 cells were grown at 37 °C in 3 liters of modified glutaminate medium (22). The following steps were carried out at 4 °C or ice-cold temperature, unless otherwise stated. Cells harvested at late logarithmic phase were washed once with the above growth medium and suspended in 20 ml of 50 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, 5 mM MgSO₄, and 20 µg/ml DNase I (14). The cells were disrupted by a single passage through an Aminco French pressure cell at 15,000 p.s.i. After broken cells were removed by centrifugation at 8,600 × g for 10 min, the supernatant was centrifuged at 51,000 × g for 60 min. The pellet was suspended in 8 ml of 50 mM potassium phosphate, pH 7.5, and 1 mM dithiothreitol and again centrifuged. The pellet was resuspended in a few milliliters of the same buffer to give a protein concentration of about 20 mg/ml. Membranes were frozen in ethanol/dry ice and stored at −80°C.

**Preparation of Liposomes**—Since *P. aeruginosa* phospholipid extracted from PML1455 cells or membranes was hard to be formed into liposomes at high lipid concentration by sonication, *E. coli* lipid sonicated easily into liposomes was used throughout this study. Liposomes were prepared by sonicating 40 mg of *E. coli* lipid in 0.8 ml of 0.1 M KCl, 20 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, and 20 mM appropriate amino acid for counterflow under N₂ gas for 10 min at room temperature using a Bransonic 12 bath sonicator (12).

**Solubilization and Reconstitution**—LIV-II transport system was solubilized with octylglucoside*¹ according to the procedure of Newman and Wilson (12) and reconstituted into liposomes by a detergent-gel filtration method (23). In a typical experiment for leucine counterflow, 100 µl of membranes thawed quickly at room temperature were added to 720 µl of a solution of 0.1 M KCl, 20 mM potassium phosphate, pH 7.5, 20 mM NaNO₃, 1 mM dithiothreitol, and 20 mM leucine. The above solution is referred to as PSDL. The membranes were mixed with 100 µl of liposomes and solubilized by the addition of 80 µl of 20% (w/v) octylglucoside. The membrane-detergent mixture was centrifuged at 110,000 × g for 1 h. The supernatant was mixed with 250 µl of liposomes and 70 µl of 20% octylglucoside to give a final detergent concentration of 2.3%. Then the mixture was:

*¹The abbreviation used is: octylglucoside, n-octyl-β-D-glucopyranoside.
applied to a Sephadex G-50 column (1.5 × 35 cm) equilibrated with PSDL and was eluted with PSDL at a flow rate of 12 ml/h at 18 °C. Turbid fractions eluting in the void volume were collected and centrifuged at 81,000 × g for 1 h. The pellet was suspended in 400 μl of PSDL, frozen in ethanol/dry ice, and stored at −80 °C. The protein content of proteoliposomes was approximately 0.055 mg/mg of phospholipid, whereas that of membranes as starting material was 1.5. For Na⁺-gradient-driven transport, leucine and NaN03 were omitted from all reconstitutions steps.

Transport Assays—Just prior to counterflow assay, frozen proteoliposomes were thawed rapidly in a water bath at room temperature, sonicated for 10 s, diluted with 4 ml of PSDL, and centrifuged at 81,000 × g for 1 h (24). The pellet was suspended in 50 μl of PSDL to obtain a protein and lipid concentration of approximately 1.0 and 30 mg/ml, respectively. For counterflow assay, 10 μl of proteoliposomes preloaded with 20 mM leucine and 20 mM NaN03 was pre-warmed at 25 °C for 5 min and diluted in 990 μl of 0.1 M KCl, 20 mM NaN03, 20 mM potassium phosphate, pH 7.5, and 50 μM [¹⁴C]leucine (2.25 μCi/ml). The final external leucine concentration was 0.25 mM. Aliquots (100 μl) were removed at intervals, diluted in 4 ml of 0.15 M LiCl, filtered through a HA-type Millipore filter with 0.45-μm pore size, and washed with 4 ml of 0.18 M LiCl. Approximately 85% of the vesicles were entrapped on the filter. (The value was measured by determining entrapped lipids.) After drying the filter, the radioactivity was counted by a gas flow counter, type LBC-451 (Aloka, Tokyo, Japan). As zero time point, 100 μl of 50 pM [¹⁴C]leucine was added to 4 ml of 0.15 M LiCl containing 1 μl of vesicles and the mixture was filtered immediately. For Na⁺-gradient-driven transport assay, the uptake was initiated by diluting 100-fold the proteoliposomes not containing amino acid and NaN03 into 0.1 M NaN03, 20 mM sodium phosphate, pH 7.5, and 2.5 μCi/ml of [¹⁴C]amino acid of 7 μM leucine, 7 μM isoleucine, or 9 μM valine.

All transport experiments were carried out at 25 °C with stirring. Analytical Procedures—Protein concentration was determined by the method of Schaffner and Weissmann (25) with bovine serum albumin as a standard. Lipid phosphorus was determined by the method of Bartlett (26) after the samples were digested with 70% perchloric acid at 170 °C for 2 h (27). The phospholipid concentration was calculated using 750 as an average molecular weight of lipid (28).

RESULTS
Transport Activities of Reconstituted Proteoliposomes—Fig. 1 shows the entrance counterflow of leucine, isoleucine, and valine by proteoliposomes preloaded with the corresponding amino acids under the condition where NaN03 was present outside and inside the vesicles. The proteoliposomes exhibited a transient uptake of leucine or isoleucine with a maximum at 3 to 5 min and both counterflow profiles were quite similar. On the other hand, the valine counterflow was smaller at the rate and lower at the maximal level than the other two counterflows. When NaN03 was replaced by KNO3, the counterflow activities were not observed. The measured counterflows of three amino acids were all dependent on the presence of Na⁺. Proteoliposomes containing no amino acids exhibited slow and monotonous uptakes, showing facilitated diffusion of the substrates (only the case of leucine was shown in Fig. 1). The counterflow of each amino acid by proteoliposomes prepared from PML1455 (LIV-11-) membranes was not observed.

The leucine transport in membrane vesicles is driven by imposition of a Na⁺-inward concentration gradient, resulting in a fast and transient accumulation of leucine (1, 3). It has been described that NaN03 is the most effective among sodium salts tested to facilitate the transient uptake of leucine (3). Thus, proteoliposomes preloaded with KCl were diluted 100-fold into a solution containing NaN03 to form a Na⁺-concentration gradient. Under the above condition, a marked and transient accumulation of leucine, isoleucine, or valine was observed (Fig. 2). The uptake of valine was slower and slightly lower at the maximal level than that of leucine and isoleucine. In the absence of the Na⁺-chemical gradient by replacing sodium salt by potassium salt or equilibrating the concentration of NaCl, the accumulation of each substrate was not observed (only the case of leucine was shown in Fig. 2). When proteoliposomes containing NaCl were diluted into a solution containing NaN03, a low and transient uptake was observed, showing a NO₃⁻-gradient-driven transport in the presence of Na⁺ ion. Proteoliposomes prepared from PML1463 (LIV-II) membranes did not exhibit the transient uptake of each substrate under the imposition of a Na⁺-concentration gradient by NaN03.

These results show that the LIV-II transport system having the activities of Na⁺-dependent counterflow and Na⁺-driven transport of leucine, isoleucine, and valine were reconstituted into liposomes from PML1455 membranes.

Site Location of Na⁺ Required for Counterflow—The counterflow activity of the reconstituted proteoliposomes shown in Fig. 1 was measured in the presence of equimolar Na⁺ at both sides of the vesicles. Whether or not the presence of Na⁺ was required at both sides for counterflow was examined by measuring the leucine counterflow (Fig. 3). Proteoliposomes containing NaN03 or KNO3 in addition to leucine were diluted into an external solution containing the same concentration of NaN03 or KNO3 with the internal salt concentration. When vesicles preloaded with either NaN03 or KNO3 were diluted in a solution containing NaN03, the leucine counterflow was observed, but when added to a solution with KNO3, no counterflow was measured.

These results show that the presence of Na⁺ outside the vesicles is necessary for the initiation of the counterflow, irrespective of the presence of intravesicular Na⁺.

Effect of Various Aminos Acids on Reconstituted Transport
were diluted in 0.1 M phosphate were added to 0.1 M and 7 M proteoliposomes containing 0.1 M leucine; 0, 7 ["C]leucine; membranes and diluted in 0.1 M 20 mM potassium phosphate were prepared from PML1455 membranes. Leucine counterflow was initiated by diluting the proteoliposomes in an external solution containing 50 mM leucine and 20 mM NaN03 or KNO3. The combinations of internal and external nitrate salts were: 0, Na+ inside and K+ outside; Na+ inside and Na+ outside; ["C]leucine. In all cases, the pH was 7.5.

Reconstitution of LIV-II Transport System

System—The transport system of LIV-II, unlike LIV-I, is specific for only branched-chain amino acids and is competitively inhibited by the presence of these amino acids (1, 3). The substrate specificity of the transport system reconstituted into the liposomes was examined by measuring the effect of various amino acids on leucine counterflow (Table I). Threonine and alanine as transported substrates of the LIV-I system were chosen to distinguish LIV-II from LIV-I (1). Glutamic acid and asparagine were selected, since these amino acids are substrates which are transported most effectively by membrane vesicles after the addition of electron donor (3).

The presence of leucine or isoleucine in the external medium inhibited the leucine counterflow almost completely. Valine partially inhibited the counterflow. On the other hand, the counterflow was not repressed by the other amino acids. The reconstituted transport system was shown to be specifically inhibited by the branched-chain amino acids that are substrates of the LIV-II transport system.

Uphill Transport of Various Amino Acids Induced by Counterflow of Branched-chain Amino Acids—Rosenberg and Wilbrandt (29) have described that in a membrane transport system containing a mobile carrier with affinities for two substrates, a concentration gradient with respect to one of the substrates is able to induce an "uphill" transport (against the concentration gradient) of the other. Based on this extended concept of counterflow, the substrate specificity of a carrier of LIV-II transport system was examined by measuring uphill transport of various amino acids induced by counterflow of leucine (Table II). The uphill transport of various amino acids was initiated by the addition of proteoliposomes preloaded with 20 mM leucine, isoleucine, or valine were diluted 100-fold in an external solution containing 50 mM ["C]leucine and 2.5 mM appropriate unlabeled amino acid.

TABLE I

| Amino acid added | Leucine counterflow* |
|------------------|----------------------|
|                  | n mole/mg protein  |
| None             | 36.2 (100)          |
| Leucine          | 0.8 (2)             |
| Isoleucine       | 0.8 (2)             |
| Valine           | 12.5 (35)           |
| Threonine        | 33.8 (93)           |
| Alanine          | 36.2 (100)          |
| Glutamic acid    | 38.6 (107)          |
| Asparagine       | 38.2 (106)          |

* Incorporated for 1 min and expressed as percentage of control in parentheses.

TABLE II

Uphill transport of various amino acids induced by counterflow of respective branched-chain amino acids

Proteoliposomes preloaded with 20 mM leucine, isoleucine, or valine were diluted 100-fold in an external solution containing 0.2 mM concentration of the appropriate ["C]-labeled amino acid (2.25 μCi/ml) to initiate uphill transport. The accumulation of various amino acids was measured at 1 min and expressed as a percentage of control.

| Transported amino acid | Leucine | Isoleucine* | Valine* |
|------------------------|---------|-------------|---------|
| Leucine                | 100     | 107         | 203     |
| Isoleucine             | 95      | 100         | 196     |
| Valine                 | 42      | 59          | 100     |
| Threonine              | 0       | -           | -       |
| Alanine                | 0       | -           | -       |
| Glutamic acid          | 0       | -           | -       |
| Asparagine             | 0       | -           | -       |

* Not determined in blanks.
with leucine to a solution containing radioactively labeled substrate to be tested. Of amino acids tested, leucine and isooleucine were transported at the same uptake rate and valine was transported at a lower rate. On the other hand, the uphill transport of other amino acids, threonine, alanine, glutamic acid, and asparagine, was not observed. The result shows that a carrier for leucine can transport only isooleucine and valine other than itself.

When the internal leucine was replaced by isooleucine or valine, each counterflow induced the uphill transport of branched-chain amino acids whose rate order was leucine \( \geq \) isooleucine \( \geq \) valine (Table II). In addition, any uphill transport was not observed in the absence of Na\(^+\).

The results obtained above indicate that the carrier(s) of the LIV-II transport system have affinity for only branched-chain amino acids and can translocate them in the presence of Na\(^+\).

**DISCUSSION**

The attempt of solubilization and reconstitution of the transport system for branched-chain amino acids (LIV-II system) from *P. aeruginosa* was successfully performed according to the modified method of Newman and Wilson who have succeeded in the reconstitution of the lactose transport system from *E. coli* (12). Octylglucoside was useful for solubilization of not only *E. coli* membranes but also *P. aeruginosa* membranes.

The rate sequence of leucine \( \geq \) isooleucine \( \geq \) valine was observed in all transport experiments of counterflow, Na\(^+\)-driven transport, and flow-induced uphill transport. The similar sequence was observed in the efficiency of inhibition of counterflow by these three amino acids. The same sequence of substrate affinity or competitive inhibition with the above has been obtained from kinetics of transport by whole cells and membrane vesicles (1, 3); the apparent \( K_m \) in membrane vesicles is 10 \( \mu M \) for leucine, 19 \( \mu M \) for isooleucine, and 130 \( \mu M \) for valine (1). Thus, the LIV-II transport system in the proteoliposomes exhibits the same transport properties as that in whole cells and membrane vesicles, although a quantitative analysis of kinetics of transport remains to be investigated.

It has been reported that NaNO\(_3\) is the most effective salt among sodium salts tested for Na\(^+\)-driven leucine uptake by membrane vesicles (3). However, the reason of effectiveness of nitrate salt has not been described yet. NO\(_3^−\), as well as SCN\(^−\), is a highly permeant anion across lipid bilayer membranes and is used to generate a membrane potential (30, 31). Therefore, it is considered that the effectiveness of NaNO\(_3\) is attributed to a generation of NO\(_3^−\)-diffusion potential (interior negative) and that the LIV-II transport system in membrane vesicles and proteoliposomes is stimulated by membrane potential in addition to Na\(^+\)-chemical potential.

The presence of Na\(^+\) in the external medium was a requisite for the occurrence of the counterflow, irrespective of the presence and absence of internal Na\(^+\). On the other hand, when proteoliposomes containing 20 \( \mu M \) Na\(^+\) were diluted 100-fold in a Na\(^+\)-free external solution, the counterflow did not occur. The external Na\(^+\) concentration of 0.2 \( \mu M \) seems to be too low to initiate the counterflow, since the LIV-II transport system in membrane vesicles requires 2.8 \( \mu M \) Na\(^+\) for half-maximal stimulation (1, 3). These findings suggest that Na\(^+\) may be required for binding of substrates to carriers as well as translocation.

The results of specific inhibition of leucine counterflow and flow-induced uphill transport strongly suggest that the LIV-II transport system should be composed of a single carrier system translocating three branched-chain amino acids. The same conclusion has been provided from genetic and kinetic research by Hoshino and his collaborators (1-3, 32). However, another possibility cannot be completely excluded that the LIV-II system may consist of three different carrier systems specific for leucine, isooleucine, or valine whose carrier has affinity for the other two amino acids. Further investigation by purification of the carrier(s) using the reconstitution system reported here will provide a confirmative conclusion on the question of whether the LIV-II transport system consists of a single species of carrier or not.

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