Mechanism and Energetics of Dipeptide Transport in Membrane Vesicles of *Lactococcus lactis*

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Alanyl-α-glutamate transport has been studied in *Lactococcus lactis* ML2 cells and in membrane vesicles fused with liposomes containing beefheart cytochrome c oxidase as a proton-motive-force-generating system. The uptake of Ala-Glu observed in de-energized cells can be stimulated 26-fold upon addition of lactate. No intracellular dipeptide pool could be detected in intact cells. In fused membranes, a 40-fold accumulation of Ala-Glu was observed in response to a proton motive force. Addition of ionophores and uncouplers resulted in a rapid efflux of the accumulated dipeptide, indicating that Ala-Glu accumulation is directly coupled to the proton motive force as a driving force. Ala-Glu uptake is an electrogenic process and the dipeptide is transported in symport with two protons. In both fused membranes and intact cells the same affinity constant (0.70 mM) for Ala-Glu uptake was found. Accumulated Ala-Glu is exchangeable with externally added alanyl-glutamate, glutamyl-glutamate, and leucyl-leucine, while no exchange occurred upon addition of the amino acid glutamate or alanine. These results indicate that the Ala-Glu transport system has a broad substrate specificity.

Lactococci (previously named group N lactic streptococci [28]) are multiple-amino-acid auxotrophs and require an exogenous nitrogen source for growth. Most strains of *Lactococcus lactis* can use amino acids or peptides or both to satisfy this requirement (14). The growth yield and specific growth rate of some *L. lactis* strains can be increased when peptides are supplied instead of amino acids (R. Otto, Ph.D. thesis, University of Groningen, Groningen, The Netherlands, 1981). In other strains no stimulation of growth on di- and tripeptides was observed. In the presence of peptides longer than three amino acids, growth was even inhibited (14).

Different models for the mechanism of peptide utilization have been proposed (18, 19). One of these models involves extracellular hydrolysis of peptides into amino acids which are subsequently taken up via specific amino acid transport systems. This mechanism of peptide utilization has been ruled out for most of the various peptides studied since high extracellular concentrations of the amino acids present in the peptides did not inhibit the uptake of the peptides (14, 25, 34). These studies do not discriminate between a model in which the peptide is transported and subsequently hydrolyzed by an intracellularly located peptidase and a model in which peptide transport is coupled to peptide hydrolysis (a transmembrane peptidase).

Studies on the mechanism of energy coupling of peptide transport in intact cells have shown that it is often very difficult to draw conclusions about the mode of energy coupling to peptide transport (33, 34). Transport studies in lactococci have shown that uptake of certain dipeptides is an energy-requiring process that can be inhibited by protonophores and inhibitors of the membrane-bound ATPase (14, 25, 33, 34). The precise mechanism of energy coupling of peptide uptake, however, is still not fully solved. The results obtained with *L. lactis* subsp. cremoris suggested that the energy for leucyl-leucine transport is supplied directly by the hydrolysis of an energy-rich phosphate bond, presumably ATP (34). A more detailed analysis of peptide transport in lactococci would require uptake studies in the absence of peptidase activity. In principle, membrane vesicles offer such an experimental system.

We have studied dipeptide transport in membrane vesicles of *L. lactis* fused with liposomes containing beef heart cytochrome *c* oxidase as a proton-motive-force (PMF)-generating system (3, 4). In these fused membranes, accumulation of intact alanyl-α-glutamate appears to be driven by the PMF. Evidence is presented that peptide transport is not directly coupled to peptide hydrolysis. The Ala-Glu transport system has affinity for both dipolar ionic dipeptides such as leucyl-leucine and negatively charged dipeptides such as alanyl-glutamic acid and glutamyl-glutamic acid.

**MATERIALS AND METHODS**

**Culture conditions.** Cultures of *L. lactis* ML2, were maintained in 10% (wt/vol) reconstituted skim milk containing 0.1% (wt/vol) tryptone (Difco Laboratories, Detroit, Mich.) and stored at −20°C. For transport studies, organisms were transferred from the milk cultures to a complex medium (2), pH 6.4, containing 0.5% (wt/vol) lactose as the carbon and energy source and incubated overnight at 30°C.

**Transport assays with intact cells.** Cells were harvested by centrifugation and washed twice in a buffer containing 50 mM potassium 2[N-morpholino]ethanesulfonic acid (MES), pH 6, and 5 mM MgSO4. Cells were de-energized according to the procedure described by Poolman et al. (23). After de-energization, cells were washed twice and resuspended in 100 mM potassium-MES–100 mM potassium 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 6–5, mM MgSO4. Transport assays were routinely performed at 28°C in 0.2 ml of incubation mixture. After addition of 2 μl of concentrated cell suspension, lactose was added to a final concentration of 0.4% (wt/vol). After 3 min of pre-energization with lactose, uptake was initiated by the addition of the radioactively labeled compounds as indicated in the figure legends. After incubation, the uptake reaction was stopped by the addition of 2 ml of 100 mM LiCl, filtered on a 0.45-μm
cellulose-nitrate filter (Schleicher & Schuell, Keene, N.H.), and washed again with 2 ml of 0.1 M LiCl. Filters were dried, and radioactivity was measured by liquid scintillation spectrometry.

**Determination of intracellular amino acid pools.** A concentrated washed cell suspension was diluted in 1 ml of 100 mM potassium-MES, pH 6.0–10 mM MgSO₄ to a final concentration of 1 mg of protein per ml. After energization of the cells with 0.4% (wt/vol) lactose, the cell suspension was incubated at 25°C in the presence of 1 mM alanyl-glutamate. At different time intervals, cells were separated from the buffer by silicon oil centrifugation (31). Extraction of the cells was performed as described before (24). Amino acids and peptides were analyzed after derivation with dansyl chloride, using methods essentially described by Tapuh et al. (30) and Wiedmeier et al. (35). The dansylated neutralized cell extracts were separated by reversed-phase high-performance liquid chromatography as described previously (24).

**Preparation of vesicles, liposomes, and fused membranes.** Membrane vesicles of *L. lactis* ML₃ were prepared as described by Otto et al. (17). Cytochrome-c-oxidase-containing liposomes were prepared by the procedure described by Driessen et al. (3). Proteoliposomes were prepared with L-α-phosphatidylethanolamine type IX from *Escherichia coli* obtained from Sigma Chemical Co., St. Louis, Mo., which was further purified by the method described by Kawaga et al. (11). Fusion between the membrane vesicles and the cytochrome-c-oxidase-containing liposomes was performed with the freeze-thaw–sonication procedure described by Driessen et al. (3).

**Transport assay in fused membranes.** The fused membranes were collected by centrifugation (280,000 × g, 45 min, 10°C) and washed once in a buffer containing 50 mM potassium phosphate (pH 6.1). The concentrated membrane suspension (5 to 8 mg of protein per ml) was diluted in 2 ml of oxygen-saturated buffer containing 50 mM potassium phosphate, pH 6.1, 0.2 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMFD), and 20 μM cytochrome c to a final concentration of 0.25 mg of protein per ml. Radioactively labeled Ala-Glu was added 2 min after the addition of 10 mM ascorbate (adjusted to the buffer pH with 10 N KOH). During incubation, the reaction mixture was gently stirred while water-saturated oxygen was flushed over the solution. At given time intervals, samples of 0.15 to 0.2 ml were taken and diluted in 2 ml of ice-cold 0.1 M LiCl. Subsequently, samples were filtered immediately on 0.45-μm cellulose-nitrate filters and washed again with 2 ml of ice-cold 0.1 M LiCl.

**Determination of electrical potential.** The electrical potential across the membrane (inside negative) was determined from the distribution of the lipophilic cation tetraphenylphosphonium (Ph₄P⁺) by using a Ph₄P⁺-selective electrode (29). Instead of radioactively labeled dipeptides, 2 μM Ph₄P⁺ was added to the reaction mixture described above for the transport assay. The membrane potential was calculated from the steady-state level of Ph₄P⁺ accumulation and was corrected for concentration-dependent binding of the probe to membranes (15). The pH gradient (ΔpH (millivolts)) = −2ΔpH across the membrane was calculated from the increase of the membrane potential upon addition of nigericin, assuming a complete interconversion of the pH gradient into the membrane potential (5).

**Synthesis of L-alanyl-o-L-[14C]glutamate.** The specific activity of 10 ml of radioactively labeled glutamic acid (285 mCi/mmol) was decreased fivefold by the addition of 4-mol equivalents of unlabeled glutamic acid. The pH of the solution was adjusted to approximately pH 8 with NaOH (2-mol equivalents compared with the amount of glutamic acid). Subsequently, the solution was concentrated by evaporation to a final volume of 0.15 ml, and an equimolar amount of N-butylxycarbonylalanine pentfluorophenyl ester dissolved in dimethylformamide (60 mg/ml) was added. N-Butyloxy carbonylalanine pentfluorophenylester was synthesized from butyloxy carbonyl alanine as described by Kisfaludy et al. (12). After addition of dimethylformamide to a final concentration of 60% (vol/vol), the reaction mixture was gently vortexed and incubated for 1.5 h at room temperature. After evaporation of the solvent and drying, 0.5 ml of ethyl acetate and aqueous 0.1 N HCl were added, resulting in a biphasic system. The ethyl acetate layer was extracted with 0.1 N HCl, separated from the water phase, and mixed, for removal of the butyloxy carbonyl group, with 75 to 90 μl of ethyl acetate (2.6 N HCl). Next the ethyl acetate fraction was gently vortexed and incubated for 20 min at room temperature. Finally, the ethyl acetate was evaporated and the product was dissolved in 5 ml of distilled water. The yield of the synthesis, as determined from counting the radioactivity of the sample, was about 12%. The purity of the sample was determined by analysis of the preparation on reversed-phase high-performance liquid chromatography and showed that no detectable levels of glutamate were present in the preparation.

**Calculations.** The apparent proton/dipeptide stoichiometry of the uptake process was calculated by using the equation

\[ n_{app} = \frac{(\Delta p - \Delta p_{pep})}{\Delta p}, \]

where Δp represents the membrane potential, Δp represents the PMF, and Δp_{pep} represents the electrochemical potential of the peptide.

**Chemicals.** [U-14C]Glutamate (285 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. All other chemicals were reagent grade and were obtained from commercial sources.

**Miscellaneous.** Protein was determined by the method of Lowry et al. (16) in the presence of sodium dodecyl sulfate (7), using bovine serum albumin as a standard.

**RESULTS**

Dipeptide uptake by intact cells. For detailed transport studies, it is necessary to use radioactively labeled substrates. Uptake of peptides is in most cases followed by rapid efflux of amino acids liberated by intracellular peptidase activity (20, 33). This exit of radiolabeled amino acids results in an underestimation of the rates of peptide uptake (9). To avoid this problem, L-alanyl-o-L-[14C]glutamate (Ala-Glu) was used. The advantage of using a dipeptide with the radiolabel on the glutamate residue is that efflux of glutamate is a very slow process in *L. lactis* (23, 24). In de-energized cells of *L. lactis*, uptake of Ala-Glu occurs at a slow rate (Fig. 1). As a control, the transport of radiolabeled glutamate was measured. No uptake of this amino acid could be detected under de-energized conditions (Fig. 1). However, a high rate of glutamate uptake was observed under energized conditions. Upon addition of the fermentable sugar lactose, the initial uptake rate of Ala-Glu increased 26-fold, indicating that uptake of the dipeptide requires metabolic energy. This metabolic energy requirement makes the operation of a transmembrane peptidase in the translocation process unlikely. The absence of glutamic acid uptake in contrast to the slow rate of Ala-Glu uptake under de-energized conditions indicates that Ala-Glu is not hydrolyzed by extracellular peptidase activity prior to uptake.

**Intracellular pools.** The slow uptake of Ala-Glu by de-energized cells could possibly be driven by an inwardly
directed Ala-Glu gradient maintained by rapid hydrolysis of the dipeptide internally or by hydrolysis directly coupled to transport via a transmembrane peptidase. To investigate the presence of peptidase activity, we determined the intracellular pools of L. lactis upon addition of Ala-Glu acid. L. lactis energized by lactose rapidly accumulated both glutamate and alanine (Fig. 2). No free intracellular pool of Ala-Glu could be detected in either energized or deenergized cells (data not shown). These results indicate that Ala-Glu is rapidly hydrolyzed by either an intracellular peptidase or a transmembrane peptidase.

**Dipeptide transport in membrane vesicles.** To differentiate between a model for dipeptide uptake via a transmembrane peptidase and a system including a dipeptide carrier and an intracellular peptidase, peptide transport was studied in a system lacking cytoplasmic peptidase activity. For this purpose membrane vesicles were isolated from L. lactis ML3 and fused with liposomes containing mitochondrial beef heart cytochrome c oxidase to incorporate a PMF-generating system (3, 4). With the electron donor ascorbate-cytochrome c-TMPD, a PMF, inside negative and alkaline, could be sustained for more than 60 min. Figure 3 shows the uptake of radioactively labeled Ala-Glu in these fused membranes. Comparable results were found with the dipolar ionic dipeptide leucyl-leucine (data not shown). Upon addition of the electron donor system ascorbate-cytochrome c-TMPD, a 40-fold accumulation of Ala-Glu could be observed. No accumulation of the dipeptide could be detected in the absence of a complete electron donor system (Fig. 3). Valinomycin, a potassium ionophore, which in the presence of K⁺ dissipates the membrane potential, decreased the Ala-Glu uptake rate and the steady-state accumulation level. Dissipation of the Δψ by the addition of both valinomycin and nigericin (a potassium/proton exchanger) (Fig. 4) caused rapid efflux of the accumulated label to equilibrium, indicating that transport and accumulation of Ala-Glu is directly coupled to the PMF. Furthermore, this observation rules out the operation of a transmembrane peptidase because glutamate exit cannot be mediated by the glutamate/glutamine transport system and therefore the extruded label must be Ala-Glu. When the fused membranes were not energized, Ala-Glu uptake continued up to equilibration (Fig. 4), indicating that these membrane vesicles lack intravesicular peptidase activity. Direct measurements of the peptidase activity on the fused membrane preparation confirmed this conclusion (data not shown).

**Role of Δψ and ΔpH in dipeptide transport.** The role of the components of the Δp in the uptake of Ala-Glu was investigated further. The initial uptake rate of Ala-Glu decreased after dissipation of the membrane potential with the potassium ionophore valinomycin (Table 1 and Fig. 3). Dissipation of the transmembrane pH gradient with nigericin had no significant effect on the Ala-Glu transport activity. Addition of both ionophores completely abolished transport activity. The same result was achieved by adding a sufficient amount of the uncoupler carbonylcyanide m-chlorophenylhydrazone, indicating direct coupling of peptide accumulation to the PMF with H⁺ as the symported cation. Inhibition of transport by valinomycin indicates that this is an electrogenic process. Because Ala-Glu is negatively charged at the experimental pH, a proton/substrate stoichiometry of >1 has to be assumed. The observation that nigericin does not inhibit peptide uptake can be explained by the fact that, upon addition of nigericin, complete interconversion of the PMF gradient into the membrane potential occurs (5). Therefore, only a minor effect on the total driving force for Ala-Glu accumulation can be expected.

For a quantitative evaluation of the proton/peptide stoichiometry, the accumulation ratio of Ala-Glu in response to the Δp was determined. A membrane potential of ~83 mV could be measured (Fig. 3). Addition of nigericin resulted in an increase of the Δψ of ~20 mV, indicating the presence of a pH gradient across the membrane of approximately the same magnitude. Assuming that the peptide accumulation is at equilibrium with the Δp, a proton/peptide stoichiometry of 1.7 to 1.8 could be calculated (see Materials and Methods for calculation).

**Kinetics of dipeptide transport.** Kinetic studies of Ala-Glu uptake have been performed in intact cells and in fused membranes. The initial rates of Ala-Glu uptake studied over a wide concentration range (0.84 to 1,500 μM) showed single phasic Michaelis-Menten-type saturation kinetics (Fig. 5), indicating the presence of one kinetically distinguishable Ala-Glu uptake system. At pH 6.1, the Michaelis-Menten constants (Kₜ) for Ala-Glu uptake were 0.69 and 0.70 mM in intact cells and fused membranes, respectively. Vₘₜₚ values of, respectively, 36 and 21 nmol/min per mg of protein were found.
The identical $K_i$ values for Ala-Glu uptake in intact cells and fused membranes show that the Ala-Glu transport system has retained its properties in the fused membrane system. 

**Substrate specificity.** The substrate specificity of the dipeptide transport system has been determined by studying the release of intravesicularly accumulated radioactively labeled Ala-Glu upon addition of an excess of unlabeled amino acids and dipeptides (Fig. 6). No release could be observed upon addition of an excess of unlabeled glutamic acid or alanine. The accumulated dipeptide, however, could be chased by an excess of unlabeled Ala-Glu (homologous exchange), Glu-Ala, Glu-Glu, and Leu-Leu (heterologous exchange). The observation that monoanionic (Ala-Glu and Glu-Ala), dianionic (Glu-Glu), and dipolar ionic (Leu-Leu) dipeptides are exchangeable with intravesicular Ala-Glu indicates a broad substrate specificity of this peptide transport system. To confirm this, we determined the inhibition kinetics of Leu-Leu on Ala-Glu transport. Ala-Glu uptake was competitively inhibited by leucyl-leucine with a $K_i$ of about 80 $\mu$M (data not shown). These results show that both dipeptides are transported via the same transport system.

**DISCUSSION**

This report is the first in which bacterial dipeptide transport has been studied successfully in isolated membranes. The studies have been performed in membrane vesicles of the fermentative bacterium *L. lactis* in which cytochrome $c$ oxidase has been incorporated by membrane fusion. Fused membranes have two major advantages over intact cells for the study of dipeptide transport. First, dipeptide transport and accumulation can be studied in the absence of peptidase activity, and second, direct information about the role of the $\Delta\psi$ as a driving force for dipeptide transport can be obtained.

Accumulation of dipeptide in fused membrane vesicles is shown to be driven by the PMF. Dipeptide utilization by *L. lactis* thus takes place by a two-step process. The first step is the translocation of the dipeptide across the cytoplasmic membrane via a specific dipeptide transport system. The second step is hydrolysis of the dipeptide by an intracellularly located dipeptidase to free amino acids, which subsequently are used by the organism for protein synthesis. The observations rule out the functioning of a transmembrane peptidase system which couples dipeptide transport directly to peptide hydrolysis.

Dipeptide utilization via a system in which an extracellular dipeptidase is functional in concert with amino acid transport systems does not play a significant role since dipeptide accumulation occurs in fused membranes and dipeptide uptake by intact cells is not inhibited by an excess of amino acids which are present in the dipeptide. Under conditions in...
transport system becomes questionable in view of our observations that Leu-Leu competitively inhibits Ala-Glu uptake and that Δp-dependent Leu-Leu uptake occurs in fused membranes. ATP could influence Δp-driven transport via the H⁺-ATPase, which is in these organisms the predominant way to generate a Δp. We conclude that both Leu-Leu and Ala-Glu are transported via only one system and that active transport of both peptides is linked to the PMF. Slow uptake of dipeptides in energy-depleted cells, i.e., in the absence of a PMF, can now be explained by transport via the secondary dipeptide transport system driven by the inwardly directed chemical gradient of dipeptide which is maintained by intracellular peptidase activity. This mechanism of dipeptide uptake and hydrolysis by intracellular peptidase activity can play an important role in peptidolytic activity of lactococci during cheese ripening (when the energy source lactose is exhausted).

The accumulation of Ala-Glu most likely occurs with a proton/substrate stoichiometry of 2:1. Since Ala-Glu is a

![Graph](image-url)

**FIG. 3.** Accumulation of L-alanyl-a-L-[14C]glutamate in *L. lactis* ML3 membrane vesicles fused with cytochrome c-oxidase-containing proteoliposomes. The fused membranes were energized with ascorbate-cytochrome c-TMPD in the absence (●) and presence (○) of 100 nM valinomycin. As a control, nonenergized membranes were incubated with L-alanyl-a-L-[14C]glutamate (□). Transport assays were performed as described in Materials and Methods.

which extracellular aminopeptidases are present (13; for a recent review, see reference 32), oligopeptides can be degraded into free amino acids and subsequently uptake of the released amino acids via specific amino acid transport systems can occur (5, 6, 23).

The mechanism of energy coupling to dipeptide transport has been investigated in detail. Dipeptide transport in *L. lactis* ML3 is directly coupled to the PMF. Some evidence for Δp-driven dipeptide transport in *Saccharomyces cerevisiae* and *Enterococcus faecalis* has been presented (21). In gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*, dipeptides are transported by binding-protein-dependent transport systems which are not driven by the Δp but most likely by ATP hydrolysis (1, 8, 27).

Recent studies in our laboratory with *L. lactis* subsp. *cremoris* indicated that the energy for leucyl-leucine transport is supplied directly by the hydrolysis of an energy-rich phosphate bond, presumably ATP (34). This conclusion was based on the observation that no Leu-Leu uptake could be detected in membrane vesicles energized with artificial diffusion gradients, while in whole cells Leu-Leu transport activity increased with increasing intracellular ATP concentration. The presence of phosphate-bond-driven Leu-Leu

![Graph](image-url)

**FIG. 4.** Uptake of L-alanyl-a-L-[14C]glutamate in fused membranes. At the time indicated, the ionophores valinomycin (100 nM) and nigericin (10 nM) were added to the membrane suspension. Dashed line indicates the level of Ala-Glu equilibration between the internal and external medium.

### TABLE 1. Effect of ionophores and uncouplers on Ala-Glu uptake activity

<table>
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<tr>
<th>Ionophore(s)</th>
<th>Uptake activity (%)</th>
<th>Δp (mV)</th>
<th>Δψ (mV)</th>
<th>Δρ (mV)</th>
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</thead>
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<tr>
<td>None</td>
<td>100</td>
<td>-103</td>
<td>-83</td>
<td>-20</td>
</tr>
<tr>
<td>Nigericin (10 nM)</td>
<td>100</td>
<td>-95</td>
<td>-95</td>
<td>0</td>
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<tr>
<td>Valinomycin (100 nM)</td>
<td>28</td>
<td>-49</td>
<td>0</td>
<td>-49*</td>
</tr>
<tr>
<td>Nigericin (10 nM) + valinomycin (100 nM)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CCCP (1 μM)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Not energized</td>
<td>0.3</td>
<td>0</td>
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</table>

* a The transport assay and Δp determination were performed as described in Materials and Methods.

* b From reference 5.

* CCCP, Carbonyl cyanide m-chlorophenylhydrazone.
The initial uptake level in energized fused membranes 2 min after addition of l-alanyl-a-l-\[^{14}\text{C}\] glutamate.

The transport process therefore involves the net translocation of one positive charge. If the same net charge translocation of one positive charge occurs in the transport of other dipeptides, a 1:1 proton/dipeptide stoichiometry would occur in the transport of neutral dipeptides such as leucyl-leucine and a 3:1 proton/dipeptide stoichiometry would occur for twofold negatively charged dipeptides such as glutamyl-glutamic acid. Preliminary results have shown that arginine-containing dipeptides, carrying a one- or a twofold positive charge, are not taken up by these bacteria and are not utilized at all (E. J. Smid, unpublished results). Rogers et al. (26) have shown that Streptococcus sanguis utilizes arginine-containing peptides via hydrolysis of the peptides by extracellular peptidases that liberate arginine, which subsequently can be taken up by a specific arginine transport system. This observation suggests that S. sanguis also does not possess a peptide transport system for arginine-containing peptides.

The dipeptide transport system of L. lactis has a relatively low affinity for Ala-Glu, and this affinity slightly increases with decreasing extracellular pH (Smid, unpublished data). Comparable results were found for leucyl-leucine transport in intact cells of L. lactis subsp. cremoris (34). The relatively low \( K_a \) (80 \( \mu \)M) of Leu-Leu on Ala-Glu transport indicates that this dipeptide is transported by L. lactis with a higher affinity. Compared with the binding-protein-dependent peptide transport systems present in gram-negative bacteria such as E. coli, the affinities of dipeptides for the secondary dipeptide transport system in lactococci are low (1, 22). Also, the di- and tripeptide transport systems in Pseudomonas aeruginosa have affinity constants which are 1 to 2 orders of magnitude lower than those found for L. lactis (10). Although the affinity for Ala-Glu is relatively low, the high

\[ V_{\text{max}} \] values indicate that the transport process can be of significant nutritional value for the organism.

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**LITERATURE CITED**