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Transport of Amino Acids in *Lactobacillus casei* by Proton-Motive-Force-Dependent and Non-Proton-Motive-Force-Dependent Mechanisms

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***Lactobacillus casei* 393 cells which were energized with glucose (pH 6.0) took up glutamine, asparagine, glutamate, aspartate, leucine, and phenylalanine. Little or no uptake of several essential amino acids (valine, isoleucine, arginine, cysteine, tyrosine, and tryptophan) was observed. Inhibition studies indicated that there were at least five amino acid carriers, for glutamine, asparagine, glutamate/aspartate, phenylalanine, or branched-chain amino acids. Transport activities had pH optima between 5.5 and 6.0, but all amino acid carriers showed significant activity even at pH 4.0. Leucine and phenylalanine transport decreased markedly when the pH was increased to 7.5. Inhibitors which decreased proton motive force (Δp) nearly eliminated leucine and phenylalanine uptake, and studies with de-energized cells and membrane vesicles showed that an artificial electrical potential ($\Delta\psi$) of at least −100 mV was needed for rapid uptake. An artificial Δp was unable to drive glutamine, asparagine, or glutamate uptake, and transport of these amino acids was sensitive to a decline in intracellular pH. When intracellular pH was greater than 7.7, glutamine, asparagine, or glutamate was transported rapidly even though the proton motive force had been abolished by inhibitors.**

Lactobacilli are gram-positive, fermentative bacteria which can rapidly lower pH (7), and many species are halotolerant (20). They are important in the preservation of foods such as cheese, sausage, and pickled vegetables; the value of these fermentations exceeds $100 billion per year (2). Lactobacilli are also able to colonize the digestive tract, and they may play a therapeutic role in human and animal health (5). The study of lactobacillus genetics was once stymied by the lack of natural gene exchange mechanisms, but their economic importance has created an interest in genetic manipulation (2).

Lactobacilli have nutritional requirements for amino acids, vitamins, fatty acids, and nucleic acids (20), but there have been few studies of solute transport. Homofermentative species (e.g., *Lactobacillus casei*) have a phosphotransferase system for glucose, but heterofermentative organisms appear to use proton-motive-force (Δp)-driven mechanisms of glucose uptake (18). Many lactobacilli require folate, and the uptake involves ATP or some other phosphate-bond intermediate (6).

Lactobacilli cannot utilize ammonia as a nitrogen source (20), and from 7 to 15 amino acids are essential for growth (9). *L. casei* requires 10 amino acids, but our results indicated that only 4 of them, glutamate, aspartate, leucine, and phenylalanine, are taken up rapidly. Glutamine and asparagine, nonessential amino acids, were taken up more quickly than other amino acids. Studies with membrane vesicles indicated that some amino acids are transported by Δp systems, while others are apparently taken up by mechanisms which do not require a Δp. Membrane vesicles of lactobacilli had not been previously used to study solute transport.

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MATERIALS AND METHODS

Organism and growth conditions. *L. casei* 393 was obtained from A. H. Romano, Microbiology Section, University of Connecticut, Storrs. The growth medium was described previously (17); the pH was adjusted to 6.7. Glucose was added as a separate solution (final concentration, 4 g/liter), and cultures were grown at 39°C. Strain 393 was unable to grow rapidly in a medium (14) containing purified amino acids and Tween 80 (E. Merck AG, Darmstadt, Federal Republic of Germany) instead of peptone (Difco Laboratories, Detroit, Mich.).

Glucose-energized cells. Batch cultures were grown overnight to an optical density at 660 nm of 1.0 (16-mm tubes) and harvested by centrifugation (1,200 × *g*, 5 min, 20°C). Cells were washed twice with 100 mM potassium phosphate (pH 6.0) containing 10 mM $MgSO_4$. Cells (approximately 32 μg of protein) were energized with 20 mM glucose for 15 min, and transport assays were conducted at 28°C in 200 μl of buffer containing 1 to 3 μM ^{14}C-amino acids (100 nCi), 10 mM $MgSO_4$, and either 100 mM potassium phosphate (pH 6.0 to 7.5) or 100 mM morpholineethanesulfonic acid (pH 4.0 to 5.5). Transport was terminated by adding 2 ml of ice-cold 100 mM LiCl to the reaction mixture and filtering this mixture through a cellulose nitrate membrane filter (0.45-μm pore size). The filters were washed once with 2 ml of 100 mM LiCl and dried for 20 min at 120°C, and radioactivity was measured by liquid scintillation. Metabolic inhibitors and ionophores were added at the same time as glucose, and the final assay volume was 210 μl. The V_{max} and K_t for each amino acid were estimated at substrate concentrations ranging from one-fifth to three times the K_t. Initial rates were determined over 30 s, and previous experiments indicated that the transport rate was linear for at least 60 s. Competing amino acids were added at the same time as unlabeled amino acid. All values reported are averages of two to four samples.

Artificial potentials. Cells were incubated for 40 min with 20 mM 2 deoxyglucose (39°C) to de-energize them. Concentrated de-energized cell suspensions were then treated with

valinomycin (2 μM, 60 min, 0°C) and incubated in 100 mM potassium phosphate (pH 6.0) to load them with K. K-loaded cells (32 μg of protein) were diluted 100-fold into sodium phosphate (pH 6.0) which contained 10 mM $MgSO_4$, and amino acid transport was conducted as described above. In some cases, cells were loaded with 200 mM potassium phosphate and extracellular KCl was varied from 0 to 20 mM to change the imposed artificial electrical potential ($\Delta\psi$).

Membrane vesicles. The method of vesicle preparation was described previously (19). As estimated from the decrease in optical density, the formation of osmotically sensitive protoplasts was approximately 50%. Membrane vesicles were resuspended in 50 mM potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$ (final protein concentration, 13 mg/ml) and frozen in liquid nitrogen until use. Membrane vesicles were loaded with 20 mM potassium phosphate and 80 mM potassium acetate (pH 6.0) by valinomycin treatment (2 μM, 60 min, 0°C), diluted into 100 mM sodium phosphate (pH 6.0) to create an artificial $\Delta\psi$ and pH gradient, and assayed for transport activity as described above. Transport assays contained approximately 50 μg of protein.

Protein. Protein from NaOH-hydrolyzed cells (0.2 N, 100°C, 15 min) was measured by the method of Lowry et al. (8) by using bovine serum albumin as a standard.

Proton motive force. Intracellular pH was measured by an acid distribution method that is based on the assumption that undissociated forms of weak acids can diffuse freely through the cell membrane in response to a pH gradient (16). Cells (105 μg of protein per 600 μl) were incubated in the presence of [7-^{14}C]benzoate (0.2 μCi), [U-^{14}C]taurine (0.2 μCi), or ^3H_2O (50 μCi). After 10 min of incubation at 28°C, the cells were centrifuged through silicon oil (0.4 ml, 2:1 mixture of AR200 and AR20) in a microcentrifuge (13,000 × *g*, 10 min, 1.5-ml tube) and supernatant samples (50 μl) were removed for scintillation counting. The tubes were then frozen, and the bottoms containing the pellets were removed with a pair of dog nail clippers. Pellets and supernatants were then dissolved in scintillation fluid compatible with aqueous samples. Intracellular volume was calculated as the difference in specific activity between ^3H_2O and [U-^{14}C]taurine. The intracellular volume was 3.7 μl/mg of protein. The membrane potential ($\Delta\psi$) was estimated from the distribution of [^{3}H]tetraphenylphosphonium chloride (TPP^+) by using the Nernst equation. Cells (105 μg of protein per 600 μl) were incubated for 10 min with [^{3}H]TPP^+ (0.2 μCi) and processed as described above. Measurements were corrected for nonspecific binding by subtracting TPP^+ bound to toluene-treated cells (6 μl of toluene per 600 μl).

Materials. Radioactively labeled substrates were obtained from the Radiochemical Centre, Amersham, United Kingdom. Only the L-amino acids were used. Silicon oils (AR20 and AR200) were obtained from Wacker Chemicals, Munich, Federal Republic of Germany; yeast extract was from BBL Microbiology Systems, Cockeysville, Md. All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Kinetics of transport. When stationary-phase cultures of *L. casei* were incubated in potassium phosphate (pH 6.0) and energized with glucose, uptake of glutamine, asparagine, glutamate, aspartate, leucine, and phenylalanine was rapid (Table 1). All of the K_t values were less than 3 μM, and V_{max} values ranged from 0.66 to 6.52 nmol/mg of protein per min. Low rates (<0.20 nmol/mg of protein per min) of transport were observed for isoleucine and valine, and no uptake of arginine, cysteine, lysine, serine, tryptophan, or tyrosine could be detected. Within 35 min the glucose was depleted, and after another 5 min more than 90% of the transport activity was lost (data not shown).

TABLE 1. Kinetics of amino acid transport for glucose-energized *L. casei* cells at pH 6.0[a]

Amino acid	V_{max}[b]	K_t[c]
Glutamine	3.81	0.99
Asparagine	6.52	2.22
Glutamate	4.63	2.99
Leucine	0.66	1.12
Phenylalanine	0.76	2.78

[a] Cells were energized with 20 mM glucose for 15 min prior to amino acid addition.
[b] Maximum transport rate (nanomoles per milligram of protein per minute).
[c] Affinity constant (micromolar).

Competition and pH. Leucine uptake was completely inhibited by a 100-fold excess of unlabeled valine or isoleucine, and a similar excess of aspartate completely inhibited glutamate uptake. Glutamine uptake was not inhibited by a 100-fold excess of unlabeled glutamate, aspartate, or asparagine, and glutamate transport was not inhibited significantly by unlabeled asparagine. Glutamine, glutamate, and asparagine uptake was greatest at pH 6.0, and there was only a modest decrease in transport activities when the pH was

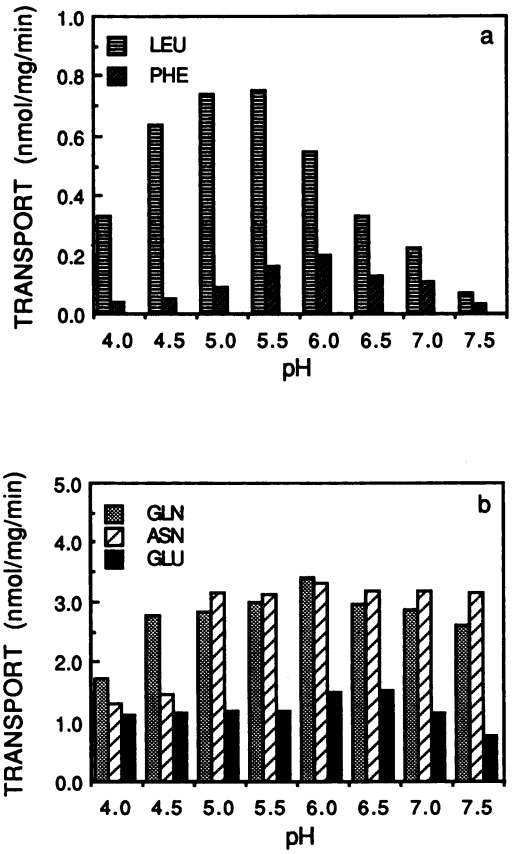

FIG. 1. Effect of external pH on the initial rate of amino acid transport by glucose-energized cells (amino acid concentrations were 1 to 3 μM).

TABLE 2. Effect of DCCD, valinomycin, and uncouplers on amino acid transport by glucose-energized *L. casei* cells at pH 6.0 and 7.5[a]

Treatment	% Inhibition of transport of [b]:					
	Glutamate at pH:		Glutamine at pH:		Asparagine at pH:	
	6.0	7.5	6.0	7.5	6.0	7.5
DCCD	41	9	31	14	92	35
CCCP	31	0	48	0	96	8
Valinomycin	5	0	30	16	17	15
Nigericin + valinomycin	73	0	71	2	80	2

[a] DCCD was added at 100 μM; CCCP and the ionophores were each added at 5 μM.
[b] Leucine and phenylalanine transport was inhibited more than 85% under all conditions.

varied from 4.0 to 7.5 (Fig. 1). Leucine transport was most rapid at pH 5.5, but neutral pH values were inhibitory. The phenylalanine transport optimum was 6.0.

Driving force for uptake. Leucine and phenylalanine uptake was almost completely eliminated when glucose-energized cells were treated with dicyclohexylcarbodiimide (DCCD), carbonyl-cyanide *m*-chlorophenyl-hydrazone (CCCP), valinomycin, or nigericin plus valinomycin (Table 2). These decreases were correlated with declines in Δp (Table 3). In de-energized cells (Fig. 2a) and membrane vesicles (Fig. 2b), artificial membrane potentials were needed for leucine and phenylalanine transport. When the magnitude of K diffusion potential ($\Delta\psi$) was decreased from −120 to −60 mV, there was a dramatic decline in the rate of leucine transport (Fig. 3).

DCCD, valinomycin, and uncouplers also decreased the transport of glutamine, asparagine, and glutamate at pH 6.0, but little or no decrease was observed when the extracellular pH was increased to 7.5 (Table 2) even though Δp was greatly decreased (Table 3). Arsenate (1 mM), an inhibitor of ATP formation, inhibited glutamine, asparagine, and glutamate uptake more than 60% (data not shown).

Intracellular pH. DCCD, CCCP, and the ionophores decreased intracellular pH when the extracellular pH was 6.0 (Table 3), and this decrease was correlated with a reduction in glutamine, asparagine, and glutamate transport (Table 2). At pH 7.5, the inhibitors did not cause a decrease in either intracellular pH or transport activity. Since there was no pH gradient in the presence of nigericin plus valinomycin, it was possible to examine the effect of the intracellular pH on the

TABLE 3. Effect of DCCD, valinomycin, and uncouplers on intracellular pH (pH_i) and proton motive force (Δp) of *L. casei* cells at an external pH (pH_e) of 6.0 or 7.5[a]

Treatment	pH_i at pH_e of:		Δp (mV) at pH_e of:	
	6.0	7.5	6.0	7.5
De-energized with 2-deoxyglucose	6.27	7.51	27	1
Nonenergized	6.29	7.76	80	87
Glucose energized	6.70	7.78	149	124
Glucose energized, DCCD	6.23	7.78	77	102
Glucose energized, CCCP	6.31	7.75	75	106
Glucose energized, valinomycin	6.36	7.77	55	54
Glucose energized, nigericin + valinomycin	6.05	7.76	3	16

[a] DCCD was added at 100 μM; CCCP and the ionophores were each added at 5 μM.

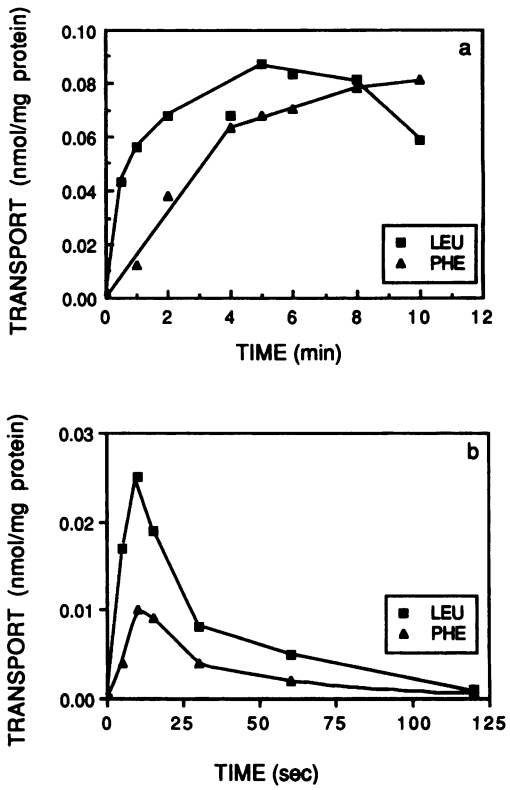

FIG. 2. (a) Transport of leucine and phenylalanine by de-energized cells which were loaded with 100 mM potassium phosphate and diluted into 100 mM sodium phosphate (pH 6.0) to create an artificial $\Delta\psi$ of −100 mV. (b) Membrane vesicles were loaded with 20 mM potassium phosphate and 80 mM potassium acetate and diluted into 100 mM sodium phosphate (pH 6.0) to create a membrane Δp of −200 mV.

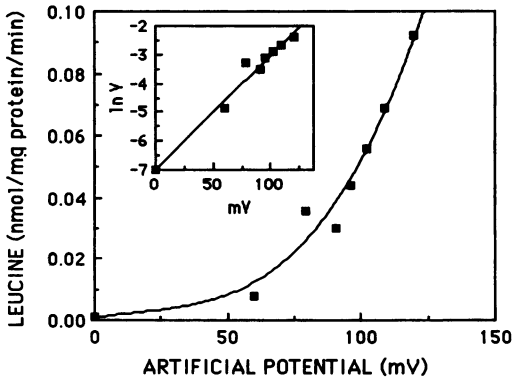

FIG. 3. Effect of imposed artificial potential on the initial rate of leucine transport by de-energized cells which were loaded with potassium phosphate (200 mM) and diluted into sodium phosphate (100 mM) containing from 20 to 0 mM KCl (pH 6.0). The inset shows the logarithmic relationship between initial velocity (V) and potential (mV).

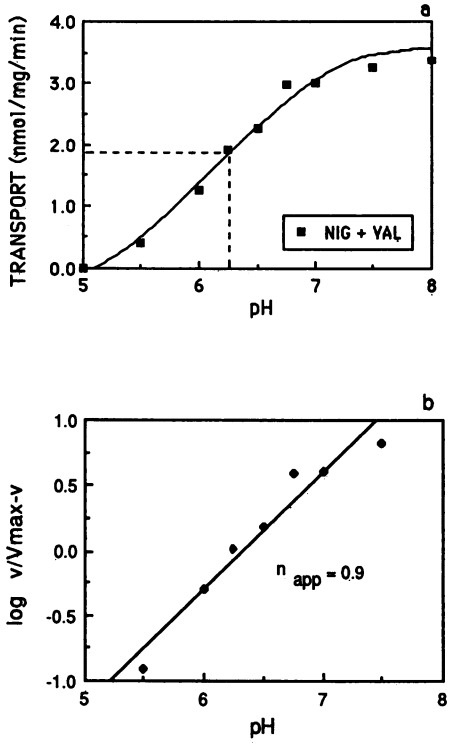

FIG. 4. (a) Effect of intracellular pH on the rate of glutamine transport by glucose-energized cells which were treated with 5 μM nigericin and 5 μM valinomycin. (b) Hill plot of the data, where v is the rate of glutamine transport (nanomoles per milligram of protein per minute) and n_{app} is the slope.

glutamine carrier. When extracellular pH was decreased to 6.25, the velocity of glutamine transport was reduced by 50% (Fig. 4a). A Hill plot of the data (Fig. 4b) indicated that the glutamine carrier had approximately one (n_{app} = 0.9) binding site for protons.

DISCUSSION

L. casei was able to transport several amino acids at a rapid rate, but some essential amino acids were taken up very slowly (Table 1). Since the cells were grown in peptone-containing medium, it is possible that peptide transport systems also provided essential amino acids. Aspartate and glutamate were taken up by the same carrier, but glutamine, glutamate, and asparagine were taken up by separate systems. Increasing the extracellular pH had little effect on the rate of glutamate transport, and since the transport assays routinely contained a concentration of glutamate (1.75 μM) that was less than the K_t (2.99 μM), it was likely that dissociated and undissociated species were transported. In *Streptococcus cremoris* and *Streptococcus lactis*, glutamine and glutamate are taken up by the same carrier, the affinity for glutamate decreases dramatically as the pH increases from 5.1 to 7.0, and it appears that only the neutral species is taken up (13). The high-affinity glutamate transport system of *Streptococcus faecalis* is also unable to transport glutamine, and it is not seriously affected by pH (21). A distinct carrier for asparagine is also found in *S. lactis* (W. N. Konings, B. Poolman, and A. J. M. Driessen, Crit. Rev. Microbiol., in press).

While *L. casei* appears to have a common carrier for branched-chain amino acids, leucine was taken up more rapidly than valine or isoleucine. Since leucine and phenylalanine transport in membrane vesicles was driven by a K and acetate diffusion potential (Fig. 2), it is likely that these amino acids were taken up by a Δp-driven mechanism. Similar results were obtained with *S. cremoris* (4).

Nonenergized cells were unable to transport leucine and phenylalanine at a significant rate (pH 6.0), even though the Δp was 80 mV (Table 3), and there was a logarithmic relationship between transport rate and the magnitude of an imposed artificial potential (Fig. 3). A similar relationship between the initial rate of leucine uptake and Δp was also noted in *S. cremoris* membrane vesicles (3), but the threshold was not as low as the one observed for *L. casei*. DCCD, CCCP, and valinomycin did not completely abolish the Δp of glucose-energized cells, but the Δp was similar to the one in nonenergized cells (Table 3). Since cells which were treated with either 2-deoxyglucose or nigericin plus valinomycin had virtually no Δp at either pH, the Δp of nonenergized cells was not solely due to a Donnan potential.

An artificial Δp was unable to drive the transport of glutamine, glutamate, or asparagine in de-energized cells or membrane vesicles, and transport by glucose-energized cells was inhibited by arsenate (data not shown). These results suggested that phosphate-bond energy was needed for uptake. However, arsenate also decreased the transport of leucine and phenylalanine, amino acids which were taken up by Δp-driven mechanisms. Because ATP and Δp are interrelated, effects of inhibitors can be ambiguous. Mechanistic studies of phosphate-bond-driven transport have been confounded by the inability of membrane vesicles to generate ATP (1).

Because DCCD (an ATPase inhibitor), valinomycin, and uncouplers such as CCCP and nigericin plus valinomycin can cause a decrease in Δp, their effects have often been taken as evidence for Δp involvement in binding-protein-dependent transport of gram-negative bacteria. Plate (10) noted that valinomycin decreased glutamine transport in *Escherichia coli* and suggested that both ATP and a membrane potential were necessary for uptake. DCCD and uncouplers inhibited glutamine, asparagine, and glutamate transport in *L. casei* but only when the external pH was less than 7.5 (Table 2). Since nigericin plus valinomycin eliminated Δp even at pH 7.5 (Table 3), it appeared that a Δp was not essential for uptake.

Recent studies have demonstrated the importance of intracellular pH as a regulator of certain transport systems (11). In *S. lactis*, the initial rate of glutamate uptake increased more than sixfold when the intracellular pH was increased from 6.0 to 6.7 (12, 13). Because valinomycin is a uniporter which does not directly facilitate proton movement, previous workers had assumed that it would not affect intracellular pH (15). However, in *L. casei* valinomycin caused a decrease of intracellular pH when extracellular pH was 6.0. The effects of the inhibitors on glutamate, glutamine, and asparagine transport are consistent with the negative impact of low intracellular pH on phosphate-bond-driven transport carriers.

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