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# Genetic and Biochemical Studies of Transport Systems for Branched-Chain Amino Acids in *Escherichia coli*

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Mutants of *Escherichia coli* K-12 requiring high concentrations of branched-chain amino acids for growth were isolated. One of the mutants was shown to be defective in transport activity for branched-chain amino acids. The locus of the mutation (*hrbA*) was mapped at 8.9 min on the *E. coli* genetic map by conjugational and transductional crosses. The gene order of this region is *proC-hrbA-tsx*. The *hrbA* system was responsible for the uptake activity of cytoplasmic membrane vesicles. It was not repressed by leucine. The substrate specificities and kinetics of the uptake activities were studied using cytoplasmic membrane vesicles and intact cells of the mutants grown in the presence or absence of leucine. Results showed that there are three transport systems for branched-chain amino acids, LIV-1, -2, and -3. The LIV-2 and -3 transport systems are low-affinity systems, the activities of which are detectable in cytoplasmic membrane vesicles. The systems are inhibited by norleucine but not by threonine. The LIV-2 system is also repressed by leucine. The LIV-1 transport system is a high-affinity system that is sensitive to osmotic shock. When the leucine-isoleucine-valine-threonine-binding protein is derepressed, the high-affinity system can be inhibited by threonine.

*Escherichia coli* K-12 is known to have specific, active transport systems for branched-chain amino acids. Biochemical studies of the systems, particularly on their substrate specificities (8, 19, 21), kinetics (9, 19), binding proteins (1, 2, 17), regulation (17, 18), and coupling energetics (12, 20), have revealed their heterogeneous nature. Several genes determining the components of the systems and their regulation have been identified (9, 10, 17). Although the classification and properties of the systems have been discussed in terms of substrate specificities (9, 19, 20) or functional interaction of the transport carrier with a periplasmic binding protein (21), the organization of the systems at a molecular level has not been elucidated, and there are some apparent contradictions in available data on this subject (3).

These discrepancies have occurred because we have little information on the genetic and biochemical properties of transport carriers in the cytoplasmic membrane. In a previous paper, we reported that isoleucine transport activity could be analyzed critically using cytoplasmic membrane vesicles prepared from *E. coli* K-12 (21).

This paper reports the isolation of mutants having a requirement for high concentrations of branched-chain amino acids for growth ($Hrb^-$). Among these strains, we found a mutant with altered transport carrier activity. The biochemical properties of transport carrier activities for branched-chain amino acids in these $Hrb^-$ mutants are described in detail. A preliminary account of this work has appeared (I. Yamato, H. Amanuma, and Y. Anraku, Japan Bioenergetics Group Abstr. 1:19-21, 1975).

## MATERIALS AND METHODS

**Bacterial strains and growth media.** The strains used in this study are listed in Table 1. Strain B76 was constructed as follows. Strain KE113 (*proB*), a generous gift from M. Tomoeda (11), was irradiated with UV light to induce mutations (15), and an isoleucine auxotroph mutant, strain 113-4, was selected after penicillin screening. A $Pro^+$ $Hrb^+$ exconjugate of strain 113-4 with 176/$F'_8$ was isolated. The exconjugate was again irradiated with UV light, and the $Pro^-$ mutants selected were tested for *proC* by complementation assay with $F'_{13}$. A *proC* mutant was transduced with P1*kc* phage grown on AB2277-1 (an isoleucine and valine prototroph [$Ilv^+$] revertant of AB2277), and an $Ile^+$ $Met^-$ transductant was selected. The $Ile^+$ $Met^-$ transductant was further transduced with P1*kc* phage grown on strain 176, and a $Met^+$ $Ilv^-$ (isoleucine and valine auxotrophy) transductant was isolated. A spontaneous mutant with resistance to T6 phage, strain B76, was isolated from one of the *proC* $Ilv^-$ $Met^+$ transductants.

Cells were grown in Davis minimal salts medium (7)

TABLE 1. *E. coli strains*

| Strain | Relevant genotype | Relevant phenotype[a] | Origin |
|---|---|---|---|
| W1-1 | F⁻ *leu-6 lacY1* | | Our collection |
| 176 | F⁻ *leu-6 lacY1* | Ilv⁻ Hrb⁻ | NTG[b] mutagenesis of W1-1 |
| KE113 | F⁻ *proB gal-2 rpsL* | | (11) |
| AN120 | F⁻ *uncA401* | | (6) |
| AB2277 | F⁻ *ilv metE rpsL* | | M. Abe |
| W3747 | $F'_{13}$ *met* | | Y. Hirota |
| E5014 | $F'_{128}$ | | H. Uchida |
| 3350$F_8$D | $F'_8$ *thy gal* | | Our collection |
| KY9216 | F'*lac ilvC7* | | Y. Hirota |
| B76 | F⁻ *rpsL tsx proC* | Ilv⁻ Hrb⁺ | Constructed in this study |
| B761 | F⁻ *rpsL tsx proC*⁺ | Ilv⁻ Hrb⁺ | Pro⁺ transductants of B76 with P1*kc* phage grown on strain 176 |
| B762 | F⁻ *rpsL tsx proC*⁺ | Ilv⁻ Hrb⁻ | |

[a] Ilv⁻, Isoleucine and valine auxotrophy; Hrb⁻, requirement for high concentrations of branched-chain amino acids to feed the existing auxotrophy in the strain's background.
[b] NTG, *N*-Methyl-*N*-nitro-*N'*-nitrosoguanidine.

supplemented with 0.5% glucose and 100 μg each of the required amino acids per ml, unless otherwise noted.

**Genetic studies.** Conjugational and transductional crosses were performed as described previously (16).

**Preparation of cytoplasmic membrane vesicles.** Cytoplasmic membrane vesicles were prepared by disrupting spheroplasts in a French press as described previously (22). Protein was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard.

**Assay of amino acid uptake by intact cells and cytoplasmic membrane vesicles.** Cells were grown in Davis minimal salts medium as mentioned above. Amino acid uptake activities of intact cells were measured by the method of Kobayashi et al. (12), with 10 mM glucose as an exogenous energy source. The uptake of substrate in the first 30 s was measured as the initial uptake activity. D-Lactate-driven uptake activity of the cytoplasmic membrane vesicles was assayed as described previously (21). The substrate was added at a concentration of 2 μM unless otherwise noted.

**Osmotic shock treatment.** Intact cells were treated by cold osmotic shock as described previously (4).

**Reagents.** L-[$^{14}$C]proline (169 mCi/mmol), L-[$^{14}$C]threonine (190 mCi/mmol), and L-[$^{14}$C]isoleucine (278 mCi/mmol) were purchased from Daiichi Chem. Co., Tokyo. D-Lactate ($Li^+$ salt) was obtained from Calbiochem, Inc. The amino acids used were all in the L-form. Other reagents used were commercial products of analytical grade.

## RESULTS

**Isolation of mutants requiring high concentrations of branched-chain amino acids and mapping of the mutation locus.** Mutants of amino acid transport activity have been isolated as cells having a phenotype of requirement for a high concentration of certain amino acids (14, 16). This phenotype seems to be more closely related with the defect of transport activity than does the phenotype of substrate analog resistance (14, 16). Thus, unlike Guardiola et al. (9), who isolated transport mutants as valine-resistant mutants, we attempted to obtain mutants requiring high concentrations of branched-chain amino acids (Hrb⁻ mutants).

After mutagenesis of a leucine auxotroph, strain W1-1, with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine and penicillin screening twice, 800 colonies of isoleucine- and valine-requiring mutants (Ilv⁻ mutants) were obtained. From these colonies, 27 strains were isolated as Hrb⁻ mutants. One of them, strain 176, could grow well in a minimal medium supplemented with 100 μg each of isoleucine and valine per ml, but could not grow with concentrations of 20 μg of these amino acids per ml.

The mutation locus was mapped to construct isogenic strains and to compare the mutation with those reported so far (9, 17). Results of conjugational and transductional crosses are summarized in Fig. 1. The Hrb⁻ mutation was near *lac* and *proB*, judging from the results of conjugational crosses (data not shown). The results of three-point transductional crosses showed that it was located at 8.9 min between *proC* and *tsx* (Fig. 1). A recipient strain B76

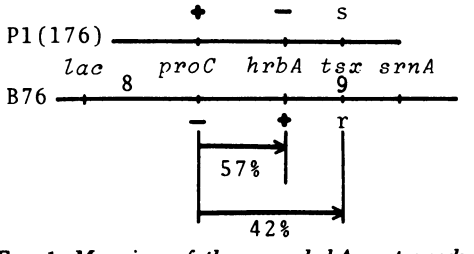


FIG. 1. *Mapping of the gene hrbA; cotransductional frequencies of hrbA and tsx with proC. Arrows indicate unselected markers. Distances are not drawn to scale.*

(*proC*⁻ *tsx*ʳ Ilv⁻ Hrb⁺) was transduced by P1*kc* phage grown on 176 (*proC*⁺ *tsx*ˢ Hrb⁻), and Pro⁺ transductants were selected. The Pro⁺ transductants were then examined for their phenotypes of Hrb and sensitivity to T6 phage. Of the Pro⁺ transductants, 42% (48/115) were T6 sensitive and 57% (65/115) were Hrb⁻ transductants, and of the T6-sensitive transductants only 2%, or 1 of 48, was Hrb⁺. With the usual assumption that this least frequent class arose from four crossing-over events, *hrbA* was placed between *proC* and *tsx*. Of the T6-resistant transductants, 27% (18/67) were Hrb⁻ and 73% (49/67) were Hrb⁺. Thus the locus of the gene (*hrbA*) responsible for the Hrb⁻ phenotype was calculated to be at 8.9 min in the genetic map of Bachmann et al. (5). Isogenic strains, B761 (*hrbA*⁺) and B762 (*hrbA*⁻), were isolated from the Pro⁺ T6ʳ transductants and used in the work described below. The *hrbA* locus is very close to *brnQ* reported by Guardiola et al. (9, 10), but is different from *brnQ* in the genetic map of Bachmann et al. (5). Moreover, Ilv⁺ transductants of strain 176 were not valine resistant even in the presence of leucine, although Guardiola et al. (9, 10) reported that this valine resistance was a characteristic of the phenotype of *brnQ*. Therefore, we cannot conclude definitely that this mutation is identical with *brnQ*, although the two seem to be very similar in terms of their genetic loci and transport properties. The mutation is tentatively called *hrbA* in this paper.

This mutation reverted spontaneously with a frequency of about $10^{-8}$, indicating that it is a point mutation. $F'_{13}$ could complement this mutation, indicating that the mutation was recessive. F′*lac* and $F'_{128}$ could not complement this mutation, consistent with the mutation locus.

Ilv⁻ mutation of strain 176 cotransduced with *metE* and *uncA* (6) with frequencies of 40 and 33%, respectively. This indicates that the Ilv⁻ mutation is in the *ilv* region. Ilv⁺ UncA⁻ transductants of B761 and B762 were isolated as BU1 and BU2, respectively. When *ilvC* of strain KY9216 was transduced to strain BU2, the phenotype of Hrb⁻ was conserved.

**Transport activities for branched-chain amino acids of the mutants.** Since a requirement for high concentrations of amino acids for growth may be attributed to a defect of transport activities (14, 16), we examined the uptake activities of strains B761 (Hrb⁺) and B762 (Hrb⁻) for branched-chain amino acids.

Figure 2 shows the kinetics of the uptakes of isoleucine and valine by strains B761 and B762 grown in the presence and absence of 90 μg of leucine per ml. The uptake activities of strain B762 grown in the presence of leucine were very low, and the $K_m$ values were 10 μM isoleucine and 60 μM valine (Table 2). On the other hand, the uptake kinetics of B761 grown in the presence of leucine showed a biphasic curve (Fig. 2) with two apparent $K_m$ values (Table 2). The lesion of *hrbA* is specific for branched-chain amino acid transport, because other amino acids, such as proline and threonine, were taken up equally well by *hrbA*⁻ and *hrbA*⁺ cells (data not shown). All the results mentioned above indicate that *hrbA* is responsible for the branched-chain amino acid transport system that is not repressed by leucine. It should be mentioned that in *hrbA* mutants the two transport components with different $K_m$ values disappeared simultaneously and a new transport component with a high $K_m$ value appeared. This strongly suggests that *hrbA* is a structural gene of the transport

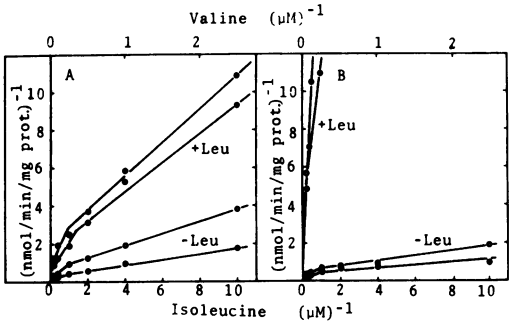


FIG. 2. *Double-reciprocal plots of isoleucine and valine uptake activities of strains B761 and B762. Concentrations of 0.1 to 5 μM isoleucine* (○) *and 0.4 to 20 μM valine* (●) *were used. (A) Strain B761 grown with (+Leu) or without (−Leu) leucine. (B) strain B762 grown with (+Leu) or without (−Leu) leucine.*

TABLE 2. *Kinetic parameters of branched-chain amino acid uptake in strains B761 and B762 grown in the presence of leucine*[a]

| Substrate | Strain B761 | | Strain B762 | |
|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (nmol/min per mg) | $K_m$ (μM) | $V_{max}$ (nmol/min per mg) |
| Leucine | 0.2 | 0.4 | | |
| | 2 | 1.3 | 20 | 1.0 |
| Isoleucine | 0.4 | 0.4 | | |
| | 3 | 1.2 | 10 | 0.5 |
| Valine | 1.4 | 0.5 | | |
| | 12 | 1.4 | 60 | 0.9 |

[a] Strains were grown in minimal medium supplemented with 0.5% glucose, 90 μg of leucine per ml, and 100 μg each of isoleucine and valine per ml. For assay of uptake by strain B762, concentrations of 0.5 to 50 μM leucine and isoleucine and 2 to 80 μM valine were used.

system(s) for branched-chain amino acids.

Strain B762 grown in the absence of leucine could take up isoleucine and valine as efficiently as strain B761 (Fig. 2), and its $K_m$ and $V_{max}$ values did not differ significantly from those of strain B761. These findings lead to the following conclusions. (i) Strain B762 (*hrbA* Hrb$^-$) has a transport system repressible by leucine as reported by Oxender and co-workers (17, 18). (ii) The transport activity for which the *hrbA* gene is responsible is expressed constitutively in the presence or absence of leucine, and supports growth of strain B761 in medium supplemented with 20 μg each of isoleucine and valine per ml. (iii) The inability of strain B762 to grow in medium supplemented with 20 μg each of isoleucine and valine per ml should be explained by causes other than differences in apparent initial velocities of uptake activities, because the apparent initial velocities of this strain did not differ significantly from those of strain B761 (see Fig. 2).

**Uptake activity of isoleucine by cytoplasmic membrane vesicles.** Cytoplasmic membrane vesicles prepared by the method of Yamato et al. (22) had high activity for uptake of branched-chain amino acids dependent upon energy derived from respiratory substrates. The coupling energy was shown to be a proton motive force (21). Table 3 summarizes the uptake activities of cytoplasmic membrane vesicles obtained from strains B761 and B762.

Cytoplasmic membrane vesicles of strain B761 grown in the presence and absence of leucine had almost the same uptake activities for isoleucine. These vesicles also showed the same uptake activities for proline. On the other hand, cytoplasmic membrane vesicles of strain B762 grown in the presence of leucine had no uptake activity for isoleucine but normal uptake activity for proline. Thus, we conclude that *hrbA* is responsible for the transport system detectable in cytoplasmic membrane vesicles. This uptake activity was inhibited by norleucine, but not by threonine (Fig. 3).

Cytoplasmic membane vesicles of strain B762 grown without added leucine showed significant uptake of isoleucine (0.016 nmol/min per mg of protein), and the $K_m$ value was 4 μM. As shown in Fig. 3, this activity was partially inhibited by norleucine, but not by threonine. Judging from these substrate specificities, this transport system is very similar to the transport system in the membrane vesicles from strain B761 described above, which is coded by *hrbA*. However, we consider that these two transport systems are not identical, because if they were, B762 (*hrbA*) should be Hrb$^+$ when grown without added leucine, and this was not the case. In addition, the transport activity in the membrane vesicles of strain B762 was shown to be repressed by leucine (Table 3).

TABLE 3. *Isoleucine and proline uptake activities of cytoplasmic membrane vesicles from strains B761 and B762 grown with or without leucine*[a]

| Cells and medium | Isoleucine (nmol/min per mg) | Proline (nmol/min per mg) |
|---|---|---|
| B761 | | |
| (−Leu) | 0.022 | 0.45 |
| (+Leu) | 0.015 | 0.30 |
| B762 | | |
| (−Leu) | 0.016 | 0.43 |
| (+Leu) | <0.002 | 0.34 |

[a] D-Lactate-dependent uptake was measured as described in the text. Cells were grown in minimal medium supplemented with 0.5% glucose and 100 μg each of isoleucine and valine per ml with (+Leu) or without (−Leu) 90 μg of leucine per ml.

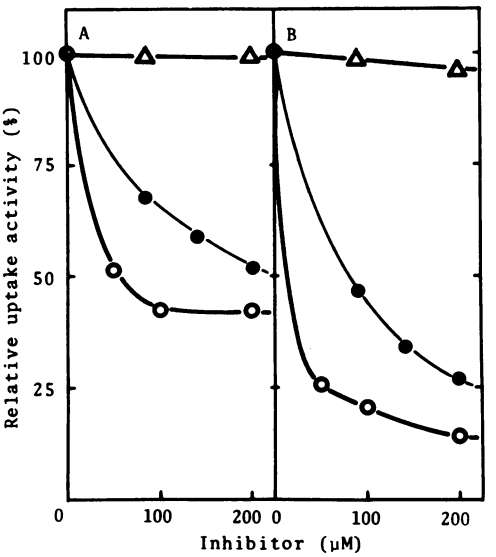


FIG. 3. *Inhibition of isoleucine uptake activity of cytoplasmic membrane vesicles from strains B761 and B762 by threonine, norleucine, and leucine. Growth conditions of the cells are described in the legend to Table 3. (A) Strain B762 grown in the absence of leucine. (B) Strain B761 grown in the presence of leucine. Inhibition of the activity by threonine (△), leucine (○), or norleucine (●).*

**Specificity of transport systems for branched-chain amino acids.** Transport systems for branched-chain amino acids have been classified in various ways (3, 17, 19). It was important to reinvestigate these systems using our mutants and analyzing not only substrate specificities but also other properties, such as

sensitivity to osmotic shock and coupling energy.

We found that threonine did not inhibit isoleucine uptake by strain B761 grown in the presence of leucine (data not shown). Synthesis of the leucine-isoleucine-valine-threonine-binding protein (LIVT-binding protein) is repressed by leucine (17). This protein is an important component of a high-affinity transport system for branched-chain amino acids. Therefore, it is conceivable that sensitivity to threonine depends primarily on the existence of LIVT-binding protein. As shown in Fig. 4, the double-reciprocal plot of the isoleucine uptake in the presence of 40 times more norleucine than isoleucine was parallel with that in the absence of norleucine. When the inhibitor-substrate ratio was held constant, as in the case of Fig. 4, this parallelism indicates that the inhibition is competitive (see Appendix). From Fig. 4, the $K_i$ values were calculated as 40 $\mu$M for the low-$K_m$ component and as 60 $\mu$M for the high-$K_m$ component. Considering the $K_m$ value (0.4 $\mu$M) of the low-$K_m$ component, we conclude that norleucine inhibition is slight ($K_i/K_m$ = 40/0.4 = 100) for this component, but high ($K_m$, 6 $\mu$M; $K_i/K_m$ = 60/6 = 10) for the high-$K_m$ component. Figure 4 also shows that osmotic shock greatly reduced the uptake activity of the low-$K_m$ component, but had little effect on that of the high-$K_m$ component, suggesting that the high-$K_m$ component was identical with the transport system detected in cytoplasmic membrane vesicles (cf. Fig. 3 and Table 3).

These differences in substrate specificities and sensitivities to osmotic shock suggest that the high-affinity transport system is independent of the low-affinity transport system. The fact that in *hrbA* cells both components disappeared simultaneously may be explained by a pleiotropic effect of *hrbA*, as is likely to be the case for mutations in the *brnR* and *brn8* loci (9). These possibilities will be discussed later. Hereafter, we designate the low-$K_m$ component as the LIV-1 system and the high-$K_m$ component as the LIV-3 system.

When strains B762 and 1761 (a Leu$^+$ revertant of strain 176 [*hrbA* Ilv$^-$]) were grown in the absence of leucine, the LIVT-binding protein could be detected with specific antibody by the Ouchterlony double-diffusion test (2). These cells showed biphasic kinetics in isoleucine uptake (Fig. 2 and 5). The activity of the high-$K_m$ component was hardly detectable in strain B762 (Fig. 2), and so strain 1761 was used for further studies. As shown in Fig. 5, threonine inhibited the activity of the low-$K_m$ component competitively ($K_i$ = 15 $\mu$M) and that of the high-$K_m$ component slightly. This slight inhibition of the high-$K_m$ component could not be due to specific inhibition of the low-$K_m$ component alone, because the $V_{max}$ value of the low-$K_m$ component was 0.2 nmol/min per mg of protein, whereas the extent of inhibition of uptake of 10 $\mu$M isoleucine by 400 $\mu$M threonine was three times more, or 0.6 nmol/min per mg of protein. Guardiola et al. reported that the high-$K_m$ component that is determined by *brnS* was inhibited by threonine (9).

As shown in Fig. 5, norleucine inhibited the low-$K_m$ component of strain 1761 slightly ($K_i$ = 40 $\mu$M), and the high-$K_m$ component greatly ($K_i$ = 60 $\mu$M). Since norleucine does not inhibit the

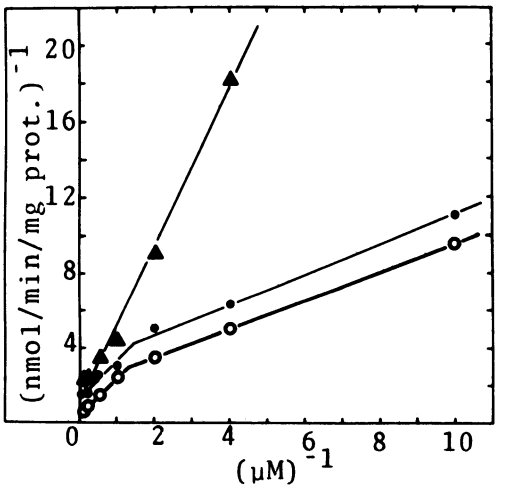


FIG. 4. *Double-reciprocal plots of isoleucine uptake activity of strain B761 grown in the presence of leucine. Growth conditions are described in the legend to Table 2.* (○) *Intact cells;* (●) *intact cells in the presence of 40 times more norleucine than isoleucine;* (▲) *osmotically shocked cells.*

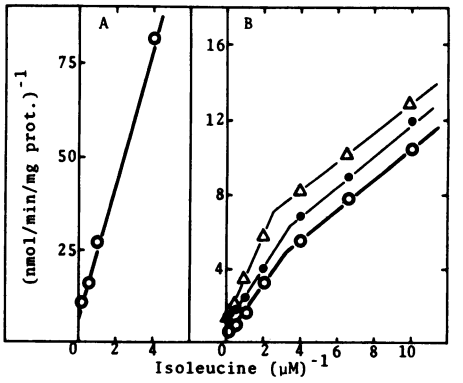


FIG. 5. *Double-reciprocal plots of isoleucine uptake activity of strain 1761 grown without added leucine. (A) Shocked cells. (B) Intact cells* (○)*; intact cells in the presence of 40 times more norleucine than isoleucine* (●)*; intact cells in the presence of 40 times more threonine than isoleucine* (△).

LIVT-binding protein (1), this indicates that norleucine inhibits carrier activity in the cytoplasmic membrane, as in Fig. 3 with strains B761 and B762. Upon osmotic shock the low-$K_m$ component disappeared almost completely and the activity of the high-$K_m$ component was reduced greatly (Fig. 5). Osmotic shock also causes release of the LIVT-binding protein (1, 4). Though the $V_{max}$ value of the uptake activity of the high-$K_m$ component observed in shocked cells was as low as that in intact cells grown in the presence of leucine, the $K_m$ value was significantly different from that in the intact cells. We found that the remaining uptake activity of the high-$K_m$ component was not inhibited by threonine (data not shown), as expected from the transport properties of cytoplasmic membrane vesicles from this strain (Fig. 3).

We designated the low-$K_m$ component of strain 1761 as the LIV-1′ system and the high-$K_m$ component as the LIV-2 system. Judging from its kinetic parameters, sensitivity to osmotic shock, and substrate specificities, the LIV-2 system seems to be identical with the activity detected in the cytoplasmic membrane vesicles from strain B762 (Table 3 and Fig. 3). The inhibitory effect of threonine on the uptake activity of the LIV-2 system suggests that the LIVT-binding protein stimulates this transport system effectively (see Fig. 5). However, it is possible that there is another transport system of low affinity, the LIV-2′ system, which is inhibited by threonine and norleucine, sensitive to osmotic shock, and repressed by leucine, although, if so, the LIV-2 system and the LIV-2′ system seem to be determined by a single gene, *brnS* (9). Furthermore, the available evidence (9, 20) suggests that the LIV-1 and LIV-1′ systems are identical, as will be discussed later.

## DISCUSSION

Several attempts have been made to explain the heterogeneous processes for transport of branched-chain amino acids in *E. coli* K-12 (3, 9, 19). In this work we isolated branched-chain amino acid transport mutants having a new phenotype and studied their genetics, substrate specificities, regulation, and sensitivity to osmotic shock. The mutation locus (*hrbA*) was found to be very close to the gene locus *brnQ* reported by Guardiola et al. (9, 10). Since the transport properties of the *hrbA* mutant so far examined were also very similar to those of the *brnQ* mutants, it seems very likely that *hrbA* is actually identical with *brnQ*, though its locus is different on the genetic map of Bachmann et al. (5).

Biochemical studies with the present mutants clearly demonstrated the existence of three distinct transport systems for branched-chain amino acids with characteristic properties. The LIV-1 system is an osmotic shock-sensitive, high-affinity transport system that is conditionally stimulated by the LIVT-binding protein. The two other systems (LIV-2 and -3), one of which is repressed by leucine (LIV-2), are detectable in cytoplasmic membrane vesicles. As mentioned in Results, the kinetics of the transport activities in the mutants apparently indicate the existence of five transport systems, LIV-1, LIV-1′, LIV-2, LIV-2′, and LIV-3. However, adopting published data with some changes in their interpretations, we prefer to consider that there are three transport systems.

In Table 4, summarizing the properties of these systems, we have tentatively attempted to correlate the systems with *brnR, S,* and *Q* (9), assuming that *hrbA* corresponds to *brnQ*. For the LIV-2 and LIV-3 systems, we have listed $K_m$ values obtained with cytoplasmic membrane vesicles. The LIV-1 system has a low $K_m$ (0.4 μM) (Fig. 2, 4, and 5). Kinetically, the existence of two high-affinity systems, LIV-1 and LIV-1′, is conceivable, but the following lines of evidence favor our conclusion. First, their uptake activities are sensitive to osmotic shock and less sen-

TABLE 4. *Characteristics of the transport systems for isoleucine*

| Transport system | $K_m$ (μM) | Energy source | Osmotic shock | Norleucine | Threonine | LIVT-binding protein | Repression by leucine | Gene |
|---|---|---|---|---|---|---|---|---|
| LIV-1 | 0.4 | ATP | Sensitive | Less inhibited | —[a] | Conditionally stimulated | Not repressed | *brnR* |
| LIV-2 | 4 | Proton motive force | Resistant | Inhibited | Not inhibited | Effectively stimulated | Repressed | *brnS* |
| LIV-3 | 6 | Proton motive force | Resistant | Inhibited | Not inhibited | (?) | Not repressed | *hrbA brnQ*[b] |

[a] When the LIVT-binding protein is derepressed, the LIV-1 system can be inhibited by threonine.
[b] We assume that *hrbA* is identical with *brnQ*, as described in the text.

sitive to norleucine inhibition. The inhibitory effect of threonine can be attributed to the existence of the LIVT-binding protein. Second, strain B761 grown in the absence of leucine, which should have all the transport systems and binding proteins, showed biphasic uptake kinetics in which the low-$K_m$ component was inhibited competitively by threonine (data not shown). This suggests that all the low-$K_m$ component(s) is coupled obligatorily with the LIVT-binding protein. Third, Guardiola et al. reported that a single gene *brnR* is responsible for the very-high-affinity transport system (9). Fourth, Wood reported that the high-$K_m$ component(s) of the transport system coupled with the proton motive force and that the low-$K_m$ component(s) coupled with an intracellular concentration of ATP (20). The LIV-2 system has a high $K_m$ (4 $\mu$M) and is resistant to osmotic shock. The possibility of the existence of a LIV-2′ system that is sensitive to osmotic shock is unlikely, because a single gene *brnS* is suggested to be responsible for both the LIV-2 and LIV-2′ systems (9). The LIV-3 system also has a high $K_m$, is resistant to osmotic shock, and is determined by the *hrbA* gene. All three transport systems are specific for leucine, isoleucine, and valine. These conclusions are consistent with all our results on the transport activities of the mutants obtained with preparations of both cytoplasmic membrane vesicles and shocked cells. It should be noted that the LIV-1 system can be distinguished kinetically only in intact cells.

Though a defect of the gene *hrbA* affects both the LIV-1 and LIV-3 transport systems (see above), these two systems seem to be independent, judging from their substrate specificities and sensitivities to osmotic shock. The pleiotropic effect of the *hrbA* mutation on the LIV-1 and LIV-3 systems may be interpreted as the result of some unknown interaction between the transport components determined by the *hrbA* and *brnR* genes, as suggested by Guardiola et al. (9). If this were the case, an alternative classification may be possible, as shown in Table 5. Here, we assume that the LIV-A system is constructed from the LIV-1 and LIV-3 systems where the low-$K_m$ component of the system is determined primarily by the gene *brnR*, but the system requires a product of the gene *hrbA* for its function. The LIV-B system is assumed to be constructed from the LIV-1′ and LIV-2 systems and requires the LIVT-binding protein as mentioned above (see Table 4). Here, the gene *brnS* may determine the LIV-2 system but not the LIVT-binding protein, though Guardiola et al. suggested that the *brnS* gene may code for the LIVT-binding protein (9).

Rahmanian and co-workers reported the existence of three independent transport systems, Ls, LIV-I, and LIV-II, by studying the transport systems kinetically using intact cells (19). The Ls and LIV-I transport systems were suggested to be high-affinity systems, and the LIV-II system was to be a low-affinity one. In the present work, although we did not examine the Ls transport system specifically, no difference in the substrate specificities of the transport carriers in the cytoplasmic membrane was detected using intact cells and cytoplasmic membrane vesicles. Furthermore, the mutation of *brnR* was suggested to affect the high-affinity systems pleiotropically (9); thus, we prefer to interpret their data as described previously (3). The Ls and LIV-I transport systems may correspond to our LIV-1 system, and the apparent differences in the substrate specificities of the Ls and LIV-I systems may be due to differences in the binding proteins that may couple conditionally to the LIV-1 system. The LIV-II transport system corresponds to our LIV-2 and LIV-3 systems.

Wood reported that the energy that drives the LIV-I and Ls systems depends on the intracellular concentration of ATP and that the LIV-II system depends on a proton motive force (20). We found that the LIV-2 and LIV-3 systems are detectable in cytoplasmic membrane vesicles and are coupled with a proton motive force as an energy source. These findings strongly suggest that the LIV-1 system receives energy through a mechanism that is coupled with the intracellular concentration of ATP. We are now investigating this interesting mechanism.

The activity for isoleucine uptake by the membrane vesicles from strain B762 grown without added leucine was only partially inhibited by norleucine or leucine (Fig. 3A). Interestingly, the remaining uptake activity was inhibited only slightly by a large excess of isoleucine, suggesting the existence of a nonspecific transport process conducting a significant flux of isoleucine in the membrane vesicles. However, no such nonspe-

TABLE 5. *Alternative classification of the transport systems for isoleucine*

| Transport system | | $K_m$ ($\mu$M) | Gene | Repression by leucine |
|---|---|---|---|---|
| LIV-A | LIV-1 | 0.4 | *brnR-hrbA* | Not repressed |
| | LIV-3 | 6 | *hrbA* | Not repressed |
| LIV-B | LIV-1′ [a] | 0.4 | *brnR-brnS* | Repressed |
| | LIV-2 | 4 | *brnS* | Repressed |

[a] The LIV-1′ system corresponds to the low-$K_m$ component of the uptake activity in strain 1761 grown without added leucine, as described in the text (see Fig. 5).

cific uptake activity was observed for proline uptake by the membrane vesicles from the same strain or for isoleucine uptake by the membrane vesicles from strain B761 grown in the presence of leucine (Fig. 3B). At present we have no explanation for the mechanism of this nonspecific transport process.

We have noticed that *hrbA* Ile$^-$ strains can grow with 20 μg of isoleucine per ml but cannot grow with a higher concentration of 90 μg of leucine per ml (i.e., when transport activities are repressed). In contrast, *hrbA*$^+$ Ile$^-$ strains can grow with 20 μg of isoleucine and 90 μg of leucine per ml. Strain B762 can grow with 20 μg of isoleucine and 40 μg of valine per ml, or with 20 μg each of isoleucine and valine and 9 μg of leucine per ml. These observations suggest that the LIV-2 system, in contrast to the LIV-3 system, cannot accumulate sufficient valine for the biosynthesis of leucine. As mentioned in Results, practically no differences were found in the substrate specificities and uptake activities of the LIV-2 and LIV-3 systems. Thus the inability of strain B762 (*hrbA*) to grow in medium supplemented with 20 μg each of isoleucine and valine per ml may be due to differences in efficiencies of energy coupling of the two transport systems.

## APPENDIX

In an enzymatic reaction of a simple Michaelis-Menten type, the initial velocity ($v$) of the reaction is represented as a function of the substrate concentration ([$S$]), as follows:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

and the reciprocal of the $v$ is given:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

When a competitive inhibitor is present at concentrations in a constant ratio ($\alpha$) to substrate concentrations, the reciprocal of the $v$ is written:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} + \frac{\alpha \cdot K_m}{V_{max} \cdot K_i}$$

Then the double-reciprocal plot in the presence of a competitive inhibitor is parallel with that in the absence of the inhibitor (see Fig. 4 and 5).

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### LITERATURE CITED

1. **Amanuma, H., and Y. Anraku.** 1974. Transport of sugars and amino acids in bacteria. XII. Substrate specificities of the branched chain amino acid-binding proteins of *Escherichia coli*. J. Biochem. **76**:1165–1173.
2. **Amanuma, H., J. Itoh, and Y. Anraku.** 1976. Transport of sugars and amino acids in bacteria. XVII. On the existence and nature of substrate amino acids bound to purified branched chain amino acid-binding protein of *Escherichia coli*. J. Biochem. **79**:1167–1182.
3. **Anraku, Y.** 1978. Transport of amino acids, p. 171–219. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, New York.
4. **Anraku, Y., H. Kobayashi, H. Amanuma, and A. Yamaguchi.** 1973. Transport of sugars and amino acids in bacteria. VII. Characterization of the reaction of restoration of active transport mediated by binding protein. J. Biochem. **74**:1249–1261.
5. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
6. **Butlin, J. D., G. B. Cox, and F. Gibson.** 1971. Oxidative phosphorylation in *Escherichia coli* K12. Mutations affecting magnesium ion- or calcium ion-stimulated adenosine triphosphatase. Biochem. J. **124**:75–81.
7. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin $B_{12}$. J. Bacteriol. **60**:17–28.
8. **Furlong, C. E., and J. H. Weiner.** 1970. Purification of a leucine-specific binding protein from *Escherichia coli*. Biochem. Biophys. Res. Commun. **38**:1076–1083.
9. **Guardiola, J., M. De Felice, T. Klopotowski, and M. Iaccarino.** 1974. Mutations affecting the different transport systems for isoleucine, leucine, and valine in *Escherichia coli* K-12. J. Bacteriol. **117**:393–405.
10. **Guardiola, J., and M. Iaccarino.** 1971. *Escherichia coli* K-12 mutants altered in the transport of branched chain amino acids. J. Bacteriol. **108**:1034–1044.
11. **Hirota, Y., M. Inuzuka, and M. Tomoeda.** 1966. Elective selection of proline-requiring mutants. J. Bacteriol. **91**:2392.
12. **Kobayashi, H., E. Kin, and Y. Anraku.** 1974. Transport of sugars and amino acids in bacteria. X. Sources of energy and energy coupling reactions of the active transport systems for isoleucine and proline in *E. coli*. J. Biochem. **76**:251–261.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Lubin, M., D. M. Kessel, A. Burdreau, and J. D. Gross.** 1960. The isolation of bacterial mutants defective in amino acid transport. Biochim. Biophys. Acta **42**:535–538.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 121–124. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Motojima, K., I. Yamato, and Y. Anraku.** 1978. Proline transport carrier-defective mutants of *Escherichia coli* K-12: properties and mapping. J. Bacteriol. **136**:5–9.
17. **Oxender, D. L., J. J. Anderson, M. M. Mayo, and S. C. Quay.** 1977. Leucine binding protein and regulation of transport in *E. coli*. J. Supramol. Struct. **6**:419–431.
18. **Quay, S. C., R. P. Lawther, G. W. Hatfield, and D. L. Oxender.** 1978. Branched-chain amino acid transport regulation in mutants blocked in tRNA maturation and transcriptional termination. J. Bacteriol. **124**:683–686.
19. **Rahmanian, M., D. R. Claus, and D. L. Oxender.** 1973. Multiplicity of leucine transport systems in *Escherichia coli* K-12. J. Bacteriol. **116**:1258–1266.
20. **Wood, J. W.** 1975. Leucine transport in *Escherichia coli*. The resolution of multiple transport systems and their coupling to metabolic energy. J. Biol. Chem. **250**:4477–4485.

21. **Yamato, I., and Y. Anraku.** 1977. Transport of sugars and amino acids in bacteria. XVIII. Properties of an isoleucine carrier in the cytoplasmic membrane vesicles of *Escherichia coli*. J. Biochem. **81**:1517–1523.

22. **Yamato, I., Y. Anraku, and K. Hirosawa.** 1975. Cytoplasmic membrane vesicles of *Escherichia coli*. I. A simple method for preparing the cytoplasmic and outer membranes. J. Biochem. **77**:705–718.