Sodium dependence of leucine transport, a measure of the Na+-coupled leucine-isoleucine-valine II (LIV-II) transport system in *Pseudomonas aeruginosa*, was compared between two wild-type strains, PAO and PML. The leucine transport activity was saturated at 0.1 mM NaCl for PAO and at 5.0 mM for PML. From kinetics experiments, the apparent $K_m$ value for Na+ with respect to leucine transport was estimated to be 3 $\mu$M for PAO and 95 $\mu$M for PML. The $K_m$ value for leucine was 6 $\mu$M for PAO and 13 $\mu$M for PML. The LIV-II carrier gene (braB) of PML was isolated for comparison of its amino acid sequence with that of the PAO carrier cloned previously. The $K_m$ values for Na+ and leucine of the cloned LIV-II carriers of PAO and PML, expressed in LIV-II defective mutants were similar to those in wild-type strains. Determination of the nucleotide and deduced amino acid sequences of the LIV-II carrier gene of PML showed an amino acid difference at position 292 between the PAO and PML carriers. The amino acid was threonine for PAO and alanine for PML. These results indicate that the substitution of the amino acid at position 292 of the LIV-II carrier causes a difference in the sodium requirement of the carriers of the PAO and PML strains.

Transport of branched chain amino acids, L-leucine, L-isoleucine, and L-valine, in *Pseudomonas aeruginosa* is mediated by two transport systems (1–3). One is a binding protein-dependent transport system (LIV-I) with high affinity for substrates. The other is Na+(Li+)/substrate cotransport system (LIV-II) with low substrate affinity (4, 5). The latter (LIV-II) transport system has been characterized using membrane vesicles prepared from the *P. aeruginosa* PML strain (4), solubilized, and reconstituted into liposomes (6). On the other hand, the genetic locus of the LIV-II system has been mapped on the chromosome of the PAO strain, which is widely used for the genetics of *P. aeruginosa* (7). The LIV-II carrier gene (braB) of PAO has been cloned, and its nucleotide sequence has been determined. The LIV-II transport activity in the PAO strain as well as the PML strain is stimulated by the addition of Na+ (1–4). During the genetic study we had considered that the property of Na+ dependence of the LIV-II transport system was identical in the two strains. Recently, we have compared the property of the LIV-II transport system in the PAO and PML

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*To whom correspondence should be sent.


2. The abbreviations used are: kb, kilobase(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
with a suspension solution of 0.1 M sucrose, 5 mM Hepes/Tris, pH 7.5, and suspended in the same solution.

For initial rate measurements, 196 µl of cell suspension containing 50 mM glucose, 10 mM alanine, and an appropriate concentration of NaCl or LiCl was incubated for 60 s at 30 °C. Alanine was added to repress LIV-I transport (2). After incubation, the uptake reaction was initiated by the addition of 4 µl of [14C]-amino acid (1.14 mM, 45 µCi/ml). After 10 s, the reaction mixture was diluted with 4 ml of 0.125 M KCl and 10 mM Tris-HCl, pH 7.5, to stop the uptake reaction, filtered immediately through a HA-type Millipore filter with 0.45-µm pore size, and washed with 4 ml of the same buffer. Residual radioactivity on the filter was counted with a liquid scintillation counter after drying it. The uptake reaction was duplicated, and the results were averaged (2).

For the time course of uptake, the reaction mixture was aerated with stirring, and 100-µl aliquots were withdrawn at appropriate time intervals, diluted, filtered, and washed as mentioned above.

**Gene Cloning and Nucleotide Sequencing—Cloning of LIV-II carrier gene (braB) from the PML strain was carried out according to the procedure of cloning of the braB gene in the PAO strain.** A gene library of *P. aeruginosa* PML14 was constructed in E. coli HB101 carrying a mobilizing plasmid RP4-4 using a cosmid-cloning vector pMBS34 after partial Sau3A digestion of PML chromosomal DNA. The cosmid-carrying insert DNA was mobilized into *P. aeruginosa* PA03536 from *E. coli* HB101 by RP4-mediated conjugation. Exconjugates were transferred onto selection plates containing a low leucine concentration of 5 µg/ml and, of them, an exconjugate that grew on the plate was isolated as a clone carrying the structural gene (braB) of the LIV-II carrier. A chromosomal DNA fragment cloned in PML14 exconjugate was 48 kb in length. Then, it was truncated with EcoRI restriction enzyme into a fragment with a length of 5.0 kb, and the 5.0-kb fragment was jointed with a broad host range vector pKT240 (see "Results" for details).

For nucleotide sequencing, a 2.5-kb AatI-HpaI restriction fragment containing the braB gene was prepared from plasmid pKT240 carrying the 5.0-kb EcoRI-EcoRI fragment and inserted into a HindIII site of vector PUC18. The remaining EcoRI site of the insert fragment was close to HpaI site in the polylinker region on pKT240. The nucleotide sequence of the 2.5-kb AatI-EcoRI fragment was determined by the dideoxy chain termination method modified by the use of dye-labeled nucleotide triphosphates (13, 14).

**Analytical Procedures—** The protein content of the cell suspension was determined by the modified method of Schaffner and Weissmann (15, 16) with bovine serum albumin as standard. The sodium content was determined by the atomic absorption method with a Varian Spectr-AA 40F atomic absorption spectrometer. Sodium concentrations of distilled water and suspension solution were approximately 0.15 and 1.5 µM, respectively.

**RESULTS**

**Effect of Sodium Concentration on Leucine Transport Activity**—The initial rate of leucine transport, a measure of LIV-II transport activity, as a function of Na+ concentration, was measured in whole cells of PA03012 and PML14 strains at constant leucine concentrations of 23 and 45 µM, respectively (Fig. 1A). The leucine transport activity in PA03012 cells increased with an increase in NaCl concentration, saturated at 0.1 mM, and gradually decreased from 0.2 up to 5.0 mM. On the other hand, the initial rate of leucine transport in PML14 cells increased gradually as the NaCl concentration increased up to 5 mM. The Fig. 1A inset shows the leucine transport activity at various NaCl concentrations lower than 0.1 mM. A function of the uptake activity against the Na+ concentration exhibited saturation in PA03012. The data in Fig. 1A were represented in a double-reciprocal fashion (Fig. 1B) and by kinetic parameters of apparent $K_a$ and $V_{max}$ were calculated (Table I). The double-reciprocal plots in terms of the initial rate and Na+ concentration exhibited linear relationships between two reciprocals with a different slope and different intercept on the ordinate. The apparent $K_a$ value for Na+ with respect to leucine transport was 2.7 µM for PA03012 and 95 µM for PML14.

These results show that the LIV-II transport systems of both strains are Na+ dependent but that their Na+ dependence and, as a result, their kinetic properties are very different.

**Effect of Substrate Concentration on Leucine Transport Activity**—Leucine transport activity at various leucine concentrations was measured at the rate-saturating Na+ concentration of 0.1 mM for PA03012 and 5.0 mM for PML14 (Fig. 2). The effects of leucine transport on transport activity were not identical for the two strains. Double-reciprocal plots of initial rates and leucine concentrations gave two different straight lines (Fig. 2, inset). The apparent $K_a$ value for leucine was calculated to be 5.6 µM for PA03012 and 13 µM for PML14. The $V_{max}$ value was approximately 10 nmol/mg protein/min for the two strains (Table I). This result shows that the apparent $K_a$ value for leucine is slightly different between PAO and PML strains.

![Fig. 1. Effect of sodium concentration on leucine transport activity in PAO and PML strains](image)
the difference in the Na⁺ requirement of the LIV-II carriers of PAO and PML is due to the nature of the carrier or to a physiological property of wild-type strains, the pKT240 plasmid carrying the 2.5-kb fragment of PAO (pKHR613), which was previously constructed,¹ was transferred into PML1460, a defective mutant of the LIV-II activity. Alternatively, the plasmid harboring that of PML (pKURI) was introduced into a LIV-II defective mutant, PAO3517. First, LIV-II transport activities of transformants with plasmids carrying braB genes were examined (Fig. 3). The PML1460 transformant with plasmid pKHR613 and the PAO3517 transformant with pKURI accumulated leucine, isoleucine, or valine, substrate for the LIV-II system, in the presence of 0.1 and 5.0 mM NaCl, respectively. PML1460 and PAO3517 cells did not transport any substrate. This shows that gene products of braB carried by plasmid pKT240 have the activity transporting leucine, isoleucine, and valine.

Next, kinetics of leucine transport for each transformant was carried out to determine the values of apparent Kₘ for leucine transport and apparent Kₘ for Na⁺ with respect to leucine transport under the same conditions as those for wild-type strains (Table I). All plots of the initial rate against leucine or Na⁺ concentration represented saturation curves, and replotting of the results in a double-reciprocal fashion gave straight lines (data not shown). The apparent Kₘ values of leucine transport and for Na⁺ in the PML transformant carrying the PML braB gene were estimated to be 6 and 3 μM, respectively. These values were the same as those in wild-type strain PAO3012. Similarly, the apparent Kₘ values for leucine and Na⁺ in the PAO transformant carrying the PML braB gene were the same as those in wild-type strain PML14. The values of Vₘax for leucine transport in the two transformants were approximately 6–7 times greater than those in the wild-type strains. These results show that the kinetic property of Na⁺-coupled leucine cotransport in the transformant carrying the braB genes of PAO or PML is the same as that in the LIV-II donor strain except for high

Cloning of the LIV-II Carrier Gene (braB) of the PML Strain—The LIV-II carrier gene (braB) of PML was cloned in E. coli HB101 (RP4-4) using a cosmid vector PMMB34 as described under “Experimental Procedures.” A clone that restored the growth at low leucine concentration was isolated, containing a chromosomal DNA fragment 48 kb in length. The DNA fragment was digested with EcoRI restriction enzyme into fragments of 0.8, 1.0, 2.3, 3.6, 5.0, 6.6, 11, and 18 kb. Its restriction map was identical to that of the chromosomal region containing the braB gene of PAO which is located in the 5.0-kb EcoRI-EcoRI region (data not shown).¹ Thus, the 5.0-kb EcoRI-EcoRI fragment was transferred into pKT240, a shuttle vector between E. coli and P. aeruginosa for subcloning of the LIV-II carrier gene of PML, and was further truncated into a 2.5-kb fragment by deleting the XhoI-AatI region. When P. aeruginosa PAO3517, a LIV-II defective mutant strain, was transformed with pKT240 carrying the 5.0- or 2.5-kb fragment, it exhibited leucine transport activity (data not shown). This shows that the LIV-II carrier gene (braB) of PML is located on the 2.5-kb fragment contained in the 5.0-kb fragment, which is the same as the braB gene of PAO.¹ The pKT240 plasmid carrying the 2.5-kb fragment is designated as pKURI.

LIV-II Transport Activity of P. aeruginosa Transformants and Their Transport Kinetics—In order to examine whether

<table>
<thead>
<tr>
<th>Strain</th>
<th>Na⁺ Kₘ</th>
<th>Leucine Kₘ</th>
<th>Vₘax</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO3012</td>
<td>2.7 μM</td>
<td>5.6 nmol/mg protein min⁻¹</td>
<td>11</td>
</tr>
<tr>
<td>PML14</td>
<td>85 μM</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>PML1460 (pKHR613)</td>
<td>2.6 μM</td>
<td>1.1</td>
<td>63</td>
</tr>
<tr>
<td>PAO3517 (pKURI)</td>
<td>91 μM</td>
<td>12</td>
<td>71</td>
</tr>
</tbody>
</table>

¹ The apparent Kₘ value for Na⁺ was determined with respect to leucine transport by PAO3012 and PML1460 (pKHR613) at 23 μM leucine and by PML14 and PAO3017 (pKURI) at 45 μM leucine.

Kinetic parameters were determined in the presence of 0.1 and 5.0 mM NaCl, respectively. Inset, double-reciprocal plot of initial rates of leucine transport by PAO3012 and PML1460 (pKHR613) and for PML14 and PAO3017 (pKURI), respectively.

Cloning of the LIV-II Carrier Gene (braB) of the PML Strain—The LIV-II carrier gene (braB) of PML was cloned in E. coli HB101 (RP4-4) using a cosmid vector PMMB34 as described under “Experimental Procedures.” A clone that restored the growth at low leucine concentration was isolated, containing a chromosomal DNA fragment 48 kb in length. The DNA fragment was digested with EcoRI restriction enzyme into fragments of 0.8, 1.0, 2.3, 3.6, 5.0, 6.6, 11, and 18 kb. Its restriction map was identical to that of the chromosomal region containing the braB gene of PAO which is located in the 5.0-kb EcoRI-EcoRI region (data not shown).¹ Thus, the 5.0-kb EcoRI-EcoRI fragment was transferred into pKT240, a shuttle vector between E. coli and P. aeruginosa for subcloning of the LIV-II carrier gene of PML, and was further truncated into a 2.5-kb fragment by deleting the XhoI-AatI region. When P. aeruginosa PAO3517, a LIV-II defective mutant strain, was transformed with pKT240 carrying the 5.0- or 2.5-kb fragment, it exhibited leucine transport activity (data not shown). This shows that the LIV-II carrier gene (braB) of PML is located on the 2.5-kb fragment contained in the 5.0-kb fragment, which is the same as the braB gene of PAO.¹ The pKT240 plasmid carrying the 2.5-kb fragment is designated as pKURI.

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LIV-II Transport Activity of P. aeruginosa Transformants and Their Transport Kinetics—In order to examine whether
V<sub>max</sub> values in the transformants, irrespective of the host strains.

Lithium ion as well as sodium ion is a coupling cation for the LIV-II carrier system (5). The kinetics of Li<sup>+</sup>-dependent leucine transport was examined using the above transformants having high transport activity. The leucine transport activities of the PAO and PML carriers increased with leucine concentration at the rate-saturating Li<sup>+</sup> concentration of 0.25 mM, and straight lines were obtained for the two carriers when the results were presented in a double-reciprocal fashion (Fig. 4). The apparent K<sub>m</sub> value for leucine was calculated to be 8 μM for PAO and 9 μM for PML (Table II), showing no difference in the apparent K<sub>m</sub> value between the two carriers. Subsequently, the initial rate of leucine transport was measured at various concentrations of Li<sup>+</sup> at a fixed leucine concentration of 45 μM to obtain the apparent K<sub>m</sub> values for Li<sup>+</sup> of the PAO and PML carriers. The result represented a saturation curve for the PML carrier but a complex profile with a high transport rate in the absence of Li<sup>+</sup> for the PAO carrier (Fig. 5). A double-reciprocal plot for the Li<sup>+</sup> concentration-dependent leucine transport of PML gave a straight line, whereas that of PAO represented a nonlinear curve (Fig. 5, inset). This means that we can estimate the apparent K<sub>m</sub> value for Li<sup>+</sup> with respect to leucine transport of the PML carrier, but we cannot calculate it for the PAO carrier in a manner similar to that described above. We will discuss the determination of the value for Li<sup>+</sup> of the PAO carrier later.

Comparison of Nucleotide Sequence of PAO and PML LIV-

**TABLE II**

**Kinetic parameters of Li<sup>+</sup>-dependent leucine transport by braB gene-carrying mutant strains**

<table>
<thead>
<tr>
<th>Li&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Leucine&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>PML1460 (pKHR613)</td>
<td>9</td>
</tr>
<tr>
<td>PAO3017 (pKUR1)</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup>The apparent K<sub>m</sub> value for Li<sup>+</sup> was determined with respect to leucine transport at 45 μM leucine.

<sup>b</sup>Kinetic parameters of leucine transport were determined in the presence of 250 μM LiCl.

**TABLE III**

Nucleotide differences and deduced amino acids in the AatI-EcoRI DNA region containing the braB gene of the PAO or PML strain

<table>
<thead>
<tr>
<th>Nucleotide difference</th>
<th>Deduced amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PAO</td>
</tr>
<tr>
<td>PAO</td>
<td>PML</td>
</tr>
<tr>
<td>140</td>
<td>T</td>
</tr>
<tr>
<td>1062</td>
<td>T</td>
</tr>
<tr>
<td>1192</td>
<td>A</td>
</tr>
<tr>
<td>2332</td>
<td>C</td>
</tr>
<tr>
<td><strong>Position</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PAO</td>
</tr>
<tr>
<td>PAO</td>
<td>PML</td>
</tr>
<tr>
<td>140</td>
<td>T</td>
</tr>
<tr>
<td>1062</td>
<td>T</td>
</tr>
<tr>
<td>1192</td>
<td>A</td>
</tr>
<tr>
<td>2332</td>
<td>C</td>
</tr>
</tbody>
</table>

<sup>a</sup>Starting from the first base of the AatI recognition sequence AGGCCT.

<sup>b</sup>Starting from the amino-terminal methionine.

**II Carrier Genes**—The nucleotide sequence of the 2.5-kb AatI-EcoRI restriction fragment containing the LIV-II carrier gene (braB) of PML14 was determined and compared with that of PAO3012<sup>1</sup>. The DNA fragment of PML as well as PAO was composed of 2452 nucleotides, but substitutions of 4 nucleotides were found in the nucleotide sequence (Table III). An open reading frame for the PML braB gene was located between nucleotide positions 319 and 1632 starting from the first letter of the AatI recognition sequence AGGCCT, showing that its location was identical to that of PAO braB, but two nucleotides were different between the sequences of their open reading frames. Those nucleotides for the PAO and PML braB genes were T and G at nucleotide position 1062 and A and G at position 1192, respectively. The amino acid sequence deduced from the nucleotide sequence of the PML braB gene showed that the LIV-II carrier of PML as well as PAO was a protein of 437 amino acids. Amino acids corresponding to the two nucleotide substitutions in the braB genes of PAO and PML were leucine and leucine at amino acid position 248 starting from the amino-terminal methionine but threonine and alanine at position 292, respectively. Only 1 amino acid difference was found at position 292 between the PAO and PML LIV-II carriers.
**DISCUSSION**

In the present study, the effect of Na\(^+\) concentration on the LIV-II transport system in *P. aeruginosa* PAO strain was examined and compared with that in the PML strain. A leucine transport activity, a measure of the LIV-II transport system, of PAO was saturated at a much lower Na\(^+\) concentration than that of PML. This indicates that Na\(^+\) dependence of the LIV-II transport system is different between the two strains. This difference in Na\(^+\) dependence was found in two transformants with pKT240 plasmids carrying LIV-II carrier genes (braB) cloned from the respective wild-type strains, independent of the host strains. Thus, it is concluded that the difference in the Na\(^+\) requirement of the LIV-II transport system between PAO and PML strains is attributed to the nature of LIV-II carriers and not to a physiological property of cells.

The two transformants carrying the braB genes of PAO and PML accumulated leucine, isoleucine, and valine in the presence of Na\(^+\), and their leucine transport activities depended on Li\(^+\). This means that the LIV-II carrier system of PAO as well as PML is a Na\(^+\)- and Li\(^+\)-coupled cotransport system (1, 4, 5).

A plot of the initial rate of leucine transport against the concentration of Na\(^+\), Li\(^+\), or substrate represented a section of a rectangular hyperbola in each case except a plot of the initial rate against Li\(^+\) concentration for the PAO LIV-II carrier. This exception is considered to be due to contamination of Na\(^+\) in the reaction mixtures, since the PAO carrier has a very small value of 3 μM for Na\(^+\). For determination of the kinetic parameter for Li\(^+\) of the PAO carrier, it is necessary to consider the contribution of Na\(^+\)-coupled transport. In consideration of the circumstance, we analyzed the kinetics of the LIV-II carrier system coupled with Na\(^+\) and Li\(^+\) on the basis of a rapid equilibrium carrier model solved and analyzed by Turner (17).

In the postulated model shown in Fig. 6, we adopted the following assumptions.

1. The carrier (C) has a binding site for substrate (S) and two separate binding sites for Na\(^+\) and Li\(^+\). The binding of Na\(^+\) to the carrier excludes the binding of Li\(^+\) and vice versa. The stoichiometry of the substrate and Na\(^+\) or Li\(^+\) is 1:1.

2. The ternary complexes such as CNaS and CLiS are formed and degraded at both sides of the membrane by an ordered mechanism such that the carrier binds cation first and substrate second as demonstrated for Na\(^+\)-coupled melibiose transport of *E. coli* (18).

3. The carrier is in rapid equilibrium with Na\(^+\), Li\(^+\), and substrate at both faces of the membrane. The rate-limiting step in the transport process is the translocation of binding sites from one membrane face to the other. The binding sites in unloaded and ternary complex states are only translocated across the membrane.

4. The carrier has reached a steady state (P) at the time of measurement.

5. The total number of carriers, free or loaded, is constant and equal to Ct.

6. Zero-trans conditions that concentrations of S, Na\(^+\), and Li\(^+\) are zero at the inside of the cell are defined as initial rate conditions.

Equations are derived from these assumptions and kinetic constants in the model shown in Fig. 6 according to the procedure of Turner (17). From Assumptions 1 and 5, we have the carrier conservation equations,

\[
[Ct] = [C] + [CNaS] + [CNaS'] + [C'] + [CLiS] + [CLiS'] + [CLi']
\]  

From Assumption 4, we have the steady-state equation,

\[
P_0[C] + P_0[CNaS] + P_0[CLiS] = P_{-d}[C'] + P_{-d}[CNaS'] + P_{-d}[CLiS']
\]

The influx of substrate, \(J_s\), is given by

\[
J_s = (P_0[CNaS] + P_0[CLiS]) - (P_{-d}[CNaS'] + P_{-d}[CLiS'])
\]

In the initial conditions defined by Assumption 6 (\([S'] = 0\), \([Na'] = 0\), and \([Li'] = 0\)) and from Assumption 3 and Equations 1 and 2, the initial in flux of substrate, \(J_b\), can be written as follows.

\[
J_b = P_0[CNaS] + P_0[CLiS] = [C][F_0]/[F_0 + \alpha_0 F_1]
\]

where \(\alpha_0 = 1 + [Na]/K_{Na} + [Na][S]/K_{Na} K_{Na} + [Li]/K_{Li} + [Li][S]/K_{Li} K_{Li}\), \(K_{Na} = 1, F_1 = P_0 + P_0[Na]/K_{Na} K_{Na} + P_0[Li]/K_{Li} K_{Li}\), \(F_0 = P_{-d}\), and \(A = P_0[/Na] + K_{Na} K_{Na} + P_0[/Li]/K_{Li} K_{Li}\).

Under a given set of experimental conditions ([S'] = 0, fixed [Na] = [Na]c), Equation 4 can be rewritten as

\[
J_b([S]) = P_0([C][S])/(B_1 + B_2[S])
\]

where \(B_1 = K_{Na} K_{Na}(1 + P_0 P_{-d}) + [Na]c /K_{Na}\) and \(B_2 = 1 + P_0 /P_{-d}\). The result has Michaelis-Menten form with maximal velocity \(V_i = P_0([C])/(1 + P_0 P_{-d})\) and Michaelis constant \(K_{Na}\) given by

\[
K_{Na} = K_i(1 + K'/[Na]c)
\]

where \(K'_i = K_i(1 + P_0 P_{-d})\), and \(K''_i = K_i(1 + P_0 P_{-d})\). Under the experimental conditions ([Li]c = 0, fixed [S] = [S]c), Equation 4 can be written in the form

\[
J_b([Na]) = P_0([C][Na])/(D_1 + D_2[Na])
\]

where \(D_1 = K_{Na} K_{Na}(1 + P_0 P_{-d})/[S]c\) and \(D_2 = 1 + P_0 /P_{-d} + K_{Na}/[S]c\). The result also has Michaelis-Menten form with maximal velocity \(V_{Na} = V_i(1 + K'/[S]c)\) and Michaelis constant \(K_{Na}\) given by

\[
K_{Na} = K_i(1 + [S]c/K''_i)
\]

The values of \(K_{Na}\), \(K_{Na} V_i\), and \(V_{Na}\) for Na\(^+\)-coupled leucine transport of PML and PAO are determined as apparent \(K_{Na}\) values for leucine or Na\(^+\) and \(V_{max}\) values experimentally from
the double-reciprocal plots of 1/v against 1/[S] and 1/v against 1/[Na]. Thus, we can obtain the values of $K_i^v$ and $K_i^v$ by solving Equations 6 and 8.

Concerning the kinetics of Li$^+$-coupled leucine transport, the initial influxes of substrate, $J_i([S])$ and $J_i([Li])$ can be derived from Equation 4 under given sets of experimental conditions of fixed [Li] = [Li]c and [Na]c ≪ [Li]c for PML and PAO and of fixed [S] = [S]c and [Na]c ≪ $K_i$ for PML, respectively. These influxes are expressed as the forms that $[Na]$ in Equation 5 and [Na] in Equation 7 are replaced by [Li]c and [Li], respectively. The results have Michaelis-Menten form. Expressions for the resulting Michaelis constants $K_{m}$, $K_{m}'$, and maximal velocity $V_{m}$, which are apparent $K_m$ values for leucine or Li$^+$ and $V_{m}$ value, are given by

$$K_{m} = K_i^v/(1 + [S]/K_i^v)$$

$$K_{m}' = K_i^v/(1 + [S]/K_i^v)$$

$$V_{m} = P_{max}$$

where $K_{m} = K_i^v(1 + P_{c}/P_{o})$ and $K_{m}' = K_i^v(1 + P_{c}/P_{o})$. The values of $K_i$ and $K_i'$ for PML or $K_{m}$ for PAO can be obtained from the experimental results as described above. For determination of values of $K_{m}$ and $K_{m}'$ for PAO, Equation 4 is rewritten under the conditions (fixed [S] = [S]c in the form

$$J_i([Li]) = [S]c (E_1 + E_2([Li]))/(H_1 + H_2([Li]))$$

where $E_1 = K_i^v\cdot\frac{[Na]c}{[K_i]}$ and $E_2 = V_m/K_i^v$, $H_1 = K_i^v + K_i^v[Na]c/(1 + [S]/K_i^v)$, and $H_2 = 1 + [S]/K_i^v$. The value of [Na]c is calculated from Equation 5 at [Li] = 0 with experimental value of $J_i([Li])$. The values of $K_i$ and $K_i'$ can be obtained by solving Equations 9 and 11. However, the $K_i$ and $K_i'$ values were finally determined from Equation 11 by the curve-fitting method after each value was calculated at given Li$^+$ concentrations and averaged (Fig. 5). The values of $K_i$, $K_i'$, $K_i$ and $K_i'$ for PAO and PML were determined as apparent dissociation constants using the experimental data as shown in Tables I and II, and the results are summarized in Table IV.

Table IV shows that the difference of kinetic parameters between the LIV-II carriers of PAO and PML is found in the values of $K_i$, $K_i'$, and $K_i$ except $K_i$ and that it is much greater for $K_i$ value, small for $K_i'$ and $K_i''$, and negligible for $K_i''$. This suggests that the differences in the kinetic property between the two carrier systems result mainly from a difference in affinity of the carrier for cations, in particular, Na$^+$, rather than in its affinity for substrate. The value of $K_i$ for PAO is 30-fold smaller than for PML, whereas the $K_i$ value for PAO is approximately 3 times greater than for PML. It is likely that the affinity of the PAO carrier for Na$^+$ is much higher than the PML carrier, whereas the affinity of the carrier for Li$^+$ is slightly lower than that of the latter. However, $K_i$, $K_i'$, $K_i''$, and $K_i'''$ are not true dissociation constants but apparent ones, since they contain factors of the true dissociation constant and translocation rates of free and loaded carrier species. This leads us to the conclusion that the difference in Na$^+$ dependence of the LIV-II carrier system observed between PAO and PML strains is due to a difference in the affinity of the carrier for cation(s) and/or translocation rates of free carrier across the membrane since it is quantitatively expressed by $K_i$ values equal to $K_i(1 + P_{c}/P_{o})$.

Differences in $K_i$ and $K_i'$ values are larger than those in $K_i^v$ and $K_i^v$. This means that the effect of the amino acid substitution on apparent dissociation constants is large for Na$^+$-coupled transport but is small for Li$^+$-coupled transport, suggesting that the binding site for Na$^+$ may be separate from that for Li$^+$. This suggestion supports the idea that the Na$^+$ and Li$^+$ recognition sites in the melibiose carrier of E. coli seem to be in separate domains.

The nucleotide sequence of the $braB$ gene of PML was determined, and its amino acid sequence was deduced from the nucleotide sequence, which enables us to compare the amino acid sequence of the PML LIV-II carrier with that of the PAO carrier reported previously. The comparison of the amino acid sequences of the PAO and PML carriers shows that a difference in amino acid at position 292 occurs in the carrier and that it is threonine for PAO and alanine for PML. This amino acid substitution is considered to be a consequence of natural mutation in the PAO or PML strain, causing a marked difference between them in the Na$^+$ dependence of the LIV-II carrier. Since the amino acid substitution results in simultaneous changes in apparent dissociation constants $K_i^v$, $K_i^v$, $K_i$, and $K_i'$ with respect to Na$^+$, substrate, and Li$^+$ binding, respectively, a structural change that can cause the changes in $K_i^v$ values of the binding sites is considered to occur in the PML carrier compared with the PAO carrier. Threonine is an uncharged polar amino acid, and alanine is nonpolar. The size of side chain group of threonine is bigger than that of alanine. Decreases in polarity and ateric compactness at position 292 may bring about the structural change in the PML carrier.

The hydropathy profiles of the two LIV-II carriers calculated by the method of Kyte and Doolittle (19) indicate that the carriers contain 12 hydrophobic regions that are considered to be transmembrane segments. The amino acid at position 292 is located in the inside of the 8th segment starting from the amino terminus. A mutation of the proline carrier of E. coli with altered cation sensitivity of substrate-binding activity and a defect in the Na$^+$-coupling activity have been identified as an alteration of arginine to cysteine at amino acid position 257 in its primary structure (11). The hydropathy profile of the proline carrier shows that the location of the altered amino acid is in a hydrophilic loop connecting the 6th and 7th hydrophobic segments (30). On the other hand, substituted amino acids in the melibiose carrier of E. coli with altered carbon specificity are located in several hydrophobic regions of the carrier, playing an important role in H$^+(Li^+)$ recognition or H$^+(Li^+)$ transport by the carrier (9, 10). Our present results suggest that the structural change caused by the amino acid substitution at position 292 may occur inside the carrier.

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**REFERENCES**

Na$^+$ Dependence of LIV-II Carriers in P. aeruginosa Strains