Osmotically Regulated Transport of Proline by *Lactobacillus acidophilus* IFO 3532

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We reported previously that, when exposed to high osmotic pressure, *Lactobacillus acidophilus* IFO 3532 cells accumulated *N,N,N*-trimethylglycine (glycine betaine), which serves as a compatible intracellular solute. When grown in medium with high osmotic pressure, these cells also accumulated one amino acid, proline. The uptake of [^{3}H]proline by resting, glucose-energized cells was stimulated by increasing the osmotic pressure of the assay medium with 0.5 to 1.0 M KCl, 1.0 M NaCl, or 0.5 M sucrose. The accumulated [^{3}H]proline was not metabolized further. In contrast, there was no osmotic stimulation of [^{3}H]leucine uptake. The uptake of proline was activated rather than induced by exposure of the cells to high osmotic pressure. Only one proline transport system could be discerned from kinetics plots. The affinity of the carrier for proline remained constant over a range of osmotic pressures from 650 to 1,910 mosM (K_t, 7.8 to 15.5 mM). The V_{max}, however, increased from 15 nmol/min/mg of dry weight in 0.5 M sucrose to 27 and 40 nmol/min/mg of dry weight in 0.5 M KCl and in 1.0 M KCl or NaCl, respectively. The efflux of proline from preloaded cells occurred rapidly when the osmotic pressure of the suspending buffer was lowered.

Many nonhalophilic osmotolerant bacteria respond to increased osmotic pressure by accumulating compatible solutes or osmolytes to high intracellular levels (reviewed in references 2, 3, and 6). Exposure of the cells to high osmotic pressure results in the rapid efflux of intracellular water, which leads to a decrease in cell volume and a decrease in turgor pressure. The accumulation of osmolytes raises the internal osmotic pressure, thus maintaining turgor. Among the most prominent compatible solutes are a relatively few amino acids, such as glutamate, glutamine, alanine, and proline, and amino acid derivatives, such as glycine betaine (*N,N,N*-trimethylglycine). Both proline and glycine betaine, when supplied in the medium, are accumulated by enteric bacteria and serve as osmoprotectants (7, 12, 13, 19, 20).

In the best understood systems, *Escherichia coli* and *Salmonella typhimurium*, a complex response in solute transport is observed when the cells are stressed by an increase in medium osmolarity. There is an initial increase in the transport and accumulation of K$^+$ ions (5, 18), as well as an increase in the synthesis of glutamate, which serves as the counterion (2). When a critical concentration of intracellular K$^+$ is reached, the expression of various osmotically responsive genes is stimulated. These genes include *proP* and *proU*, whose products are the transport systems for proline and glycine betaine (betaine). The stimulation of *proU* gene expression by potassium glutamate in an in vitro system has been shown to occur at the transcriptional level (9, 14). Betaine, when available, is the compatible solute of choice for these organisms. In addition to induction, high osmotic pressure increases the activity of the transport systems, both in intact cells and in membrane vesicles (10). The gram-positive bacterium *Bacillus subtilis* also responds to increased osmotic pressure by the initial uptake of K$^+$ ions, followed by the accumulation of endogenously synthesized proline (19). When betaine is present in the medium, proline

biosynthesis is inhibited and betaine is accumulated to high levels in preference to proline.

We reported previously that, when exposed to high osmotic pressure, *Lactobacillus acidophilus* IFO 3532 cells accumulated betaine, which serves as a compatible intracellular solute (8). Betaine uptake was found to be activated but not induced by exposure of the cells to high osmotic pressure. Furthermore, these organisms did not accumulate carbohydrates (reducing sugars), K$^+$, or amino acids, except for proline. In cells growing in MRS medium containing 1.0 M KCl or NaCl, the intracellular proline concentration increased to levels five- to sixfold higher than those observed in unstressed cells (8).

In this report, we present evidence that *L. acidophilus* IFO 3532 cells possess a low-affinity proline transport system that, like the betaine transport system, is activated but not induced by increased osmotic pressure.

MATERIALS AND METHODS

Growth and preparation of bacteria. *L. acidophilus* IFO 3532 (currently *Lactobacillus casei* subsp. *rhamnosus* Hansen IFO 3532) cells were grown to the mid-exponential phase (optical density at 600 nm, 0.6 to 0.9) after 1% (vol/vol) inoculation from an overnight culture grown in MRS medium (Difco Laboratories, Detroit, Mich.). The cultures (30 to 40 ml) were incubated at 37°C without shaking in MRS medium containing, where indicated, 0.5 to 1.0 M KCl or NaCl. The cells were harvested at 4°C by centrifugation at 8,000 × g for 10 min and washed twice in 15 to 20 ml of the buffers used in the experiments. Washed cells were suspended to an optical density at 600 nm of approximately 1.0 in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, and the pH was adjusted to 6.5 with NaOH or with Tris base. The buffers were supplemented with KCl or NaCl at the concentrations used in the experiments.

Measurement of [^{3}H]proline uptake. To 1.1 ml of cell suspension was added 25 µl of 2 M glucose (final concentra-

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tion, 40 mM), and the cells were preincubated at 26°C for 5 min. To start the reaction, we added 60 µl of [³H]proline (L-[3,4-³H]proline; 30.8 Ci/mmol; Dupont-NEN Research Products, Boston, Mass.) and up to 60 µl of 1 M nonradioactive proline to yield final concentrations of proline of up to 50 mM. The reaction mixtures (total volume, 1.25 ml) contained approximately 1,000 to 2,000 cpm of [³H]proline per µl. Samples (0.2 ml) were removed at various times after incubation at 26°C. The cells were separated by filtration through polycarbonate filters (0.4-µm-pore size; Nuclepore Corp., Pleasanton, Calif.). The filtered cells were washed with 2 to 3 ml of the appropriate buffer and then counted for radioactivity in a liquid scintillation counter. For K_t and V_{max} determinations, the initial rates of proline uptake were determined over the first 5 min of the assay. Statistical analyses were performed by the analysis of variance test.

The transport of betaine and leucine was measured as described for proline. [¹⁴C]betaine ([*methyl*-¹⁴C]glycine betaine) was added to a final concentration of 2 mM and a specific activity of 0.15 mCi/mmol (8), and [³H]leucine (L-[2,3,4,5-³H]leucine; ICN Radiochemicals, Irvine, Calif.) was added to a final concentration of 0.1 mM and a specific activity of 8 mCi/mmol.

Extraction and chromatography of accumulated proline. The chemical nature of transported proline was examined by allowing MRS–0.8 M KCl-grown cells suspended in 0.5 M KCl buffer to accumulate [³H]proline for 20 min. Samples (1.2 ml) of the incubation mixtures were centrifuged, and the cells were resuspended in 100 to 200 µl of water. The tubes were placed in a boiling water bath for 15 min and centrifuged, and the pellets were reextracted with water as described above. The extracts were pooled and dried under vacuum, and the dried material was dissolved in 20 µl of water and chromatographed on silica gel thin-layer chromatography plates (Si250-PA; J. T. Baker Chemical Co., Phillipsburg, N.J.) with water-saturated phenol-ethanol-water (10:4:4) and n-butanol-acetic acid-water (12:3:5) as the mobile phases (4). One-centimeter sections were scraped off the dried plates and counted for radioactivity. The only peak of radioactivity, accounting for 95% of the applied material, migrated like genuine [³H]proline and nonradioactive proline, which was located by staining with isatin. The extracted radioactivity was found to comigrate with genuine [³H]proline.

Efflux of proline. Cells grown in MRS-KCl were prepared as described above, resuspended in 50 mM [³H]proline in MES buffer containing 0.5 M KCl and 40 mM glucose, and incubated at 26°C for 20 min. One-milliliter samples were centrifuged at 12,000 × g in a microcentrifuge at room temperature. The pellets were resuspended rapidly (5 to 10 s) in 1.0 ml of MES buffer containing 40 mM glucose, 0.5 M KCl, and, where indicated, 50 mM nonradioactive proline. The cell suspensions were incubated at 26°C, and at various intervals 0.2-ml samples were filtered, washed, and counted for radioactivity.

Other measurements. Osmolality measurements were carried out with a model 3L osmometer (Advanced Industries, Needham Heights, Mass.). The relationship 1 ml of cell suspension at an optical density at 600 nm of 1 = 0.39 mg (dry weight), determined previously, was used for uptake calculations. Solute uptake was calculated on a cell dry-weight basis because the intracellular volumes probably varied as the cells accumulated osmolytes.

RESULTS

Effect of medium osmolarity on proline uptake by *L. acidophilus* IFO 3532. In initial experiments, we compared the uptake of proline, whose intracellular concentration was increased by growth in high-osmotic-pressure medium, with that of leucine, which is unaffected by medium osmolarity (8), and that of betaine, which is activated by increased osmotic pressure (8). The uptake of proline by MRS-KCl-grown cells occurred in response to increased osmotic pressure. When 0.5 M KCl was added to the assay buffer, proline uptake was faster than that seen in buffer alone (Fig. 1A). Proline (50 mM) responded to osmotic pressure in the same way as betaine (2 mM). Activation of the transport of both substrates was seen whether the cells were grown in MRS medium (approximately 450 mosM) or in MRS medium supplemented with 0.5 to 0.8 M KCl (1,380 to 1,890 mosM). When transport systems were saturated with substrates, the difference in osmotically stimulated uptake by cells grown in MRS or MRS-KCl was less than twofold. This result indicates that activation and not induction was the major effect of increased osmotic pressure on transport rates.

Like betaine transport in this organism, proline uptake required the addition of glucose as an energy source (data not shown). The two systems are separate, however, since betaine (2 mM) did not affect the uptake of 0.1 to 25 mM proline in buffer containing 1 M KCl and proline (20 mM) did not affect the uptake of 0.2 mM betaine (8).

In contrast to the uptake of proline and betaine, which remained linear for at least 20 to 40 min in these experiments, leucine uptake reached a plateau within 5 to 10 min. More important, with leucine uptake, there was no pattern of stimulation of uptake by increased medium osmolarity.

The stimulation of proline uptake was not due to a specific effect of KCl but was caused by the increase in medium osmolarity, since NaCl and LiCl had the same effect. Moreover, when cells were grown with 0.8 M NaCl instead of 0.8 M KCl added to MRS medium, the same stimulation of proline uptake was seen when either NaCl or KCl was added to the assay buffer. In addition, sucrose also stimulated proline uptake (Table 1), indicating that the effect was due to osmotic pressure and not to increased ionic strength. It should be noted that under the conditions used in these experiments, higher concentrations of sucrose resulted in problems with filtering of the cells.

Intracellular proline. To determine whether the uptake of radioactive proline represented transport (translocation across the cell membrane) or included further metabolism, we allowed cells to accumulate [³H]proline from buffers containing high (50 mM) or low (2.5 mM) concentrations of proline. The accumulated radioactive material was extracted and chromatographed for comparison with genuine proline. Essentially all the internalized proline was found to comigrate with genuine proline.

Kinetic constants for proline uptake. Only one transport system was discernible in kinetics plots of uptake rates versus extracellular proline concentrations. In contrast to the betaine transport system in this organism, which has a K_t of 50 µM (8), the affinity of the proline transport carrier for its substrate was much lower. K_t values of 7.8 to 15.5 mM proline were determined over a wide range of osmotic pressures (Table 1). No significant differences, as tested by analysis of variance, were found among the K_t values measured in buffers containing 0.5 or 1.0 M KCl, 1.0 M NaCl, or 0.5 M sucrose. This result indicates that the affinity
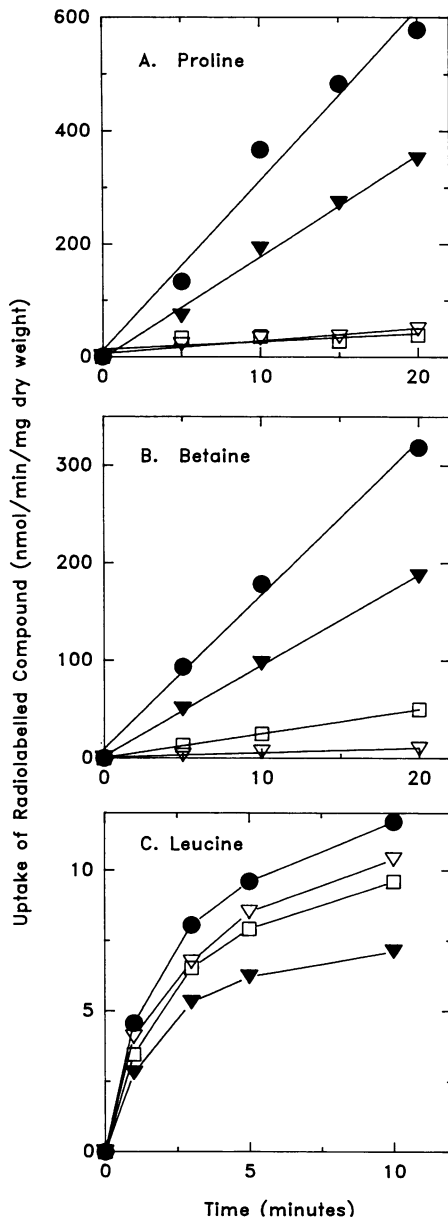
FIG. 1. Effect of KCl on the uptake of proline and leucine. The cells were grown and assayed as described in the text under the following conditions: ●, grown in MRS containing 0.8 M KCl (MRS-KCl), assayed in 25 mM MES buffer (pH 6.5) containing 0.5 M KCl (MES-KCl); ▼, grown in MRS, assayed in MES-KCl; ∇, grown in MRS-KCl, assayed in MES; □, grown in MRS, assayed in MES. The reactions were initiated with 50 mM [^{3}H]proline (A), 2 mM [^{14}C]betaine (B), or 90 μM [^{3}H]leucine (C).

of the transport carrier for proline does not change with increasing osmotic pressure.

The rate of transport, however, was affected by the medium osmolarity (Table 1). With 0.5 M sucrose the V_{max} (14.8 nmol of proline taken up per min per mg of cell dry weight) was significantly lower than with 1 M salt or with 0.5 M KCl (40 to 45 or 27 nmol/min/mg of cell dry weight, respectively). The rate of uptake of proline observed in buffer without added osmolytes was too low to be accurately measured, even at saturating concentrations of substrate

TABLE 1. Effect of medium osmolarity on the kinetic constants of proline transport by *L. acidophilus* IFO 3532[a]

Addition	mosM	K_t (mM)[b]	V_{max} (nmol/min/mg of cell dry weight)[c]
0.5 M Sucrose	650	10.4 ± 1.9 (2)	14.8 ± 0.6
0.5 M KCl	930	15.5 ± 3.1 (7)	27.3 ± 6.7
1.0 M KCl	1,850	11.0 ± 1.4 (6)	41.2 ± 2.6
1.0 M NaCl	1,910	7.8 ± 0.7 (2)	44.9 ± 1.0

[a] All cultures were grown to the mid-exponential phase in MRS medium with 0.8 M KCl, harvested by centrifugation, and washed at least twice in the buffer used in the assay.
[b] Means ± standard errors of the means, with the number of separate cell cultures shown in parentheses.
[c] Significantly different from other values, as determined by analysis of variance.

(Fig. 1A); therefore, kinetic parameters could not be measured without carrier activation.

Activation of proline uptake. Activation of proline uptake by increasing the medium osmotic pressure was found to be similar whether the osmolyte saturated the transport system or not (Fig. 2). Thus, 50% activation was seen with approximately 0.45 M KCl added to the buffer (840 mosM), whether proline uptake was tested at 90 μM, 2.5 mM, or 20 mM. No activation was seen below 0.3 M KCl.

Efflux of proline. In contrast to the slow uptake of proline in response to an increase in osmotic pressure, the internal concentration of proline was found to respond rapidly to decreases in medium osmolarity (Fig. 3). Proline which had been accumulated in buffer containing 0.5 M KCl was found to efflux within 1 min when preloaded cells were centrifuged and resuspended in buffer without added salt. The cells retained less than 10% of the proline. Rapid but less extensive efflux was seen when the dilution buffer contained 0.5 M KCl. In this case, 70 to 80% of the proline was retained. This rapid efflux occurred in glucose-energized cells, whether the cells were resuspended in buffer containing 50 mM proline or not.
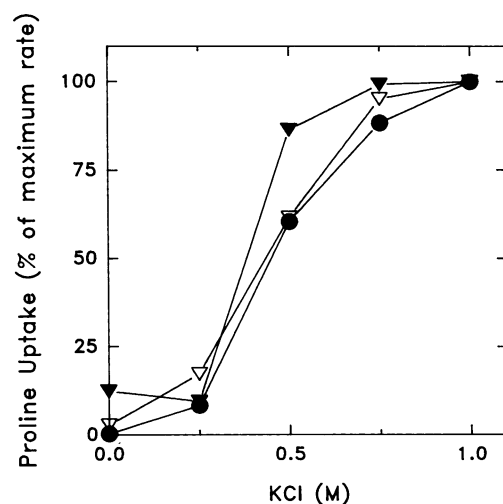

FIG. 2. Activation of proline uptake by a high medium osmotic pressure. Cells were grown in MRS-KCl, washed, and assayed as described in the text. The initial rates of proline transport were measured with 20 mM (●), 2.5 mM (∇), and 90 μM (▼) proline in MES buffer containing the indicated concentrations of KCl.
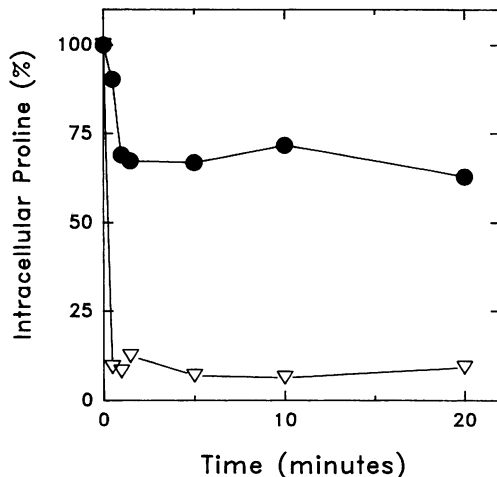
FIG. 3. Effect of medium osmolarity on proline efflux from preloaded cells. Cells were allowed to accumulate [^{3}H]proline as described in the text and centrifuged. At zero time, the cells were resuspended in MES buffer (pH 6.5) with 0.5 M KCl (●) or without KCl (▽), and then samples were removed, filtered, washed, and counted for radioactivity as described in the text.

DISCUSSION

The osmotically activated transport systems for proline and betaine in *L. acidophilus* IFO 3532 (renamed *L. casei* subsp. *rhamnosus* Hansen IFO 3532) are distinct but share a number of characteristics. (i) Both systems are activated, not induced, by increased osmotic pressure. (ii) Although the affinities for their substrates differ by a factor of approximately 300, both systems transport over a long time course (20 to 40 min), compared with the uptake of leucine, which is complete within 5 to 10 min. Leucine is not an osmolyte and is presumably used for nutrition. (iii) The V_{max} values of both the proline and the betaine transport systems increase under the influence of increased osmotic pressure, while the affinity for at least one substrate, proline, is not affected. (iv) Both systems appear to catalyze unidirectional transport. The accumulated radioactive proline or betaine is not exchanged with nonradioactive substrate, whether the cells are starved or glucose energized.

Adjustment to decreased osmotic pressure does not seem to involve the proline transport system. Rapid efflux of accumulated proline was seen when the osmotic pressure of the medium was decreased suddenly. The efflux took place in fully energized cells and was unaffected by high concentrations of external proline. The resulting decrease in internal osmotic pressure presumably resulted in the reestablishment of cellular turgor. The efflux of osmolytes has been observed in other organisms subjected to osmotic downshock, including the halophile *Ectothiorhodospira halochloris* (17) and *E. coli* (16). In *E. coli* a rapid efflux, lasting less than 2 s, resulted in the loss of intracellular solutes, endogenous nucleotides, and K$^+$ but not of acid-precipitable macromolecules. The nonspecific leak was followed by a rapid restoration of the permeability barrier of the cell membrane. It is possible that proline and betaine efflux by osmotically downshocked lactobacilli is due to a similar nonspecific leak in the cell membrane.

L. acidophilus IFO 3532 was found to possess only a single proline transport system that is osmotically activated and has a low affinity for proline. In other organisms capable

of accumulating proline as a compatible solute, there is generally more than one transport system for this solute and at least one system is responsive to changes in osmotic pressure. In *Staphylococcus aureus*, there are two proline transport systems, one of which is osmotically activated (1). *E. coli* transports proline by the PutP system and also by the osmotically induced and activated ProU and ProP systems (3, 6, 7, 20).

In *Lactococcus* spp., proline is a poor nutrient and peptides containing proline serve a more significant function in nutrient acquisition. The uptake of proline by a passive diffusion system (or by a system that is not saturated by 10 mM proline) was recently reported for *Lactococcus* spp. (15). This system was not activated by 0.5 M NaCl. The low-affinity transport system of *L. acidophilus* IFO 3532 did not function unless the concentration of salt in the buffer exceeded 0.3 M, indicating that the proline carrier is involved in regulation rather than nutrition. It is known that lactobacilli, which are nutritionally fastidious, do not generally require proline as a nutrient (11).

The physiological role of the osmotically activated proline transport system in this nutritionally fastidious *Lactobacillus* species remains obscure. In MRS medium containing a high salt concentration, only betaine was accumulated to sufficiently high levels to compensate for the increased osmotic pressure (8). In the same cells, the intracellular proline concentrations increased significantly in response to increased osmotic pressure but only reached approximately 20 mM. Osmocompensatory levels of intracellular proline were accumulated by these cells only at high extracellular proline concentrations (50 mM). Indeed, under those conditions proline was accumulated at a rate similar to that of betaine and to a final intracellular concentration comparable to that of betaine. Therefore, proline accumulation by this osmoactivated transport system is likely to contribute significantly to the osmotic tolerance of this organism only in media containing relatively high proline concentrations, particularly if betaine is absent.

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