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Purification and Properties of a Binding Protein for Branched-Chain Amino Acids in *Pseudomonas aeruginosa*

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A binding protein for branched-chain amino acids was purified to a homogeneous state from shock fluid of *Pseudomonas aeruginosa* PML14. It was a monomeric protein with an apparent molecular weight of 4.3×10^4 or 4.0×10^4 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or gel filtration, respectively. The isoelectric point was determined to be pH 4.1 by electrofocusing. Amino acid analysis of the protein showed that aspartic acid, glutamic acid, glycine, and alanine were major components and that the protein contained only one residue each of tryptophan and cysteine per molecule. The binding protein contained no sugar. The binding activity of the protein was specific for the branched-chain amino acids. The protein also bound alanine and threonine with lower affinity. The dissociation constants of this protein for leucine, isoleucine, and valine were found to be 0.4, 0.3, and 0.5 μM, respectively. Mutants defective in the production of the binding protein were identified among the mutants deficient in a transport system for branched-chain amino acids (LIV-I). The revertants from these mutants to LIV-I-positive phenotype simultaneously recovered normal levels of the binding protein. These findings suggest strongly the association of the binding protein with the LIV-I transport system.

In some bacterial transport systems, periplasmic binding proteins are found to associate with the transport of amino acids, sugars, and ions (10, 24). Transport by such systems is sensitive to osmotic shock (9) and is not operative in membrane vesicles (31). The mechanism of energy coupling of such systems appears to differ from that in shock-resistant transport systems (11, 18, 31). However, such an association of binding proteins with transport systems in *Pseudomonas aeruginosa* has not been well established, although Stinson et al. (27, 28) have recently reported that the glucose-binding protein (GBP) is associated with glucose transport by the organism.

P. aeruginosa accumulates the branched-chain amino acids by at least two systems with different substrate specificities, LIV-I and LIV-II (16, 17). The LIV-I system, which has a high affinity, is operative in the absence of Na^+ and may be specific for alanine and threonine in addition to branched-chain amino acids. The LIV-II system, which has a lower affinity, is operative only when Na^+ is present and is specific only for branched-chain amino acids. Transport activities for branched-chain amino acids by the two systems can be assayed separately with whole-cell suspensions. Our previous study (16) also suggested that a binding protein(s) might be required for the LIV-I transport system. Osmotic shock treatment of cells caused a preferential decrease in the activity of the LIV-I system, which was lost in membrane vesicles (17). Leucine-binding activity was found in the shock fluid of *P. aeruginosa*. The binding activity of the shock fluid was similar to the activity of the LIV-I transport system in affinity for leucine and in substrate specificity (16). However, no conclusive evidence has been obtained for the association of such a binding protein with the LIV-I transport system of *P. aeruginosa*.

In this report, we describe the purification and properties of the leucine-binding protein from *P. aeruginosa* cells. We also describe the isolation and characterization of mutants defective in the LIV-I system with normal LIV-II activity and their revertants to LIV-I-positive phenotype. The results convince us of the association of the binding protein with the LIV-I transport system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were PML14 (prototroph) and its derivatives. Bacteria were grown aerobically at 37°C in a synthetic medium (D-medium) described previously (16) with 0.5% D-glucose as an energy source unless otherwise indicated.

Mutagenesis. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was done according to the procedure of Fargie and Holloway (13) with modifications. Cells were harvested at the midexponential phase, suspended to 2×10^8 cells per ml in 50 mM

potassium phosphate buffer, pH 7, containing 20 μg of NTG per ml, and incubated at 37°C for 60 min. Cell survival under the above conditions was approximately 5%. After mutagenesis, cells were centrifuged, suspended in nutrient broth and cultured overnight at 30°C. The nutrient broth culture was diluted in a 100-fold volume of D-medium, cultured at 30°C for several hours, and then plated.

Elimination of Na^+ from agar. Agar powder from a commercial source (Iwai Kagaku Co., Tokyo) was found to contain about 0.2% Na^+ as determined by atomic absorption spectroscopy. If agar plates (usually containing 1.5% agar powder) are prepared with this agar powder, about 1.3 mM Na^+ contaminates the plates, which is enough for the LIV-II system of *P. aeruginosa* to operate (16). For preparing Na^+-free agar plates, Na^+ was eliminated from agar powder as follows. Agar powder was washed 10 times on a glass filter with 1 liter of 1 M KCl per 100 g of powder, followed by washing with distilled water. The washed powder was dried in a warm air stream. The agar powder thus washed contained only 0.004% Na^+, giving a concentration of only 0.03 mM Na^+ in the agar plates. Agar plates used in this study were all prepared with this washed powder. When needed, NaCl was added to plates to make the final concentration 20 mM.

Extraction of leucine-binding protein from cells. Extraction of periplasmic proteins was performed basically by the same method as described previously (16). However, the osmotic shock stage after the $MgCl_2$ extraction was omitted, for almost all the binding activity was recovered in the $MgCl_2$ extract. Cells harvested at the midexponential phase were suspended in 50 mM Tris-hydrochloride, pH 7.3, containing 0.2 M $MgCl_2$ at a concentration of 0.1 to 0.2 g (wet weight) of cells per ml. After incubating for 10 min at 30°C, the cell suspension was rapidly chilled in an ice bath and was left standing for 15 min. The temperature shift was repeated once more. The suspension was centrifuged twice at 8,000 × *g* for 10 min at 4°C to remove cells. The supernatant was recentrifuged at 20,000 × *g* for 10 min at 4°C. The resulting fluid was concentrated to about 4 mg of protein per ml by ultrafiltration (Diaflo PM10 filter, Amicon Corp.). This concentrated fluid was stored at −70°C until used.

Binding assays. Binding activities were measured by the equilibrium dialysis method, using dialysis bags (7, 16). A dialysis bag filled with 0.3 ml of a sample was dialyzed for 20 h at 4°C against 10 mM Tris-hydrochloride, pH 7.3, containing 0.02% NaN_3 and 1 μM ^{14}C-labeled amino acid unless otherwise indicated. No chemical alteration of [^{14}C]leucine during dialysis under these conditions was found by paper chromatography with [^3H]leucine as an internal standard.

Transport assays. Transport activities of the LIV-I and the LIV-II systems were assayed separately at 37°C as described previously (16).

Polyacrylamide gel electrophoresis. Urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the method of Uemura and Mizushima (30). Samples solubilized in a solution of 1% SDS, 1% β-mercaptoethanol, 0.0025% bromophenol blue, and 8 M urea were layered onto 8% polyacrylamide gel columns (0.5 by 7.5 cm) containing 0.5% SDS and 8 M urea and developed at 4 mA per gel. The gels were fixed with 20% (wt/wt) sulfosalicylic acid and stained with Coomassie brilliant blue R250 according to the method of Maizel (21).

SDS slab-gel electrophoresis using a discontinuous buffer system was conducted according to the method of Laemmli (19) with modifications by Mizuno and Kageyama (22). Gels were fixed and stained with 10% acetic acid, 25% isopropanol, and 0.1% Coomassie brilliant blue R250 at 37°C for 1 h. Cytochrome *c* (12,400), β-lactalbumin (18,400), α-chymotrypsinogen A (25,000), hen egg albumin (45,000), and bovine serum albumin (68,000) were used as molecular weight standards.

Polyacrylamide gel isoelectric focusing was performed by the procedure of Wrigley (32) with modifications. A purified sample (5 μg) suspended in a solution of 30% glycerol and 2% ampholite was layered on a 7.5% polyacrylamide gel column (0.5 by 7 cm) containing 2% ampholite of desired pH ranges. A solution of 15% glycerol and 2% ampholite was overlaid onto the sample solution. Ampholite used was a mixture of ampholine with pH ranges 3.5 to 5, 5 to 7, and 3.5 to 10 (LKB Instruments, Inc.) in the ratio of 4:4:2. The samples were developed at 200 V for 3 h at 4°C. The gels were removed and sliced into 2-mm widths. After the pH was determined with a flat-type combination pH electrode model 6210-05T (Horiba, Kyoto), each slice was fixed overnight with 15% trichloroacetic acid, stained for 2 h at 37°C with 0.25% Coomassie brilliant blue G250, and destained with 7.5% acetic acid.

Amino acid analysis. Samples were hydrolyzed in twice-distilled 6 N HCl containing 0.02% phenol at 105°C for 24, 48, and 72 h in sealed evacuated tubes. To determine sulfur-containing amino acids, samples were oxidized with performic acid according to the procedure of Moore (23) and hydrolyzed for 18 h. Amino acid analyses were performed with a Durrum type D-500 automatic amino acid analyzer. Tryptophan content was determined spectrophotometrically with the unhydrolyzed sample according to the method of Goodwin and Morton (15).

Other methods. Hexose content was determined by the phenol-sulfuric acid method of Dubois et al. (12), with D-galactose as a standard. Protein content was determined by the method of Lowry et al. (20), with bovine serum albumin as a standard.

Chemicals. ^{14}C-labeled L-amino acids and ^3H-labeled L-leucine were all purchased from Radiochemical Centre. Acrylamide was obtained from Eastman Kodak. SDS and Coomassie brillant blue R250 and G250 were from Merck & Co. NTG was purchased from Aldrich Chemical Co., 5′,5′,5′-trifluoro-DL-leucine was from Fairfield Chemical Co., and DL-4-azaleucine was from ICN Pharmaceuticals. L-Leucine amide and L-leucine ethyl ester were from Protein Research Foundation. All other compounds used were of reagent grade.

RESULTS

Purification of leucine-binding protein. (i) Ammonium sulfate fractionation. The shock fluid was prepared from 400 liters of a

culture of *P. aeruginosa* PML14 cells. The concentrated fluid containing 2.2 g of protein in 550 ml was used for purification. All the purification steps described below were carried out at 4°C. Ammonium sulfate (215 g) was added to the concentrated shock fluid (4 mg of protein/ml) over a period of 1 h to bring the concentration to 60% saturation. After 1 h, the precipitate was centrifuged for 20 min at 8,000 × *g*. The supernatant (650 ml) was adjusted to 95% saturation with 176 g of ammonium sulfate. After 1 h, the precipitate was collected by centrifugation. The precipitate was suspended in 80 ml of 10 mM Tris-hydrochloride, pH 7.3, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (TMC buffer). About 90% of the leucine-binding activity was recovered in the 60 to 95% ammonium sulfate fraction. This fraction was dialyzed overnight against TMC buffer.

(ii) DEAE-cellulose column chromatography. The dialyzed ammonium sulfate fraction (136 ml, 10 mg of protein/ml) was subjected to DEAE-cellulose column chromatography (2.2 by 40 cm) which had been equilibrated with TMC buffer. The column was washed with TMC buffer at a flow rate of 50 ml per h, followed by elution with an NaCl gradient (0 to 0.2 M). A profile of the chromatography is shown in Fig. 1. The fractions containing leucine-binding activity (181 to 212) were pooled, concentrated by ultrafiltration through a PM10 filter, and dialyzed overnight against TMC buffer containing 50 mM NaCl. The dialysate was rechromatographed on a DEAE-cellulose column (1.5 by 38 cm) with an NaCl gradient of 0.05 to 0.15 M. The fractions with activity were collected, concentrated, and dialyzed against TMC buffer containing 0.125 M NaCl. About 12-fold purification was attained, with a recovery of 74%, by these two chromatography steps (Table 1).

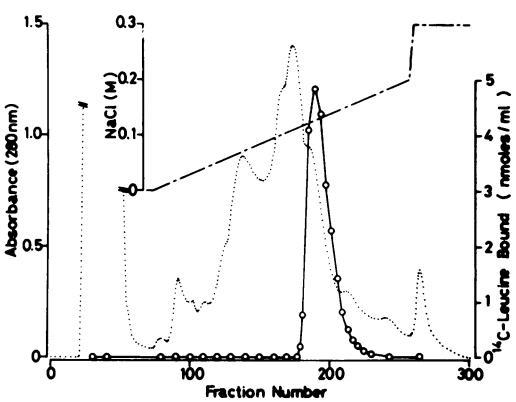

FIG. 1. *Elution profile of the ammonium sulfate fraction on a DEAE-cellulose column (2.2 by 40 cm). The sample solution (136 ml) was loaded onto the column, which had been equilibrated with TMC buffer. After the column had been washed with 300 ml of the same buffer, the leucine-binding protein was eluted with 1 liter of linear gradient of 0 to 0.2 M NaCl in TMC buffer. The fraction volume and flow rate were 6 and 50 ml/h, respectively. Symbols: (·····) absorbance at 280 nm; (—○—) leucine-binding activity; (— - —) NaCl concentration.*

(iii) DEAE-Sephadex A-50 column chromatography. The concentrated sample after DEAE-cellulose column rechromatography was loaded on a DEAE-Sephadex A-50 column (1.5 by 37 cm) which had been equilibrated with TMC buffer containing 0.125 M NaCl. After the column was washed with the same solution, materials adsorbed to the column were separated with an NaCl gradient of 0.125 to 0.25 M in TMC buffer. The leucine-binding protein was eluted at an NaCl concentration of about 0.18 M. The fractions containing leucine-binding activity were pooled, concentrated, and dialyzed against 0.01 M Tris-hydrochloride buffer, pH 7.3. This preparation had a specific activity of 7 nmol per mg of protein and was purified 21-fold over the concentrated shock fluid (Table 1). The purified sample showed only one band when analyzed by urea-SDS-polyacrylamide gel electrophoresis (Fig. 2), SDS slab-gel electrophoresis, or polyacrylamide gel isoelectric focusing (data not shown). The purified sample of the binding protein was stored at −70°C until use.

Molecular weight and isoelectric point. The apparent molecular weight of the leucine-binding protein determined by SDS slab-gel electrophoresis was 43,000 (Fig. 3). Estimation of molecular weight was also made with the gel filtration method described by Andrews (6). The molecular weight determined by gel filtration on a column (1.6 by 90 cm) of Sephadex G-75 (superfine grade) was 40,000 (Fig. 3), which was very similar to that by SDS-polyacrylamide gel electrophoresis, indicating that the leucine-binding protein is a monomeric protein. An isoelectric point of 4.1 was determined by electrofocusing in acrylamide gel with a pH gradient of 3.5 to 7.

Chemical composition. The amino acid composition of the leucine-binding protein is shown in Table 2. Aspartic acid, glutamic acid, glycine, and alanine were found to be major components. Based on one residue of cysteine per mol of the protein, a minimal molecular weight of 41,000 was calculated. Table 2 also shows that the purified protein contained only one residue of tryptophan per molecule. The amino acid composition of the leucine-binding protein of *P. aeruginosa* was very similar to that of the LIVT-binding protein (formerly called the leucine-binding protein) of *Escherichia coli* purified by Anraku (8). Of particular interest in the

TABLE 1. *Purification of leucine-binding protein*

Purification step	Vol (ml)	Protein (mg)	Total act (U)[a]	Sp act (U/mg)	Yield (%)
Concentrated shock fluid[b]	550	2,180	710	0.33	100
Ammonium sulfate fractionation (60–95%)	136	1,340	634	0.47	89
First DEAE-cellulose chromatography	191	138	569	4.1	80
Second DEAE-cellulose chromatography	147	84	466	5.6	66
DEAE-Sephadex A-50 chromatography	15.7	51	351	6.9	49

[a] One unit of binding activity is equivalent to 1 nmol of substrate bound.
[b] Extracted from 700 g (wet weight) of cells.

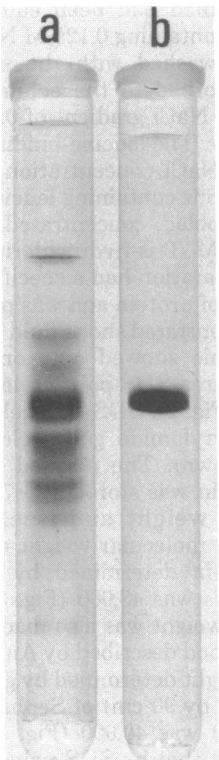

FIG. 2. *Urea-SDS-polyacrylamide gel electrophoresis of the leucine-binding protein. Gels: (a) 100 μg of the concentrated shock fluid; (b) 25 μg of the purified protein.*

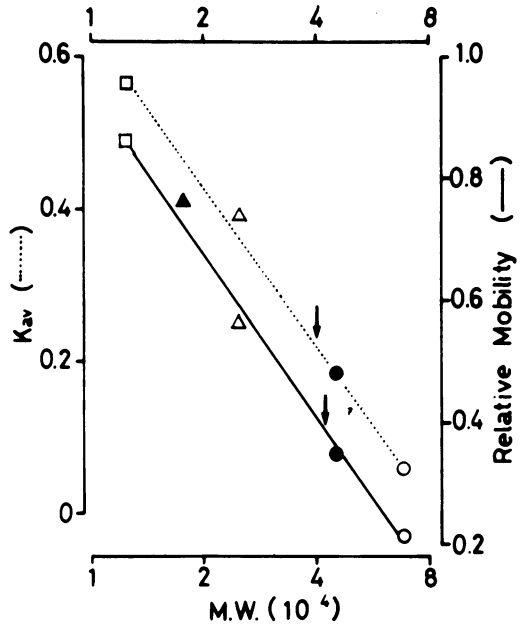

FIG. 3. *Molecular weight determination of the leucine-binding protein. Relative mobility* (——) *signifies the relative migration of a protein on SDS-polyacrylamide gel to that of bromophenol blue. The K_{av} value* (- - -) *indicates the ratio $(V_e - V_0)/(V_t - V_0)$, where V_0, V_t, and V_e represent the void, total, and elution volumes, as determined by Sephadex G-75 gel filtration. Proteins:* (○) *bovine serum albumin;* (●) *hen egg albumin;* (△) *chymotrypsinogen A;* (▲) *β-lactalbumin;* (□) *cytochrome c. Arrows indicate the positions to which the leucine-binding protein migrated.*

chemical composition is whether the leucine-binding protein contains carbohydrate, for it has been reported that the GBP of *P. aeruginosa* contains carbohydrate, which constitutes nearly 16% of the molecule (28). However, no hexose was detected in the leucine-binding protein by the phenol-sulfuric acid method, suggesting that the protein contains no carbohydrate.

pH optimum and stability. The effect of pH on leucine-binding activity was measured under standard assay conditions. The results indicated that the activity has a broad pH optimum for binding: it was active in the pH range of 5 and 10. However, a decrease in activity was observed at pH values below 5. No activity was detected at pH 3.5.

The thermal stability of the leucine-binding activity was also examined (Fig. 4). The leucine-binding protein was very stable: no loss of activity was observed by heating at 80°C for 10 min (Fig. 4A). However, the protein was inactivated by 25% or completely after boiling for 10 min or 2 h, respectively (Fig. 4B).

Effects of various compounds on binding. Effects of cations and other compounds on the leucine-binding activity were examined in 50 mM Tris-hydrochloride buffer, pH 7.3. The

TABLE 2. *Amino acid composition of leucine-binding protein*

Amino acid	Relative composition (residues/100 residues)
Aspartic acid	10.5
Threonine	5.7
Serine	2.7
Glutamic acid	11.4
Proline	4.7
Glycine	10.9
Alanine	12.6
Half-cystine	0.26
Valine	8.2
Methionine	2.6
Isoleucine	5.8
Leucine	5.9
Tyrosine	2.8
Phenylalanine	4.1
Lysine	8.1
Histidine	0.93
Arginine	2.5
Tryptophan	0.29

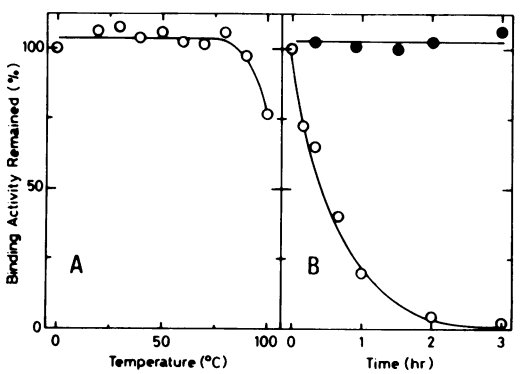

FIG. 4. *Thermal stability of the leucine-binding activity. (A) Purified protein (0.3 mg/ml) was heated for 10 min at the temperature indicated and then chilled in an ice bath. (B) Purified sample in a sealed tube was heated at 30°C (●) or 97°C (○) for the time indicated. Residual binding activities for leucine were assayed under standard conditions and expressed as percentage of the control.*

binding of leucine by the purified protein was unaffected by 5 mM NaCl or KCl, 1 mM multivalent cations ($MgCl_2$, $CaCl_2$, $ZnCl_2$, $MnCl_2$, $CuCl_2$, $FeSO_4$, or $FeCl_3$), 1 mM EDTA, or 5 mM NaN_3. It was also unaffected by 0.1 mM sulfhydryl reagents such as $HgCl_2$, iodoacetate, and *p*-chloromercuriphenyl sulfonic acid, or by 5 mM β-mercaptoethanol.

Dissociation constants and substrate specificity. The binding of leucine, isoleucine, or valine as a function of substrate concentration was examined. The data showed straight lines when plotted in a double-reciprocal fashion (data not shown). The K_d values for leucine, isoleucine, and valine were found to be 0.4, 0.3, and 0.5 μM, respectively, which were very similar to the values obtained with a concentrated shock fluid (16).

The substrate specificity of the purified protein was examined by the binding assay with various ^{14}C-labeled amino acids (Table 3). Leucine, isoleucine, and valine were bound to the protein to the same extent, as expected from their K_d values. Alanine and threonine were also bound to the protein, although less effectively than branched-chain amino acids, suggesting that the protein has much higher K_d values for these amino acids than for branched-chain amino acids. No other amino acids tested were bound to the protein. These results indicate that the leucine-binding protein of *P. aeruginosa* is specific for branched-chain amino acids, and also for alanine and threonine to a lesser extent. Therefore, we designate this protein as LIVAT-BP (leucine, isoleucine, valine, alanine, and threonine-binding protein). The inhibition experiments further confirmed the above finding. The leucine-binding activity of the purified protein was completely inhibited by 100-fold amounts of nonlabeled leucine, isoleucine, or valine. Considerable inhibition by alanine or threonine was also observed, but no inhibition by any other amino acid was observed. The same inhibition pattern has been reported for the leucine-binding activity of the concentrated shock fluid (16).

The effects of various leucine analogs on leu-

TABLE 3. *Binding activity of the purified binding protein for various* L*-amino acids*[a]

Amino acid examined	Binding activity[b] (nmol/ml)
Leucine	2.6
Isoleucine	3.3
Valine	2.9
Alanine	0.6
Threonine	0.3
Glycine, Serine, Aspartic acid, Glutamic acid, Asparagine, Methionine, Lysine, Arginine, Phenylalanine, Tyrosine, Histidine, Proline	0.0

[a] A dialysis bag containing the purified sample (0.2 mg/ml) was dialyzed against 10 ml of Tris-hydrochloride buffer with 1 μM ^{14}C-labeled amino acid as indicated.

[b] Expressed as the amount of substrate bound per milliliter of the sample solution.

cine binding were also examined (Table 4). The addition of 100-fold amounts of D-leucine caused no inhibition, suggesting that the leucine-binding protein has a stereospecificity for the L-configuration. No analog was found which caused notable inhibition of the leucine-binding activity of LIVAT-BP. Trifluoroleucine and azaleucine, which inhibited the growth of this strain, showed no inhibition under these conditions.

Isolation of transport mutants defective in the production of LIVAT-BP. We found two distinct systems for the transport of branched-chain amino acids in *P. aeruginosa* (16, 17): LIV-I, which has a high affinity, and LIV-II, which has a low affinity and requires Na^+ for its activity. The LIV-I system is shock sensitive, and its specificity is very similar to that of the purified leucine-binding protein as described above. These findings suggest that the binding protein purified in this study may be an essential component of the LIV-I transport system. To clarify the role of LIVAT-BP, LIV-I transport-negative mutants were sought, using selection for resistance to trifluoroleucine (TFL).

We have found that TFL, a leucine analog, is a potent inhibitor of *P. aeruginosa* growth. Growth inhibition by TFL without Na^+, however, was suppressed not only by the addition of leucine, isoleucine, or valine but also by the addition of alanine or threonine. On the other hand, in the presence of Na^+, growth inhibition by TFL was suppressed only by branched-chain amino acids, but not by alanine or threonine. Amino acids effective in the suppression were those transported under respective conditions. Therefore, mutants defective in the LIV-I transport system should be resistant to TFL in the absence of Na^+. In fact, such mutants of *P. aeruginosa* were isolated by selection for resistance to TFL as described below.

TABLE 4. *Effect of various analogs on leucine binding*[a]

Addition	Inhibition (%)
L-Leucine	98
D-Leucine	0
DL-Norleucine	9
DL-Azaleucine	0
DL-Trifluoroleucine	0
Leucyl glycine	6
Leucine amide	0
Leucine ethyl ester[b]	0
Glycyl leucine	11
N-Acetyl leucine	15

[a] Activity was measured with 1 μM [^{14}C]leucine in the presence of a 100-μM concentration of the unlabeled compound indicated except leucine ethyl ester.

[b] This compound was contaminated by leucine (1%), so that the effect of this compound was examined at the concentration of 10 μM.

LIV-I transport-defective mutants were isolated from a prototrophic strain, PML14. NTG-mutagenized cells (10^7 cells) were plated on a D-medium-based agar plate containing 50 μg of TFL per ml and grown at 37°C for 48 h. Colonies resistant to TFL appeared at a frequency of 10^{-5}. Such colonies (total, 164) were picked and checked for resistance to TFL (10 μg/ml) on agar plates with or without 20 mM Na^+. About half of them (67 colonies) were resistant to TFL without but not with Na^+. They were examined for LIV-I and the LIV-II activity. Fifteen strains had no LIV-I activity but showed normal levels of LIV-II transport. Most of these mutants (13 strains) showed a severe decrease in leucine-binding activity in the shock fluid. Two such mutants, PML1451 and PML1453, were chosen for further study.

Revertants of strains PML1451 and PML1453 to LIV-I-positive phenotype were also isolated. The wild-type strain PML14 can feed on L-isoleucine as a carbon source with a doubling time of about 4 h. This suggests that cultivation with isoleucine as a carbon source in the absence of Na^+ would distinguish LIV-I mutants from revertants to the LIV-I-positive phenotype. A 0.05-ml portion of the cell suspension (5×10^8 cells/ml) was plated on a D-medium-based agar plate containing 0.2% isoleucine as a carbon source and incubated at 37°C. After 3 to 5 days, faster-growing colonies were found in the background growth. Such colonies were picked, reisolated, and examined for their sensitivity to TFL. Most of the colonies which reverted to TFL-sensitive phenotype without Na^+ simultaneously regained activity of the LIV-I transport system. Strains PML1452 and PML1454, revertants of PML1451 and PML1453, respectively, were chosen for further study.

(i) Transport activity. It was shown previously (16) that two systems for the transport of branched-chain amino acids can be assayed separately with the same cell suspension: LIV-I without Na^+, and LIV-II with Na^+ and excess alanine. Two activities of the relevant strains are shown in Table 5. Strains PML1451 and PML1453 showed very low levels of leucine transport by LIV-I, with normal activity of the LIV-II system. Strains PML1452 and PML1454, revertants to the TFL-sensitive phenotype of PML1451 and PML1453, respectively, showed normal levels of transport by LIV-I and by LIV-II, as demonstrated by wild-type strain PML14.

(ii) LIVAT-BP production. The ability of the above strains to synthesize functionally active LIVAT-BP was examined with cells grown

in D-medium. Binding activities of the shock fluids of these strains were determined (Table 6). Little leucine-binding activity was detected with the shock fluids of PML1451 or PML1453. However, the shock fluids of PML1452 and PML1454 showed normal leucine binding, as high as that of PML14. The proteins in the shock fluids of these strains were also analyzed by SDS slab-gel electrophoresis (Fig. 5). The leucine-binding protein was one of the major proteins in the shock fluid of wild-type strain PML14 (Fig. 5a). Strains PML1451 and PML1453, the LIV-I-defective mutants, were defective in the band corresponding to LIVAT-BP (Fig. 5b, d), although very low levels of protein at the LIVAT-BP band were still found. However, the shock fluids of PML1452 and PML1454, revertants to LIV-I-positive phenotype, regained the band corresponding to LIVAT-BP (Fig. 5c, e). The shock fluid of PML1453 had lost another band, X, with the concomitant appearance of bands Y and Z (Fig. 5d). Strain PML1454 regained LIV-I transport activity without band X (Table 6; Fig. 5e). Therefore, band X is probably not involved in leucine transport. These findings show a parallelism of the activity of LIV-I with the activity and/or production of LIVAT-BP, suggesting that the binding protein is associated with the LIV-I transport system.

TABLE 5. *Transport activities of LIV-I and LIV-II of P. aeruginosa mutant strains*

Strain	Relative rate[a] (%)	
	LIV-I[b]	LIV-II[c]
PML14	100	100
PML1451	14	116
PML1452	111	124
PML1453	9	121
PML1454	108	130

[a] Expressed as percentage of the control (PML14). Control values of the initial rates of LIV-I and LIV-II by PML14 cells were 32 and 18 nmol per mg of protein per min, respectively.

[b] Assayed in the absence of Na^+ with 18 μM [^{14}C]-leucine.

[c] Assayed in the presence of 30 mM NaCl and 10 mM alanine with 17 μM [^{14}C]leucine.

TABLE 6. *Leucine-binding activities of shock fluids of P. aeruginosa mutant strains*[a]

Strain	Binding activity[b]	
	nmol/mg	%
PML14	0.28	100
PML1451	0.04	14
PML1452	0.28	100
PML1453	0.03	11
PML1454	0.29	104

[a] Shock fluid (about 4 ml) was prepared from 250 ml of the culture of each strain. About 1.5 mg of protein was contained in the fluid.

[b] Assayed with 1 μM [^{14}C]leucine under standard conditions.

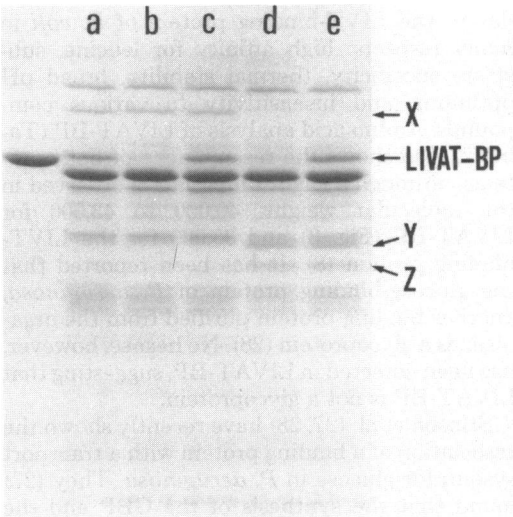

FIG. 5. *SDS-polyacrylamide gel electrophoresis of shock fluids from strains PML14 (a), PML1451 (b), PML1452 (c), PML1453 (d), and PML1454 (e). Samples containing about 4 μg of protein were loaded on a 12% acrylamide gel. The direction of migration is from top to bottom. Purified LIVAT-BP (0.7 μg) is shown on the left.*

DISCUSSION

The present study shows that the properties of the leucine-binding protein (LIVAT-BP) purified from the shock fluid of *P. aeruginosa* are very similir to those of the leucine-binding activity of the whole fluid (16). Similar dissociation constants for branched-chain amino acids and similar substrate specificities were found with these two preparations. No other protein having leucine-binding activity was found in the process of purification (Fig. 1), suggesting that the leucine-binding protein purified here is the only one that is responsible for leucine binding by the shock fluid of *P. aeruginosa*. The purified sample was homogeneous in both molecular size and electrophoretic behavior. In *E. coli*, however, at least three leucine-binding proteins have been found in osmotic shock fluid: LIVT-binding protein (initially called the leucine-binding protein) (7, 25), leucine-specific binding protein (1, 14), and a second LIVT-binding protein (2). The leucine-binding protein of *P. aeruginosa* is sim-

ilar to the LIVT-binding protein of *E. coli* in many respects: high affinity for leucine, substrate specificity, thermal stability, broad pH optimum, and insensitivity to various compounds. Amino acid analysis of LIVAT-BP (Table 2) also shows the resemblance of both proteins, although a slight difference is observed in the molecular weight: 40,000 to 43,000 for LIVAT-BP (Fig. 3) and 36,000 for the LIVT-binding protein (8). It has been reported that the glucose-binding protein of *P. aeruginosa*, another binding protein purified from the organism, is a glycoprotein (28). No hexose, however, has been detected in LIVAT-BP, suggesting that LIVAT-BP is not a glycoprotein.

Stinson et al. (27, 28) have recently shown the association of a binding protein with a transport system for glucose in *P. aeruginosa*. They (27) found that the synthesis of the GBP and the glucose transport system were coregulated. A GBP-deficient mutant was defective in glucose transport, and a revertant to GBP-positive phenotype regained the normal level of transport activity (28). Binding activities for dicarboxylic acids (27) or glycerol (29) have also been found in the shock fluid of *P. aeruginosa*. Furthermore, coregulation of these activities and their corresponding transport systems has been suggested (27, 29). However, the molecular properties of such binding activities have not been elucidated, and no conclusive evidence has yet been obtained which shows the association of those activities with their corresponding transport systems.

The present study suggests strongly that the leucine-binding protein of *P. aeruginosa* is associated with the LIV-I transport system, which is sensitive to osmotic shock. Its substrate specificity and the K_d values for branched-chain amino acids were very similar to those of the LIV-I system. Furthermore, parallelism of the production of LIVAT-BP with the activity of the LIV-I system was found: mutants defective in LIVAT-BP had severely decreased LIV-I transport activity, and their revertants to LIV-I-positive phenotype had regained the binding protein. These findings, together with those described previously (16), suggest that the leucine-binding protein of *P. aeruginosa* is required in some way for the transport of the substrates via the LIV-I system. In this respect, Ames and Spudich (4) have proposed an interesting model in which the histidine-binding protein of *Salmonella typhimurium* has two distinct sites in the molecule: one is the binding site for the substrate, and the other is the interaction site for the *hisP* protein, which has been recently identified as a cytoplasmic membrane protein (3). However, further investigation, particularly into the genetic elements, is needed to clarify the mechanism by which LIVAT-BP takes part in the LIV-I transport system of *P. aeruginosa*.

With regard to transport systems for branched-chain amino acids in *E. coli*, a system with high affinity, referred to as LIV-I by Rahmanian et al. (26) or LIV-1 by Yamato et al. (33), is associated with a periplasmic binding protein (LIVT-binding protein) (5, 10, 26, 33). Oxender and his co-workers have determined by genetic analysis that the LIVT-binding protein coded by *livJ* is an essential component for the high-affinity system (5). On the other hand, Anraku (10) and Yamato et al. (33) found that the transport activity with high affinity (LIV-1) was retained even after the entire repression of the LIVT-binding protein, although a considerable decrease was observed in transport activity, suggesting that the binding protein is not an essential component but conditionally stimulates the high affinity system.

In this study, LIV-I-defective mutants were isolated by the selecting for resistance to TFL. Although TFL showed no apparent inhibition of leucine-binding by LIVAT-BP (Table 4), mutants defective in LIVAT-BP were obtained by selection under Na^+-free conditions. The mutants thus obtained were still sensitive to TFL in the presence of Na^+. However, TFL was much more inhibitory to leucine transport by the LIV-II system (Na^+ dependent) than by LIV-I (data not shown). These findings suggest that selection for TFL resistance is a very useful tool for isolating LIV-defective mutants of *P. aeruginosa* with various phenotypes. Indeed, LIV-II mutants with normal LIV-I activity have been isolated by selecting for TFL resistance with Na^+ and excess amounts of alanine, and mutants defective in both LIV-I and LIV-II have been isolated by selecting for TFL resistance in the presence of Na^+. These mutants are now being characterized.

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