Transport of Basic Amino Acids by Membrane Vesicles of Lactococcus lactis

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The uptake of the basic amino acids arginine, ornithine, and lysine was studied in membrane vesicles derived from cells of Lactococcus lactis which were fused with liposomes in which beef heart mitochondrial cytochrome c oxidase was incorporated as a proton motive force (PMF)-generating system. In the presence of ascorbate N,N,N',N'-tetramethylphenylendiamine-cytochrome c as the electron donor, these fused membranes accumulated lysine but not ornithine or arginine under aerobic conditions. The mechanism of energy coupling to lysine transport was examined in membrane vesicles of L. lactis subsp. cremoris upon imposition of an artificial electrical potential (ΔΨ) or pH gradient or both and in fused membranes of these vesicles with cytochrome c oxidase liposomes in which the ΔΨ and ΔpH were manipulated with ionophores. Lysine uptake was shown to be coupled to the PMF and especially to the ΔΨ, suggesting a proton symport mechanism. The lysine carrier appeared to be specific for L and D isomers of amino acids with a guanidine or NH₂ group at the C6 position of the side chain. Uptake of lysine was blocked by p-chloromercuribenzenesulfonic acid but not by malemides. Counterflow of lysine could not be detected in L. lactis subsp. cremoris, but in the arginine-ornithine antiporter-containing L. lactis subsp. lactis, rapid counterflow occurred. Homologous exchange of lysine and heterologous exchange of arginine and lysine were mediated by this antiporter. PMF-driven lysine transport in these membranes was noncompetitively inhibited by arginine, whereas the uptake of arginine was enhanced by lysine. These observations are compatible with a model in which circulation of lysine via the lysine carrier and the arginine-ornithine antiporter leads to accumulation of arginine.

Lactococcus lactis subsp. lactis and many other lactic acid bacteria contain an arginine catabolism specific transport system, i.e., the arginine-ornithine antiporter (the genus Lactococcus encompasses all previously named lactic streptococci, i.e., Streptococcus lactis, S. diacetilactis, and S. cremoris [20]) (9, 14). The prime function of this arginine-ornithine antiporter is to mediate electroneutral exchange between exogenous arginine and intracellular ornithine under conditions of arginine degradation by the arginine deiminase pathway. Lysine is a low-affinity substrate for the antiporter (9, 17). The arginine deiminase pathway yields 1 mol of ATP per mol of arginine converted into ornithine (1, 2). The tightly controlled stoichiometric exchange reaction catalyzed by the antiporter requires the formation of 1 mol of ornithine per mol of arginine accumulated. Cells of L. lactis subsp. lactis grown in the presence of arginine maintain a high intracellular concentration of ornithine (14, 17, 18) for reasons which are probably related to the kinetic properties of the antiporter. Rapid exchange rates can only be achieved when the carrier is saturated with ornithine on the cytoplasmic side. Since the arginine-ornithine antiporter is only present in arginine-grown cells (14) and arginine and lysine are essential amino acids for many lactococci (16), other mechanisms for basic amino acid transport have to be operational.

We studied the mechanism of basic amino acid transport in membrane vesicles derived from L. lactis fused with proteoliposomes containing beef heart mitochondrial cytochrome c oxidase. These membrane vesicles were isolated from L. lactis subsp. cremoris, which is unable to hydrolyze arginine (1, 20). All strains of L. lactis subsp. cremoris lack arginine deiminase, and some strains lack the catabolic ornithine carbamoyltransferase as well (1). L. lactis subsp. cremoris also lacks the arginine-ornithine antiporter (W. N. Konings, B. Poolman, and A. J. M. Driessen, Crit. Rev. Microbiol. [In press]), which excludes a possible interference of the uptake of basic amino acid by this system. Of the three basic amino acids lysine, arginine, and ornithine, only lysine is accumulated to a significant level in response to a proton motive force (PMF). Lysine uptake appears to occur in symport with a proton(s). The lysine carrier has a low affinity for ornithine and arginine. PMF-driven lysine uptake was also observed with membrane vesicles derived from cells of L. lactis subsp. lactis in which the arginine-ornithine antiporter was either repressed or induced. Net uptake of arginine during growth on arginine-containing medium may occur by a shuttle which consists of the H⁺-lysine symporter and the arginine-ornithine antiporter. Accumulated arginine can be directed into the biosynthesis of cell components or used for the maintenance of a high intracellular ornithine pool and the synthesis of ATP by substrate-level phosphorylation.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and isolation of membranes. L. lactis subsp. cremoris Wg2 (protease and arginine-ornithine antiport deficient) was grown anaerobically at a controlled pH of 6.4 on MRS broth (3) with 2% (wt/vol) lactose as described previously (13). L. lactis subsp. lactis ML3 was grown on a chemically defined medium with 2% (wt/vol) galactose and 20 mM arginine (14). Right-side-out membrane vesicles were prepared by osmotic lysis (13) and stored in liquid nitrogen in a solution containing 50 mM potassium phosphate (pH 7.0) with 10 mM MgSO₄. For some experiments, membrane vesicles were derived from cells grown on a chemically defined medium in which arginine was omitted and 2% (wt/vol) glucose served as the energy source. The arginine-ornithine antiporter is not expressed

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Phospholipids, preparation of cytochrome c oxidase proteoliposomes, and membrane fusion. Crude Escherichia coli and soybean phospholipids were acetone-ether washed as described previously (22) and stored as stock solutions of 100 mg of lipid per ml in chloroform supplemented with 1 mM dithiothreitol. Cytochrome c oxidase was isolated from beef heart as described previously (23). For reconstitution, a dry lipid film was suspended in 50 mM potassium phosphate (pH 6.0) to a concentration of 20 mg/ml and n-octyl-β-D-glucopyranoside was added to a final concentration of 30 mM. The suspension was sonicated with a probe-type sonicator (MSE Scientific Instruments, West Sussex, United Kingdom) at an amplitude of 2 to 3 μm (peak to peak) under a flush of N₂ for 200 s at 4°C. Burst cycles of 15 s of sonication and 45 s of rest were used. Cytochrome c oxidase (4.5 nmol of heme a per ml) was added, and the mixture was dialyzed against 500 volumes of 50 mM potassium phosphate (pH 6.0) at 4°C for 4 h. Dialysis was continued overnight against 500 volumes of the same buffer. For membrane fusion (5, 6), cytochrome c oxidase proteoliposomes (10 mg of lipid) and bacterial membrane vesicles (1 mg of protein) were mixed and rapidly frozen in liquid nitrogen. Frozen membranes were slowly thawed at room temperature, and the suspension was sonicated for 8 s as described above. Fused membranes were collected by centrifugation (45 min at a maximum of 280,000 × g at 4°C; Beckmann 75 Ti) and suspended to a protein concentration of 8 to 10 mg/ml in 50 mM potassium phosphate (pH 6.0).

Transport assays. Amino acid transport by membrane vesicles of L. lactis fused with cytochrome c oxidase proteoliposomes was performed essentially as described previously (4–9). Imposed PMF-driven lysine transport was assayed as described previously (4, 8). Membrane vesicles of L. lactis were equilibrated with 20 mM potassium phosphate of the indicated pH containing 100 mM potassium phosphate in the presence of 2 μM valinomycin. Membranes were collected by centrifugation (30 min at 48,000 × g and 4°C) and concentrated as far as possible in 20 mM potassium phosphate–100 mM potassium phosphate (typically 20 to 25 mg of protein per ml). For imposition of a PMF with the inside alkaline and negative, samples of 4 μl were then dialyzed against 200 μl of 20 mM potassium phosphate of the indicated pH, supplemented with 100 mM sodium pipes [piperazine-N,N’-bis(2-ethanesulfonic acid)] and 1.5 μM [¹³C]lysine at 22°C. For imposition of Δψ, inside negative, samples were diluted into 20 mM sodium phosphate supplemented with 100 mM sodium PIPES. A ΔpH, inside alkaline, was imposed by diluting the membranes into 20 mM potassium phosphate–100 mM PIPES at the pH indicated. At timed intervals, 2 ml of ice-cold 0.1 M LiCl was added to the samples and subsequently filtered over 0.45-μm-pore-size cellulose-nitrate filters (Millipore Corp., Bedford, Mass.). The filters were washed once with 2 ml of ice-cold 0.1 M LiCl, and the radioactivity retained on the filters was measured by liquid scintillation spectrometry. Exchange, efflux, and counterflow experiments were performed essentially as described previously (4, 9).

Other analytical procedures. Protein was measured by the method of Lowry et al. (11), with bovine serum albumin as a standard.

Materials. L-[¹⁴C]lysine dihydrochloride (12.4 TBq/mol), L-[²,³-H]lysine dihydrochloride (1.1 TBq/mmol), and L-[¹⁴C]arginine dihydrochloride (11 TBq/mol) were obtained from Amersham Corp. (Buckinghamshire, England).

FIG. 1. PMF-driven transport of basic amino acids by fused membranes obtained from membrane vesicles of L. lactis subsp. cremoris Wg2 (A) and L. lactis subsp. lactis ML3 (B) fused with cytochrome c oxidase proteoliposomes. Uptake of lysine (○), ornithine (●), and arginine (□) is shown. L. lactis subsp. cremoris Wg2 was grown on complex broth with lactose. L. lactis subsp. lactis ML3 was grown on chemically defined medium with galactose and arginine. In panel A, the final lysine, ornithine, and arginine concentration was 2.5 μM. In panel B, amino acid concentrations of 0.7 μM were used. For clarity, the negligible uptake of basic amino acids in the absence of cytochrome c is not shown. Uptake was assayed at pH 6.0.

L-[²,³-H]ornithine dihydrochloride (1.1 TBq/mmol) was purchased from Dupont. NEN Research Products (Boston, Mass.). n-Octyl-β-D-glucopyranoside and E. coli phospholipids were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of reagent grade and were acquired from commercial sources.

RESULTS

Uptake of basic amino acids by fused membranes. Uptake of the basic amino acids lysine, ornithine, and arginine was examined in fused membranes obtained from membrane vesicles of L. lactis subsp. cremoris and cytochrome c oxidase proteoliposomes. These membranes lack the arginine-ornithine antiporter. In the presence of the electron donor system ascorbate-N,N,N’,N’-tetramethylphenylenediamine-cytochrome c, lysine was accumulated more than 100-fold (Fig. 1A). Accumulated [¹³C]lysine was slowly chased by the addition of an excess of unlabeled lysine (data not shown). Uptake of ornithine and arginine was virtually undetectable (Fig. 1A).

Artificially imposed PMF-driven uptake of lysine. The effect of an artificially imposed PMF on lysine transport was investigated. An electrical potential across the membrane (Δψ), inside negative, can be created by diluting K⁺-loaded membranes into a K⁺-free (i.e., Na⁺) buffer in the presence of the K⁺ ionophore valinomycin. Dilution into a Na⁺ buffer results in both the generation of a chemical gradient of Na⁺ across the membrane (ΔpNa) and a Δψ. A pH gradient across the membrane (ΔpH), inside alkaline, can be imposed by
diluting acetate-loaded membranes into a solution containing a less permeable anion, such as PIPES. Since acetate is only membrane permeable in its protonated state, i.e., acetic acid, exit of acetic acid will result in the quantitative removal of protons from the intravesicular space. Simultaneous imposition of both a K⁺ and acetate diffusion gradient results in the transient generation of a PMF, inside negative and alkaline. At pH 6.0, lysine is rapidly accumulated in response to an imposed Δψ (Fig. 2). No uptake of lysine was observed when valinomycin was omitted, i.e., ΔpNa alone (data not shown). Uptake was higher when in addition to a Δψ, a ΔpH was present. With a ΔpH alone, the rate of lysine uptake is low but several fold higher than in the absence of a PMF (Fig. 2). A similar picture emerged when lysine uptake was assayed at pH 5.0 and 7.0 (Fig. 2, inset). These results indicate that both the Δψ and the ΔpH can act as a driving force for lysine uptake, suggesting that transport occurs in symport with a proton(s).

**Effect of ionophores on lysine transport.** The relation between the PMF and lysine transport was studied in more detail in membranes containing cytochrome c oxidase. The composition of the PMF can be easily varied by the use of the ionophores nigericin and valinomycin, which allows a detailed investigation of the mode of energy coupling to transport of this positively charged amino acid. Nigericin mediates electroneutral K⁺-H⁺ exchange, thereby collapsing ΔpH. Under those conditions, a compensatory increase in Δψ occurs (6-8). At pH 6.0 and 7.0, uptake of lysine is stimulated by nigericin (Fig. 3). However, at pH 5.0 lysine uptake was dramatically reduced by this ionophore. Valinomycin, an ionophore that catalyzes electrogenic K⁺ transport, can be used for decreasing Δψ. In the fused membrane system, reduction of the Δψ is only partially compensated by an increase in ΔpH. Valinomycin strongly inhibited lysine transport at all pH values tested (Fig. 3), although this effect is less pronounced at pH 5.0. At pH 5.0, Δψ is low and the ΔpH is the major component of the PMF. These results support the conclusion that lysine uptake occurs in symport with a proton(s).

**Kinetics analysis of lysine transport.** Lysine uptake displayed monophasic saturation kinetics. Calculation of the Michaelis-Menten constants from the initial rate of lysine uptake (time points taken within the first 10 min) with a concentration range of 3 to 70 μM gave an affinity constant (Kₐ) of 16 μM and a maximal velocity (Vₘₐₓ) of 0.28 nmol/min per mg of protein. Lysine transport was assayed at two concentrations, 2.9 and 29.8 μM, in the presence of unlabeled ornithine or arginine. The concentrations of these amino acids varied between 50 and 300 μM. Ornithine is a very weak competitive inhibitor of lysine transport with an inhibitor constant (Kᵢ) of about 0.5 mM. Arginine exhibits a Kᵢ which is greater than 1 mM.

**Inhibition of transport of lysine by analogs.** The specificity of the lysine carrier was assessed from the extent of inhibition of lysine transport by a 33-fold excess of a wide range of basic amino acid analogs. The initial rate of artificially imposed PMF-driven L-lysine uptake was markedly reduced by the lysine analogs S-aminoethyl-L-cysteine and δ-hydroxy-DL-lysine (Table 1). Uptake was also strongly inhibited by the D enantiomer of lysine and by the structural analog of arginine, L-homoarginine. The results indicate that
specificity of the lysine carrier is restricted to amino acids with a guanidino or NH₂ group at the C6 position.

Inhibition of transport by sulfhydryl-specific reagents. The sensitivity of the lysine carrier for sulfhydryl group-specific agents was tested. Membrane vesicles of L. lactis subsp. cremoris were incubated with various sulfhydryl group-specific agents at different concentrations, and the residual imposed PMF-driven rate of lysine uptake was determined. The lysine carrier was not inhibited by maleimides, i.e., N-ethylmaleimide and the lipophilic N-[1-naphthyl]maleimide (data not shown). Strong inhibition at low concentrations was observed with the organomercurial p-chloromercuribenzenesulfonic acid. Approximately 3 to 5 nmol of p-chloromercuribenzenesulfonic acid per mg of membrane protein was sufficient for complete inhibition of lysine transport. Full recovery of lysine transport activity was observed when dithiothreitol was added in a 10-fold excess to p-chloromercuribenzenesulfonic acid (500 μM). Dithiothreitol alone had no effect on lysine transport.

Exchange and efflux of lysine. Countercurrent experiments were conducted with membrane vesicles of L. lactis subsp. cremoris to test whether the lysine carrier mediates homologous lysine exchange. Membrane vesicles were equilibrated with 10 to 500 μM lysine and diluted 50-fold into a medium containing [¹⁴C]lysine (3 μM). Under those conditions, an outwardly directed lysine concentration gradient is imposed, and transient accumulation of the [¹⁴C]lysine should occur as a result of two competing processes: (i) exchange between [¹⁴C]lysine and unlabeled lysine and (ii) efflux of lysine. In none of these cases could significant uptake of lysine be detected (data not shown). In another experiment, fused membranes were allowed to accumulate lysine in the presence of a PMF, and the release of [¹⁴C]lysine was followed upon addition of the ionophores valinomycin and nigericin, which dissipate the PMF. Lysine was slowly released under those conditions. However, efflux of [¹⁴C]lysine was not accelerated by an excess of unlabeled lysine added on the outside (data not shown). These results suggest that the lysine carrier catalyzes exchange at a rate which is even slower than or equal to the rate of efflux.

Transport of basic amino acids by membrane vesicles of L. lactis subsp. lactis. Transport of lysine, ornithine, and arginine was also examined in membrane vesicles isolated from cells of L. lactis subsp. lactis grown on a chemically defined medium under conditions in which the arginine-ornithine antiporter is expressed (Driessen et al., submitted). In membrane vesicles fused with cytochrome c oxidase proteoliposomes, upon generation of a PMF a more than 1,000-fold accumulation of lysine could be reached at low external lysine concentration (Fig. 1B). Ornithine and arginine were accumulated to a much lower level. Membrane vesicles derived from cells in which the arginine-ornithine antiporter was completely repressed (Driessen et al., in preparation) displayed a similar response. The Vₘₐₓ and Kₛ for PMF-driven lysine transport were 1.5 nmol/min per mg of protein and 1 μM, respectively. Arginine is a strong inhibitor of PMF-dependent uptake of lysine by membrane vesicles derived from cells of L. lactis subsp. lactis which are induced for the arginine-ornithine antiporter but not in L. lactis subsp. cremoris membrane vesicles. Uptake of lysine was inhibited by low concentrations of arginine, whereas under the same conditions, uptake of arginine was enhanced (Fig. 4A). This phenomenon was only observed when the lysine concentrations did not exceed 10 μM (not shown). The extent of inhibition of lysine uptake was almost quantitatively compensated by an increase in arginine uptake.
suggesting that lysine accumulated by the lysine carrier is rapidly released in exchange for external arginine. A kinetic analysis of the inhibitory effect of arginine on PMF-driven lysine transport indicates that arginine acts as a noncompetitive inhibitor in the lower concentration range (1 to 50 μM) (data not shown).

Heterologous exchange of lysine and arginine in membrane vesicles in *L. lactis* subsp. *lactis*. Exchange was directly assayed by diluting membrane vesicles of *L. lactis* subsp. *lactis* loaded with lysine into a buffer containing [14C]lysine (data not shown) or [14C]arginine (Fig. 4B). Both amino acids were rapidly accumulated, whereas only a low level of uptake was observed with membrane vesicles which were not preloaded. These results support previous observations that lysine is a substrate for the arginine-ornithine antiporter (9, 17). The inhibiting effect of arginine on PMF-driven lysine transport suggests that circulation of lysine via the lysine carrier and the arginine-ornithine antiporter can lead to accumulation of arginine.

**DISCUSSION**

In this report, we presented evidence that uptake of lysine in lactococci is mediated by a highly specific PMF-dependent transport system. Separate PMF-driven arginine uptake appears to be absent.

Lysine and arginine are major constituents of lactococcal cell wall and protein and are essential for growth of some *L. lactis* strains (16). A transport system for (di-)peptides containing positively charged arginyl or lysyl residues is absent in *L. lactis* (21), indicating that free amino acids are required for growth. An understanding of the mechanism(s) of basic amino acid transport in lactococci is also of interest because growing cells of *L. lactis* subsp. *lactis* are able to maintain an intracellular ornithine concentration of up to 5 mM under conditions of arginolysis (14). In the arginine deiminase pathway, uptake of arginine and efflux of the end product ornithine occur by an electroneutral arginine-ornithine exchange system (9, 14, 17). The strict stoichiometric coupling of this process prevents a rise in the intracellular ornithine concentration and requires a stoichiometric interconversion of accumulated arginine into ornithine. *L. lactis* subsp. *lactis* can easily resist high concentrations of arginine (up to 40 mM) in the medium, whereas growth of *L. lactis* subsp. *cremoris*, which lacks the arginine-ornithine antiporter, is almost completely inhibited under those conditions (unpublished data). These observations indicate that other transport mechanisms for basic amino acids may be functional in *L. lactis* and prompt us to study the mechanism of basic amino acid transport in more detail by the use of lactococcal membrane vesicles.

When the arginine-ornithine antiporter is repressed or absent, lysine is only transported with a reasonable affinity and to appreciable levels by a PMF-dependent mechanism. H+–amino acid stoichiometries can be estimated from the steady-state level of amino acid accumulation and the PMF. Although we have not undertaken a quantitative determination of this parameter, the more than 1,000-fold accumulation of lysine at pH 7.0 (lysine concentration of 0.7 μM) with a PMF of about −100 mV (7, 8) suggests a H+-lysine stoichiometry of at least 0.8. Since the apparent pK of the C-6 NH2 group of lysine is around pH 10.5, lysine is positively charged in the pH range of 5 to 7. Consequently, lysine transport should be mainly driven by the Δψ component of the PMF, which is in line with the observation that the ionophore nigericin stimulates lysine transport at most pHis. At low pH, other factors may interfere, such as the internal pH (8, 15) or the incomplete interconversion of ΔpH into Δψ.

Two transport mechanisms for lysine have been recognized in *L. lactis* subsp. *lactis*, the lysine carrier and the arginine-ornithine antiporter. Interplay between both systems offers an alternative way for arginine to enter the cell (Fig. 5). Lysine accumulated in response to a PMF by the lysine carrier can be excreted by the arginine-ornithine antiporter in exchange for external arginine, causing the net accumulation of arginine (Fig. 4A). Accumulated arginine can be used for biosynthesis or is immediately converted into ornithine to maintain a high intracellular ornithine pool. Lysine (up to 10 mM) and ornithine (10 to 15 mM) are major constituents of the intracellular amino acid pool of freshly harvested cells of *L. lactis* subsp. *lactis* (14, 17). These values drop below 1 and 2.5 mM in the presence of exogenous arginine, which supports the notion that the antiporter also catalyzes exchange between arginine and lysine in vivo. Whether the putative lysine-arginine shuttle (Fig. 5) significantly contributes to net uptake of arginine in vivo remains to be established.

The high intracellular ornithine pool in arginine-grown cells (14, 18) may also originate from ornithine biosynthesis by the N2-acetylglutamate–N2-acetylornithine pathway (12). Cells of *L. lactis* subsp. *lactis* growing in an arginine-deficient medium use exogenous glutamic acid or ornithine as the precursor for the biosynthesis of arginine (2, 12, 19). Glutamic acid is converted into ornithine by the N2-acetylglutamate–N2-acetylornithine pathway. Ornithine in turn can be converted into arginine, most likely by the arginosuccinyl route. It is not known whether these anabolic routes are also operational when arginine is present in the growth medium, whereas their presence may be a strain-dependent property (16). The energetic costs for the synthesis of 1 mol of arginine from 1 mol of glutamic acid (12) is at least 2 mol
of ATP and 1 mol of carbamoylphosphate (2), which is much higher than the energy required for uptake by the proposed lysine-arginine shuttle. It should be emphasized that the operation of this putative shuttle cannot apply to L. lactis subsp. cremoris. Since the arginine-ornithine antiporter is absent in these species (Konings et al., in press), other means are required to obtain arginine such as (i) conversion of ornithine into arginine by the argininosuccinyl route or (ii) accumulation of arginine by a PMF-independent mechanism, for instance, by a mechanism which involves ATP or a derivative thereof (Konings et al., in press).

LITERATURE CITED