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Transport Systems for Branched-Chain Amino Acids in *Pseudomonas aeruginosa*

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The cells of *Pseudomonas aeruginosa* showed high activity for leucine transport in the absence of Na^+, giving a K_m value of 0.34 μM. In the presence of Na^+, however, two K_m values, 0.37 μM (LIV-I system) and 7.6 μM (LIV-II system), were obtained. The former system seemed to serve not only for the entry of leucine, isoleucine, and valine, but also for that of alanine and threonine, although less effectively. However, the LIV-II system served for the entry of branched-chain amino acids only. The LIV-II system alone was operative in membrane vesicles, for the transport of branched-chain amino acids in membrane vesicles required Na^+ and gave single K_m values for the respective amino acids. When cells were osmotically shocked, the activity of the LIV-I system decreased, whereas the LIV-II system remained unaffected. The shock fluid from *P. aeruginosa* cells showed leucine-binding activity with a dissociation constant of 0.25 μM. The specificity of the activity was very similar to that of the LIV-I system. These results suggest that a leucine-binding protein(s) in the periplasmic space may be required for the transport process via the LIV-I system of *P. aeruginosa*.

Transport of sugars and amino acids in bacteria is complex, often involving several systems with different affinities for a particular substrate. Such multiplicity has been demonstrated in the transport of branched-chain amino acids, L-leucine, L-isoleucine, and L-valine, in *Escherichia coli* K-12 (5, 12, 21, 25), or in a marine pseudomonad (10, 20). At least three systems for branched-chain amino acids have been shown in both organisms by kinetic or genetic analyses.

As reported in a previous paper (13), membrane vesicles of *Pseudomonas aeruginosa* have an Na^+-dependent transport system common to branched-chain amino acids. The paper also showed that no other system was found in the vesicle preparation for the transport of these amino acids. Kay and Gronlund (14) reported that branched-chain amino acids were taken up via a common process by the whole cells of *P. aeruginosa*. They, however, did not report the properties of the system such as kinetic behavior or cation requirement. Therefore, it is uncertain whether the system observed in our vesicle preparation corresponds to that reported by Kay and Gronlund. Thus, the nature of the transport systems for branched-chain amino acids in *P. aeruginosa* has remained undescribed except for that of the vesicle preparation. No evidence has been provided of *P. aeruginosa* demonstrating multiplicity of the transport systems for branched-chain amino acids.

In this report, we describe the evidence indicating that at least two systems are operating in *P. aeruginosa* for the transport of branched-chain amino acids. We also suggest that a leucine-binding protein(s) found in a shock fluid may be responsible for one of the transport systems of these amino acids.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *P. aeruginosa* PML14 (formerly P14), a prototrophic strain, was grown aerobically at 37°C in a synthetic medium, pH 6.8, which contained 4.4 g of K_2HPO_4, 3.4 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 20 ml of a salt mixture, and 5 g of D-glucose as an energy source per liter. The composition of the salt mixture was described elsewhere (13). In the case of the preparation of membrane vesicles, cells were grown in another synthetic medium, glutamate medium, which was previously described (13).

Preparation of the cell suspension. Cells were harvested in middle exponential phase by centrifugation, washed twice, and suspended to a concentration of about 5×10^8 cells per ml in 10 mM Tris-hydrochloride, pH 7.4, containing 1 mM KCl and 1 mM $MgCl_2$ (TMK buffer). The cell suspension was kept at room temperature (about 22°C) before assay.

Osmotic shock treatment of whole cells. Osmotically shocked cells were prepared by the method described by Cheng et al. (9) with modifications. For this purpose, 10 ml of cell culture was subjected to the treatment. Cells were harvested in middle exponential phase, washed once by TMK buffer, suspended in 10 ml of 10 mM Tris-hydrochloride, pH 8.4, containing 0.2 M $MgCl_2$ (extraction buffer), and incubated for 20

min at room temperature. Cells were collected by centrifugation, rapidly dispersed into 10 ml of distilled water, and incubated for 20 min at room temperature. Cells were recollected and suspended in TMK buffer. The viability of the cells was unaffected by this treatment.

For obtaining a shock fluid, a liter-scale culture was used, and a temperature shift from 30 to 4°C was introduced into the stage of $MgCl_2$ extraction, which resulted in higher yields of binding activity. Harvested cells were suspended in extraction buffer at a concentration of 1 g (wet weight) of cells per 20 ml. After incubating for 5 min at 30°C, the cell suspension was rapidly chilled in an ice bath and incubated for 15 min. The temperature shift was repeated once more. Cells were collected by centrifugation, rapidly dispersed into distilled water at the same concentration, and incubated for 20 min at room temperature. The $MgCl_2$ extract and the subsequent water extract were combined and centrifuged twice at 30,000 × *g* for 30 min at 4°C to remove whole cells. The supernatant was concentrated by ultrafiltration (Diaflo PM10 filter, Amicon Corp., Lexington, Mass.). The concentrated fluid was dialyzed overnight at 4°C against TMK buffer and stored at −70°C until the binding assay.

Transport assays. The initial rate of leucine transport by whole cells was determined by the following method. A portion (usually 0.2 ml) of the cell suspension was preincubated for 2 min at 37°C with 50 mM D-glucose and with 40 mM KCl or NaCl. The reactions were initiated by the addition of [^{14}C]leucine (46 μCi/μmol) at a final concentration of 20 μM. After 10 s, reaction mixtures were diluted with 3 ml of 10 mM Tris-hydrochloride, pH 7.4, containing 125 mM KCl, filtered instantaneously through membrane filters (type HAWP, Millipore Corp., Bedford, Mass.), and washed once with another 3 ml of the buffer. The filters were removed and dried, and their radioactivities were measured by a gas flow counter, type LBC-451 (Aloka, Tokyo). In some experiments, [^{14}C]leucine of higher specific radioactivity (342 μCi/μmol) was used to determine the rates of the transport at lower concentrations. Transport assays for isoleucine and valine were performed in the same way, using [^{14}C]-isoleucine (79 μCi/μmol) or [^{14}C]valine (35 μCi/μmol).

The time course of leucine uptake (see Fig. 1) was measured as follows. One milliliter of the cell suspension in a test tube was preincubated with stirring for 2 min at 37°C in the presence of D-glucose and of KCl or NaCl, and the reactions were started by the addition of [^{14}C]leucine at a final concentration of 20 μM. At intervals, 70-μl portions of the mixture were withdrawn, diluted, and washed as mentioned above. To determine the incorporation into a trichloroacetic acid-insoluble fraction, 70-μl portions of the reaction mixture were diluted with 3 ml of cold 10% trichloroacetic acid, left standing for 10 min, filtered, and washed once with 3 ml of 5% trichloroacetic acid.

Transport activity by membrane vesicles was assayed at 25°C as previously described (13).

Binding assays. Binding activities were measured by the equilibrium dialysis method using dialysis bags (3). A dialysis bag filled with 0.3 ml of sample was dialyzed for 20 h at 4°C against 10 ml of TMK buffer containing 0.02% NaN_3 and 1 μM ^{14}C-labeled amino acid. After the dialysis, 40-μl samples were withdrawn from inside or outside of the bag. Their radioactivities were counted with 0.5 ml of NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and 5 ml of toluene-based scintillator by a liquid scintillation counter, Beckmann LS-250.

Other methods. Membrane vesicles were prepared as previously described (13). Protein content was determined by the method of Lowry et al. (17) with bovine serum albumin as a standard.

Chemicals. ^{14}C-labeled L-amino acids and ^{3}H-labeled L-leucine were all purchased from the Radiochemical Centre, Amersham, England, via Japan Radioisotope Association. All other compounds used were of reagent grade.

RESULTS

Effect of Na^+ on leucine uptake by cells and its cellular distribution. As reported previously (13), leucine transport in the vesicle preparation of *P. aeruginosa* required Na^+ for its activity. If this transport system is the sole pathway for the entry of leucine into whole cells, leucine uptake by cells should also be dependent on Na^+. The time course of leucine uptake and its distribution within the cells in the presence or absence of NaCl is shown in Fig. 1. The difference between the total uptake and the trichloroacetic acid-insoluble fraction was regarded as free leucine pool in these experiments. This approximation was justified by the following experiment. A cell suspension was incubated for 6 min in the presence of [^{14}C]leucine. After the cells were trapped on a membrane filter, the intracellular pool was extracted with cold 10% trichloroacetic acid. From the acid-soluble fraction, acid was removed with cold ether and amino acids were separated by paper chromatography with a solvent system of *n*-butanol–acetic acid–water (4:2:1, vol/vol/vol). On the paper chromatogram, 90% of total radioactivity was recovered at the position of [^{3}H]leucine added as an internal standard. Therefore, leucine incorporated into the trichloroacetic acid-soluble fraction remained unchanged under the conditions for uptake assay.

Whole cells, unlike the vesicle preparation, showed very high activity for leucine uptake even in the absence of NaCl (Fig. 1), suggesting that some other system(s) which is independent of Na^+ should operate in the whole cells of *P. aeruginosa*. However, the addition of NaCl caused considerable stimulation of the initial rate of leucine uptake. Figure 1 also shows that the addition of NaCl caused a considerable increase in the steady-state level of leucine uptake, although the pool formation by the cell saturated very rapidly and reached a steady-state level within only 2 min whether Na^+ was present or absent. On the other hand, leucine incorpo-

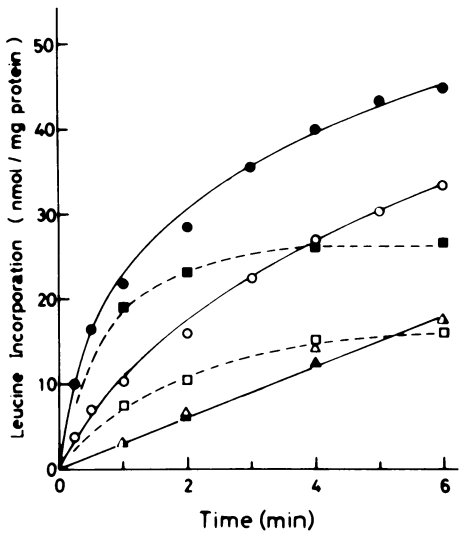

FIG. 1. *Time course of [^{14}C]leucine distribution in whole cells. [^{14}C]leucine was added at time 0 to the cell suspension with 40 mM KCl (open symbols) or with 40 mM NaCl (solid symbols). Symbols: circle, incorporation into whole cells; triangle, incorporation into trichloroacetic acid-insoluble fraction; square, incorporation into free leucine pool.*

ration into the trichloroacetic acid-insoluble fraction was not affected at all by NaCl. Leucine uptake by whole cells was inhibited completely by the addition of 10 mM KN_3 whether NaCl was present or absent (data not shown), indicating that leucine uptake shown in the figure is an energy-dependent process.

The effect of some monovalent cations on the initial rate of leucine uptake by whole cells was studied. The results are shown in Fig. 2. Addition of NaCl up to a concentration of 40 mM increased the rate of leucine uptake considerably. The stimulatory effect was of a saturable nature. Concentration of 10 mM NaCl was enough to cause maximal stimulation. On the other hand, addition of KCl was not effective on leucine uptake, indicating that the stimulatory effect is specific for Na^+. However, the partial substitution of Li^+ for Na^+ was also observed. These effects of monovalent cations on leucine uptake by cells are very similar to those on leucine uptake by the vesicle preparation (13).

Biphasic kinetics of leucine transport in the presence of Na^+. As described above, whole cells of *P. aeruginosa* showed considerable activity for leucine uptake even without addition of Na^+, and the addition of Na^+ resulted in the stimulation of leucine uptake. To clarify the nature of the transport system(s) for leucine in *P. aeruginosa*, leucine uptake by the whole cells was kinetically investigated. The initial rates of uptake were measured for the concentration range of 0.075 to 30 μM leucine with or without Na^+. Results are presented in a double reciprocal fashion (Fig. 3). Without addition of Na^+, a transport system with very high affinity was observed. The plot gave a straight line with a K_m of 0.34 μM and a V_{max} value of 30 nmol/mg of protein per min (Fig. 3A). Such a system with high affinity was not observed in the vesicle preparation with or without Na^+ (13; see Table 2). On the other hand, in the presence of Na^+, a biphasic curve was revealed for the kinetics of leucine transport by the cells (Fig. 3B). The data were analyzed by the method of Neal (18), giving two K_m and two V_{max} values. For the purpose of identification, we will refer to the low K_m (0.37 μM) component of leucine uptake as the LIV-I transport system and the high K_m (7.6 μM) component as the LIV-II transport system. The V_{max} values for LIV-I and for LIV-II were 25 and 45 nmol/mg of protein per min, respectively. The K_m value and the V_{max} value of the LIV-I system obtained with Na^+ were the same as those of the system observed without Na^+, indicating that the LIV-I system can operate whether Na^+ is present or absent. On the other hand, the LIV-II system was observed only when Na^+ was present, suggesting that this system may be responsible for leucine transport by the vesicle preparation.

Substrate specificity of LIV-I and LIV-II. The effects of various amino acids on leucine uptake by whole cells were examined to make the specificity of LIV-I and LIV-II systems clear. [^{14}C]leucine uptake with or without Na^+ was severely inhibited by the addition of a 20-fold

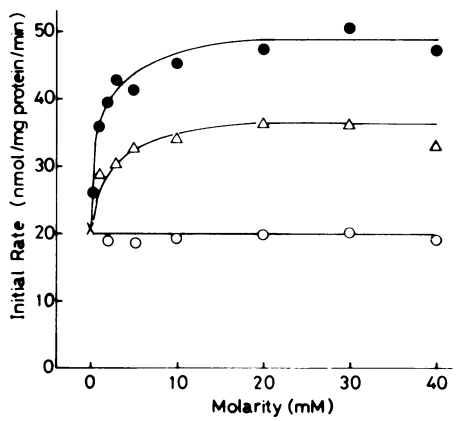

FIG. 2. *Effect of monovalent cations on leucine uptake. The cell suspension was preincubated for 2 min in the presence of KCl (○) or NaCl (●) or LiCl (△) at the final concentration indicated. [^{14}C]leucine was then added to the cell suspension to initiate reactions.*

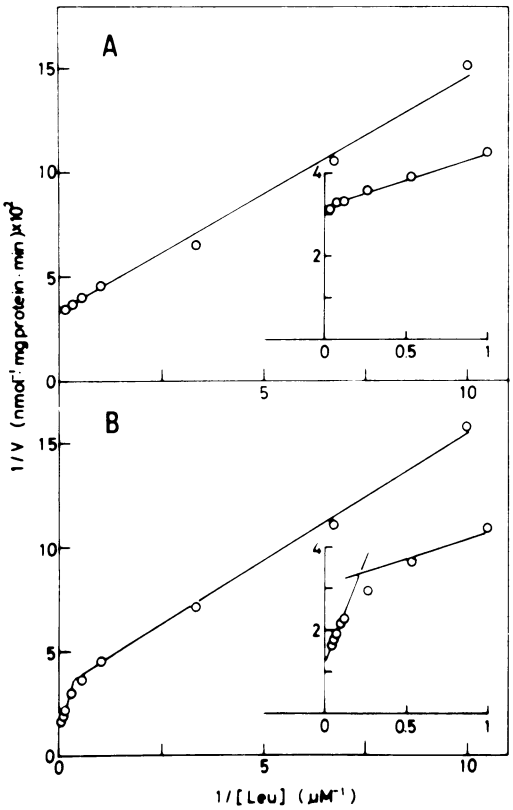

FIG. 3. *Double-reciprocal plot of initial rate of leucine uptake by the cells in the absence (A) or presence (B) of 40 mM NaCl.*

amount of cold leucine or isoleucine or valine, which are structurally related amino acids (Table 1). However, less effective inhibition of leucine uptake by valine was observed in the presence of Na^+. Alanine and threonine also showed complete inhibition of leucine uptake in the absence of Na^+, but higher concentrations of these amino acids than those of branched-chain amino acids were required for the inhibition. On the other hand, leucine uptake in the presence of Na^+ was not completely inhibited by alanine or by threonine even at 1 mM. The other amino acids caused no or little inhibition of leucine uptake with or without Na^+ even at the concentration of 1 mM.

To investigate the nature of inhibition of leucine uptake by the above amino acids more precisely, the following experiments were done. The effects of these amino acids on the initial rate of leucine uptake in the presence or absence of Na^+ was examined with increasing concentrations of these amino acids. Leucine uptake was wholly sensitive to isoleucine or valine whether Na^+ was present or absent (Fig. 4A). These results suggest that both the LIV-I and the LIV-II system may serve for the transport of these amino acids. However, a slightly higher concentration of valine was needed for the inhibition of leucine uptake in the presence of Na^+, suggesting that the LIV-II system may serve for valine transport less effectively.

On the other hand, alanine and threonine showed different patterns of inhibition (Fig. 4B). In the absence of Na^+, alanine and threonine inhibited leucine uptake completely, although about five times as much was needed to give the same extent of inhibition as isoleucine or valine. The results suggest the idea that the LIV-I system may also serve for the transport of alanine and threonine with lower affinity. In the presence of Na^+, however, a saturable component of leucine uptake remained, even after the inhibition by alanine or threonine reached a maximal level. The level remaining after the maximal inhibition by alanine was the same as that remaining after the maximal inhibition by threonine. As the LIV-I system is wholly sensitive to alanine and threonine, the results indicate that the LIV-II system is entirely insensitive to these amino acids. In other words, the LIV-II system does not serve for the transport of alanine and threonine.

The above observations enabled us to measure the activity of the LIV-II system alone using whole cells. The results by kinetic analyses in the presence of Na^+ and an excess amount of alanine are shown in Fig. 5 and 6. The initial

TABLE 1. *Effect of various amino acids on leucine transport by whole cells*

Amino acid added[a] (μM)	Initial rate[b]	
	−NaCl	+NaCl
None	100	100
Leucine (100)	0	0
Isoleucine (100)	2	0
Valine (100)	0	20
Alanine (100)	25	44
Alanine (1,000)	0	32
Threonine (100)	34	58
Threonine (1,000)	0	33
Glycine (1,000)	98	101
Serine (1,000)	50	68
Methionine (1,000)	77	77
Aspartic acid (1,000)	96	89
Asparagine (1,000)	98	94
Glutamic acid (1,000)	104	78
Glutamine (1,000)	96	89
Proline (1,000)	101	104

[a] Each cold amino acid as indicated was added concomitantly with [^{14}C]leucine at the final concentration given in parentheses.

[b] Assayed at the concentration of 5 μM [^{14}C]leucine and expressed as percentage of control.

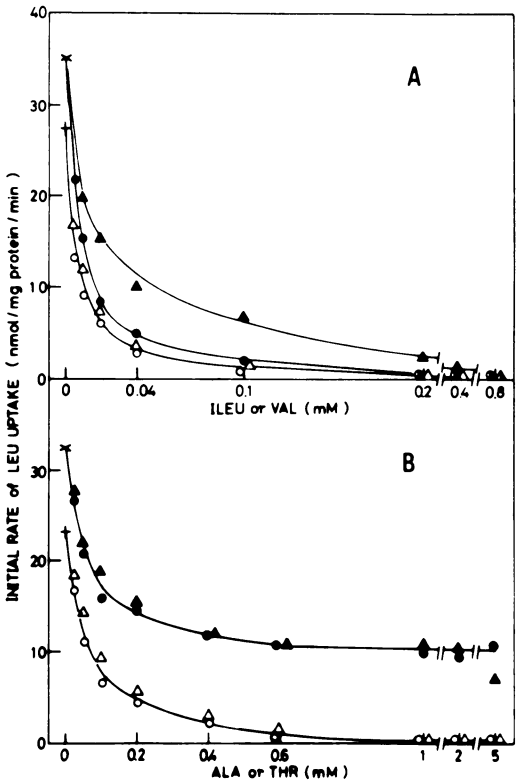

FIG. 4. *(A) Effects of isoleucine (circle) and valine (triangle) on leucine uptake. (B) Effects of alanine (circle) and threonine (triangle) on leucine uptake. The cells were preincubated in the presence (solid symbols) or absence (open symbols) of Na^+. Each cold amino acid was added concomitantly with [^{14}C]leucine at the final concentrations indicated. The final concentration of [^{14}C]leucine was 5 μM.*

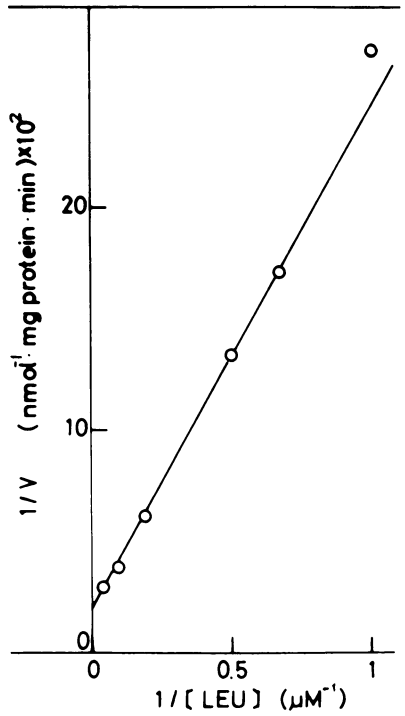

FIG. 5. *Effect of excess amount of alanine on leucine uptake in the presence of NaCl. Alanine was added concomitantly with [^{14}C]leucine to give a final concentration of 10 mM. Initial rates of leucine uptake were determined as a function of leucine concentration with NaCl at 40 mM. Results are presented in a double reciprocal fashion.*

rates of leucine uptake in the presence of 10 mM alanine were determined as a function of leucine concentration (Fig. 5). The double reciprocal plot gave a single K_m of 11 μM with a V_{max} value of 50 nmol/mg of protein per min, characteristic of the LIV-II system. Figure 6 shows the saturable nature of a stimulatory effect of Na^+ on leucine uptake, giving an apparent K_m value of 1.7 mM for Na^+ with respect to leucine uptake. These characteristics of leucine uptake of the cells under the above condition were the same as those in the vesicle preparation (13; see Table 2).

Finally, the transport of isoleucine and valine was directly assayed using ^{14}C-labeled compounds. The results are summarized in Table 2. Both amino acids showed behavior similar to that of leucine. In the absence of Na^+, a system with low K_m only was observed for the transport of respective amino acids. On the other hand, biphasic curves were revealed for the transport

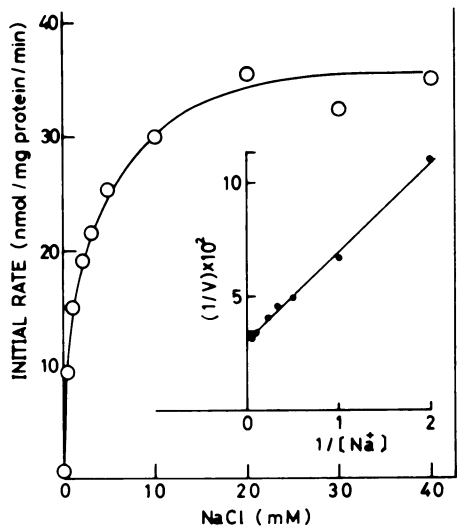

FIG. 6. *Stimulatory effect of Na^+ on leucine uptake in the presence of excess amount of alanine. Initial rates of leucine uptake were determined as a function of NaCl concentration with a constant concentration of [^{14}C]leucine (20 μM). (Inset) Double reciprocal plot.*

TABLE 2. *Apparent K_m values for the transport of branched-chain amino acids under the various conditions*

Prepn	Conditions[a]	K_m (μM) for the transport of:			K_m[b] (mM) for Na^+
		Leu	Ile	Val	
Cells	+KCl	0.34	0.19	0.18	
Cells	+NaCl	0.37 7.6	0.25 14.9	0.23 59	
Cells	+NaCl and alanine	11	13.3	105	1.7
Vesicles	+NaCl	10	19	130	2.8

[a] KCl or NaCl was added to give the final concentration of 40 mM. Alanine was added concomitantly with ^{14}C-labeled amino acid to give the final concentration of 10 mM.

[b] The apparent K_m value for Na^+ was determined with respect to leucine transport.

of isoleucine and valine in the presence of Na^+, corresponding to LIV-I (low K_m) and LIV-II (high K_m). Furthermore, in the presence of Na^+ and an excess amount of alanine, only a system of the higher K_m was observed for each amino acid. These K_m values were very similar to those obtained with membrane vesicles, respectively. K_m values for Na^+ with respect to leucine transport were also similar in cells and in vesicles. The K_m value of LIV-II for valine was considerably higher than that for leucine or isoleucine. This fact, as well as the results of competition experiment (Fig. 4), indicates that the transport of valine by LIV-II is less effective than that of leucine or isoleucine.

Effect of osmotic shock on leucine uptake by the cells. The activity of the LIV-I system was not found in membrane vesicles (13; Table 2). This fact suggests that some periplasmic factor(s) such as a binding protein may be requisite for the system. Therefore, the effect of osmotic shock treatment on leucine uptake by the cells was studied. The method of Cheng et al. (9) has been used for extracting periplasmic enzymes (8) and binding proteins (22, 23) from the cells of *P. aeruginosa* without damaging the cell viability. Thus, the cells were subjected to osmotic shock by the $MgCl_2$ method. The activity of the LIV-I system was lowered considerably after the treatment (Table 3). On the other hand, the activity of the LIV-II system remained almost unaffected. In other words, LIV-I was shock sensitive and LIV-II was shock resistant. The LIV-I system might involve some periplasmic factors.

Leucine-binding activity found in the shock fluid. To examine the role of periplasmic components, binding activities for various amino acids were assayed with the shock fluid of *P. aeruginosa*. A few amino acids were bound (data not shown). Among these amino acids, the binding activity for leucine was the highest, giving a value of about 0.3 nmol/mg of protein. The dissociation constant (K_D) for leucine was estimated to be 0.25 μM, which was very similar to the K_m value of the LIV-I system. The substrate specificity was also investigated by inhibition assays with various amino acids (Table 4). Leucine-binding activity of the shock fluid was nullified completely by the addition of a 100-fold amount of cold leucine, isoleucine, or valine. The addition of alanine and threonine also caused inhibition of the activity to a lesser extent. Other amino acids were ineffective for the activity. These results show that one or more binding proteins exist in the shock fluid of *P. aeruginosa*, which have similar properties to the LIV-I trans-

TABLE 3. *Effect of osmotic shock on LIV-I and LIV-II*

Expt	Prepn	Relative rate[a] (%) of	
		LIV-I[b]	LIV-II[c]
1	Control cells	100	100
	Shocked cells	32	91
2	Control cells	100	100
	Shocked cells	34	80

[a] Expressed as percentage of control. Control values of the initial rates of LIV-I and LIV-II were 21.2 and 20.1 nmol/mg of protein per min for experiment 1, or 22.6 and 22.3 nmol/mg of protein per min for experiment 2, respectively.

[b] Assayed in the absence of Na^+.

[c] Assayed in the presence of 30 mM Na^+ and 10 mM alanine.

TABLE 4. *Effect of various amino acids on leucine-binding activity of the shock fluid*

Amino acid[a] added	Leucine-binding activity[b] (% of control)
None	100
Leucine	5
Isoleucine	4
Valine	4
Glycine	99
Alanine	13
Serine	93
Threonine	15
Methionine	93
Aspartic acid	99
Glutamic acid	130
Asparagine	129
Glutamine	128
Lysine	96
Phenylalanine	107
Proline	102

[a] Added at the final concentration of 100 μM.

[b] Assays were performed with 1 μM [^{14}C]leucine in the presence of the indicated nonlabeled amino acid. Each dialysis bag contained 300 μg of protein. Binding activity in the absence of any inhibitor was 0.34 nmol/mg of protein.

port system in affinity for leucine and in substrate specificity. In combination with the sensitivity of the LIV-I transport system to osmotic shock, it is suggested that the binding protein(s) found in the shock fluid is responsible for the recognition of substrates in the transport process via the LIV-I transport system in *P. aeruginosa*.

DISCUSSION

The present study shows that at least two transport systems operate in the whole cells of *P. aeruginosa* for branched-chain amino acids. Two transport systems showed distinct properties from each other in many respects. The LIV-I system with higher affinity was operative even in the absence of Na^+ and may be specific for alanine and threonine in addition to branched-chain amino acids. The LIV-II system with lower affinity, however, was operative only when Na^+ was present, and was specific only for branched-chain amino acids. Transport activities for branched-chain amino acids by the two systems could be assayed separately with the whole cell suspension: LIV-I without Na^+ and LIV-II with Na^+ and an excess amount of alanine. The features of the LIV-II system were very similar to those of the vesicle preparation (13). Substrate specificity and the apparent K_m values for branched-chain amino acids and for Na^+ with respect to leucine transport were the same as those of the vesicle system (Table 2). Therefore, it seems that the LIV-II system alone is retained in the membrane vesicles, whereas the LIV-I system is lost during the preparation of membrane vesicles.

Biphasic kinetics for the transport of branched-chain amino acids has also been found in *E. coli* K-12 (6, 12, 21, 25) and in a marine pseudomonad (10, 20). Rahmanian et al. (21) kinetically distinguished two systems for the transport of branched-chain amino acids in *E. coli*, designating them the LIV-I system with a high affinity (K_m = 0.2 μM) and the LIV-II system with a low affinity (K_m = 2 μM). Both the LIV-I and the LIV-II systems of *E. coli* have some properties in common with those of *P. aeruginosa*. Rahmanian et al. (21) showed that the LIV-I system of *E. coli* served for the transport of alanine and threonine in addition to branched-chain amino acids. Transport activity specific for the LIV-I system was lost in membrane vesicles of *E. coli* (25). Furthermore, sensitivity to osmotic shock was also observed in the LIV-I system of *E. coli* (4, 21, 25). These properties of the LIV-I system of *E. coli* are very similar to those of the LIV-I system of *P. aeruginosa* as described in this study. The LIV-II system of *E. coli*, on the other hand, serves for the transport of branched-chain amino acids alone (21). The system was insensitive to osmotic shock (21, 25). Transport activity found in membrane vesicles of *E. coli* was also attributed to the LIV-II system (5, 25, 26), indicating that the LIV-II system of *E. coli* is basically analogous to that of *P. aeruginosa*. However, a striking difference between the LIV-II of *E. coli* and that of *P. aeruginosa* is that the system of *P. aeruginosa* operates only when Na^+ is present. The difference would be due to the nature of energy coupling of the two systems. Yamato and Anraku (26) have shown that isoleucine transport by cytoplasmic membrane vesicles of *E. coli* is driven by proton motive force, implying that solute accumulation via the LIV-II system in *E. coli* is mediated by co-transport of H^+. On the other hand, leucine transport by membrane vesicles of *P. aeruginosa* is not directly driven by proton motive force but by sodium motive force which is generated by proton motive force (13). Therefore, leucine accumulation via the LIV-II system in *P. aeruginosa* is probably mediated by co-transport of Na^+. In contrast to the organisms mentioned above, *Salmonella typhimurium* has quite different features of the transport of branched-chain amino acids. Kiritani and Ohnishi (15) reported that a single system for branched-chain amino acids appeared to be present in the wild-type strain of *S. typhimurium*, whereas two transport systems were shown by kinetic analysis in the transport mutants of the organism. In *E. coli* and *S. typhimurium*, some LIV transport systems are known to be repressible by leucine added in the growth medium (5, 15, 21). In *P. aeruginosa*, however, only slight repression by leucine was observed for the transport activity (data not shown).

In bacterial transport systems, periplasmic binding proteins are sometimes found to associate with the transport of amino acids, sugars, and ions (5, 19). Transport by such systems is sensitive to osmotic shock, and the mechanism of energy coupling appears to differ from the shock-resistant transport systems (7, 16, 25). In transport systems for branched-chain amino acids in *E. coli*, the LIV-I system is associated with a periplasmic binding protein (LIVT-binding protein) (4, 21, 25). Another system which requires a binding protein specific for leucine alone (Ls system) was also found in *E. coli* K-12, although it was a minor route and was kinetically indistinguishable from the LIV-I system (11, 21). The correlation of these binding proteins to respective transport systems has recently been shown by genetic analysis (1, 2). Stinson et al. (22, 23) have recently shown such a correlation in a transport system for glucose in *P. aeruginosa*. They found co-regulation of the synthesis of glucose-binding protein and the glu-

cose transport system (22). They also found that a glucose-binding protein-deficient mutant was defective in the corresponding transport system and that a revertant to glucose-binding protein-positive phenotype simultaneously recovered normal levels of the transport system (23). Such a correlation has also been suggested in glycerol transport in *P. aeruginosa* (24). However, no system has been described in the amino acid transport of *P. aeruginosa* for which a certain binding protein is requisite.

The present study suggests that a binding protein(s) may be responsible for the LIV-I system of *P. aeruginosa*. Osmotic shock treatment of cells of *P. aeruginosa* caused a preferential decrease in the activity of the LIV-I system (Table 3). Binding activity for leucine was observed in the osmotic shock fluid (Table 4). The K_D value for leucine binding approximated the K_m value for leucine transport via the LIV-I system. Furthermore, the inhibition patterns by various amino acids were very similar in two assays, leucine binding and LIV-I transport. Our investigation now in progress suggests that only one binding protein for branched-chain amino acids exists in the shock fluid, specificity of which is consistent with the LIV-I transport system. Therefore, it is probable that only one binding protein is responsible in *P. aeruginosa* for the high affinity system. However, further investigation is necessary for clarifying the role of the binding protein in the LIV-I system of *P. aeruginosa*.

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