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Uniport of Anionic Citrate and Proton Consumption in Citrate Metabolism Generates a Proton Motive Force in *Leuconostoc oenos*

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The mechanism and energetics of citrate transport in *Leuconostoc oenos* were investigated. Resting cells of *L. oenos* generate both a membrane potential ($\Delta\Psi$) and a pH gradient (ΔpH) upon addition of citrate. After a lag time, the internal alkalinization is followed by a continuous alkalinization of the external medium, demonstrating the involvement of proton-consuming reactions in the metabolic breakdown of citrate. Membrane vesicles of *L. oenos* were prepared and fused to liposomes containing cytochrome *c* oxidase to study the mechanism of citrate transport. Citrate uptake in the hybrid membranes is inhibited by a membrane potential of physiological polarity, inside negative, and driven by an inverted membrane potential, inside positive. A pH gradient, inside alkaline, leads to the accumulation of citrate inside the membrane vesicles. Kinetic analysis of ΔpH-driven citrate uptake over a range of external pHs suggests that the monovalent anionic species (H_2cit^-) is the transported particle. Together, the data show that the transport of citrate is an electrogenic process in which H_2cit^- is translocated across the membrane via a uniport mechanism. Homologous exchange (citrate/citrate) was observed, but no evidence for a heterologous antiport mechanism involving products of citrate metabolism (e.g., acetate and pyruvate) was found. It is concluded that the generation of metabolic energy by citrate utilization in *L. oenos* is a direct consequence of the uptake of the negatively charged citrate anion, yielding a membrane potential, and from H^+-consuming reactions involved in subsequent citrate metabolism, yielding a pH gradient. The uptake of citrate is driven by its own concentration gradient, which is maintained by efficient metabolic breakdown (metabolic pull).

Lactic acid bacteria (LAB) are fermentative organisms that rely largely on substrate-level phosphorylation in the glycolytic pathway to obtain the metabolic energy necessary for growth (for a recent review on energy transduction in LAB, see reference 18). The mechanisms in these organisms that supply the cells with additional means of ATP generation, e.g., the arginine deiminase pathway (19), have been described. Other mechanisms for energy conservation have also been discovered, such as end product excretion (15) and, more recently, the precursor-product exchange mechanism (16, 20). In the latter process, the precursor is taken up in exchange for the product of the internal decarboxylation reaction. A proton motive force (PMF) is generated as a result of the electrogenic nature of the transport step and proton consumption in the decarboxylation reaction. Subsequently, the PMF is used to drive ATP synthesis or other metabolic energy-requiring processes. Examples of energy conservation by precursor-product exchange are oxalate fermentation in *Oxalobacter formigenes* (1), malolactic fermentation in *Lactococcus lactis* (20), and the conversion of histidine to the biogenic amine histamine in *Lactobacillus buchneri* (16).

Details on the biochemistry of citrate metabolism by dairy LAB are now available (3, 22, 24, 28). However, the energetics of citrate uptake is still poorly understood. The ability to transport citrate is plasmid encoded in these organisms (4, 5, 8). The genes coding for the citrate permeases (*citP*) of *Lactococcus lactis* and *Leuconostoc lactis* have been cloned and sequenced and shown to be almost identical (4, 5). A smaller but significant degree of identity exists with the sodium-dependent citrate transporter of *Klebsiella pneumoniae* (27). Initial characterization of the mechanism of citrate uptake mediated by the lactococcal citrate carrier revealed a dependency of citrate transport on both components of the PMF, the pH gradient and the membrane potential, indicating that protons are cotransported during the process (5). Therefore, these LAB seem to use the free energy stored in the PMF to accumulate citrate inside the cell. In contrast to these findings, it was recently suggested that *Lactococcus lactis* subsp. *lactis* biovar diacetylactis is able to generate metabolic energy from citrate utilization (7). Addition of citrate to resting cells was immediately followed by the generation of a membrane potential. The authors have proposed that the mechanism of energy generation is of the precursor-product exchange type; uptake of citrate in its divalent state ($Hcit^{2-}$) is coupled to the exit of one of the monovalent anionic products of citrate metabolism, i.e., acetate or pyruvate.

Here, we present the results of our studies on the kinetics and energetics of citrate transport in *Leuconostoc oenos*, a lactic acid bacterium that plays an important role in wine production. It performs malolactic fermentation and metabolizes citrate, a precursor of aroma compounds (e.g., diacetyl), at the low pHs normally found in wine (9). Our results show that citrate uptake and metabolism result in the formation of an electrochemical gradient of protons across the cytoplasmic membrane. To assess the mechanism of citrate transport, membrane vesicles were isolated and the effect of the pH

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gradient and of the transmembrane electrical potential on citrate uptake was evaluated. A model that explains the generation of metabolic energy from citrate uptake and metabolism is presented.

MATERIALS AND METHODS

Chemicals. [1,5-^{14}C]citrate (110 mCi/mmol), L-[U-^{14}C] leucine (319 mCi/mmol), [carboxyl-^{14}C]benzoic acid (22 mCi/mmol), and [^{14}C]potassium thiocyanate (55 mCi/mmol) were obtained from Amersham. All other chemicals were of reagent grade.

Organism and growth conditions. *L. oenos* GM (Microlife) was grown at 30°C in FT80 medium (2), modified by omission of Tween 80, containing 2.5 g of citric acid and 8 g of glucose per liter. Cultures were harvested in the late exponential growth phase, washed twice, and resuspended in 50 mM potassium phosphate, pH 5.

Isolation of membrane vesicles. Membrane vesicles of *L. oenos* were prepared by osmotic lysis as described previously (17), with the following modifications. Cells were harvested in the late exponential growth phase (A_{600} = 0.5 to 0.6), and the amounts of lysozyme, DNase, and RNase were doubled. The vesicles were resuspended in 50 mM potassium phosphate (pH 6) without $MgSO_4$. Membrane vesicles were stored under liquid nitrogen, in aliquots of 1 ml containing 1 mg of protein (12).

Fusion of membrane vesicles with proteoliposomes and liposomes. Cytochrome *c* oxidase isolated from beef heart mitochondria was reconstituted by detergent dialysis into liposomes consisting of acetone–ether-washed *Escherichia coli* lipids and egg phosphatidylcholine in a ratio of 3:1, as described previously (6). The proteoliposomes contained 1 nmol of cytochrome *c* oxidase per 10 mg of lipid. Hybrid membranes were obtained by fusion of cytochrome *c* oxidase proteoliposomes with *L. oenos* membrane vesicles (1 mg of membrane protein per 10 mg of lipid). Fusion was achieved by freeze-thaw sonication (microtip at an amplitude of 4 μm for 8 s) (6) or, alternatively, by freeze-thaw extrusion. Frozen and thawed membranes were extruded successively through 0.4- and 0.2-μm-pore-size polycarbonate filters with an extrusion apparatus (Avestin, Inc.) (13). The internal volume of the hybrid membranes was assumed to be 8 μl/mg (6). Membrane vesicles were fused with liposomes devoid of cytochrome *c* oxidase by the same procedures.

Measurement of membrane potential and pH gradient across the cell membrane. The intracellular pH was estimated from the distribution of [carboxyl-^{14}C]benzoic acid (50 mCi/mmol), using the silicon oil centrifugation technique (21). The membrane potential was inferred from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+). External TPP^+ was measured with a TPP^+-selective electrode, as described elsewhere (11). The membrane potential was calculated from the distribution of the probe after correction for nonspecific binding of TPP^+ to cell components (23). All experiments were carried out at 30°C.

Transport assays. (i) PMF-driven uptake. Membrane vesicles were fused with proteoliposomes containing cytochrome *c* oxidase. Hybrid membranes were concentrated by centrifugation (250,000 × *g* for 30 min at 4°C) and resuspended in 50 mM potassium phosphate, pH 5, to a protein concentration of 0.3 mg/ml. The hybrid membranes were incubated in potassium phosphate, pH 5, containing 200 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 20 μM cytochrome *c*, and 10 mM potassium ascorbate. After 1 min of incubation under a flow of water-saturated air, the radiolabeled substrates were added, and uptake was assayed as follows. At various times, samples were taken, immediately mixed with 2 ml of ice-cold 0.1 M LiCl, and filtered over 0.45-μm-pore-size cellulose-nitrate filters. The filters were rinsed with 2 ml of the LiCl solution and transferred to scintillation vials containing 2 ml of scintillation fluid, and radioactivity was counted.

(ii) Uptake driven by artificially imposed electrical gradients. Membrane vesicles were fused with liposomes devoid of cytochrome *c* oxidase. For the generation of a membrane potential ($\Delta\Psi$), inside negative, hybrid membranes were prepared in 100 mM potassium phosphate, pH 6, concentrated by centrifugation (250,000 × *g* for 30 min at 4°C) to a protein concentration of approximately 40 mg/ml, and diluted 100-fold into 100 mM sodium phosphate buffer, pH 6, in the presence of valinomycin (1 μM). To generate a $\Delta\Psi$, inside positive, hybrid membranes were incubated for 1 h on ice in 100 mM sodium phosphate, pH 6, washed once with the same buffer, and concentrated by centrifugation. Subsequently, these Na^+-equilibrated membranes were diluted 100-fold into 100 mM potassium phosphate, pH 6, in the presence of valinomycin. For the control experiment (no $\Delta\Psi$), K^+-loaded membranes were diluted into 100 mM potassium phosphate, pH 6, also in the presence of valinomycin. In all cases, the dilution media were supplemented with 5 μM [1,5-^{14}C]citrate.

(iii) pH jump experiments. A pH gradient (alkaline inside) was imposed by addition of sulfuric acid. Hybrid membranes were resuspended in 50 mM potassium phosphate, pH 6.5, containing 5 μM [1,5-^{14}C]citrate at a protein concentration of 0.3 mg/ml. A small drop of 0.5 N sulfuric acid was carefully positioned on the wall of the tube, just above the level of the liquid in the tube. At time zero, the reaction was started by vigorous vortexing. Samples were taken at intervals after the pH jump, and the stopping procedure was as described above. Thiocyanate distribution was monitored in the same way, but in this case the dilution medium was supplemented with 20 μM [^{14}C]thiocyanate. All transport assays were performed at 30°C.

RESULTS

PMF generation in whole cells. The effect of citrate metabolism on the membrane potential and the pH gradient was established by adding citrate to resting cells of *L. oenos*. The membrane potential ($\Delta\Psi$) was inferred from the distribution of the lipophilic cation TPP^+, which was monitored by the use of a TPP^+-selective electrode (11). The endogenous $\Delta\Psi$ of resting cells of *L. oenos* was found to be very low. The electrode response observed following the addition of cells is due mainly to dilution and nonspecific binding of TPP^+ to cell components (Fig. 1). A transient transmembrane electrical potential was generated immediately upon the addition of citrate (Fig. 1, top track). Preincubation of the cells with the F_0F_1 ATPase-blocking agent *N,N*-dicyclohexylcarbodiimide (DCCD) resulted in a higher $\Delta\Psi$ that was stable for a longer period of time (Fig. 1, bottom track). The $\Delta\Psi$ generated was immediately dissipated by the addition of valinomycin, a potassium ionophore that collapses the membrane potential. The maximal $\Delta\Psi$ generated after citrate addition was calculated to be −50 mV. The magnitude of the $\Delta\Psi$ increased with increasing citrate concentrations up to 1 mM (data not shown).

The pH gradient across the membrane of *L. oenos* was inferred from the distribution of benzoic acid (Table 1). Resting cells resuspended in 50 mM potassium phosphate, pH 4, maintained a ΔpH of 1.3 units for a prolonged period of time. Addition of citrate led to an increase of the gradient up to 2 pH units, and this gradient was stable for at least 10 min. Treatment of the cells with DCCD lowered the pH gradient

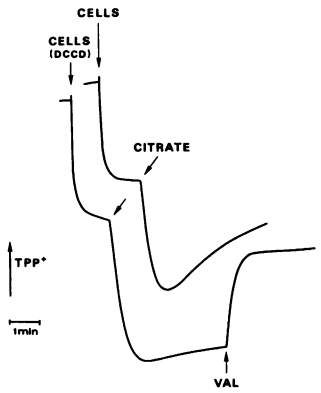

FIG. 1. Membrane potential generation by citrate metabolism in resting cells of *L. oenos*. TPP^+ uptake by cell suspensions of *L. oenos* was monitored with a TPP^+-selective electrode. A total of 0.2 ml of untreated cells (top track) or cells treated with 100 μM DCCD for 1 h at 30°C (bottom track) resuspended at a protein concentration of 9 mg/ml was added to 1 ml of 50 mM potassium phosphate (pH 5) containing 4 μM TPP^+. Citrate was added to a final concentration of 1 mM. Valinomycin (VAL) was added at a concentration of 1 μM.

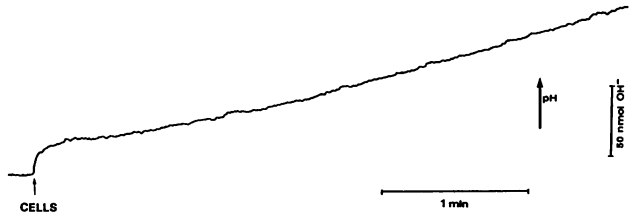

FIG. 2. Alkalinization induced by citrate metabolism. Cells were washed and resuspended in 5 mM potassium phosphate (pH 5), and 0.1 ml of cell suspension (9.2 mg/ml) was added to 1.9 ml of the same buffer containing 1 mM citrate. The external pH was measured continuously. The pH change was calibrated by the addition of small aliquots of 20 mM KOH. Addition of cells caused an electrode response that corresponds to a small increase in the initial pH. The overall change in pH was a few hundredths of a pH unit.

somewhat in both the presence and absence of citrate. The citrate-induced pH gradient in DCCD-treated cells increased from 1.6 pH units at 3 min to 1.9 pH units at 10 min.

The overall effect of proton-consuming and/or proton-producing reactions in the utilization of citrate by *L. oenos* was investigated by monitoring the changes in external pH upon addition of resting cells to a weakly buffered medium containing 1 mM citrate. Figure 2 shows that a short lag time is followed by a constant rate of alkalinization of 30.5 nmol of OH^- min^{-1} (mg of protein)$^{-1}$. This alkalinization was completely absent when citrate was omitted from the medium (data not shown). Apparently, citrate metabolism is associated with a continuous consumption of protons, which provides a mechanism for the observed generation of the pH gradient by citrate utilization (see Discussion).

Uptake of citrate in membrane vesicles. To study the mechanism of citrate uptake, membrane vesicles of *L. oenos* were prepared and fused with proteoliposomes containing cytochrome *c* oxidase. In this hybrid system, a PMF can be generated by addition of the electron donor system ascorbate-TMPD-cytochrome *c*. Addition of [^{14}C]citrate and [^{14}C] leucine to the hybrid membranes in the absence of the electron donor system leads to equilibration of the substrates across the membrane (Fig. 3A and B, respectively). Energization results in the generation of a PMF (inside alkaline and negative) and accumulation of leucine inside the membranes, showing the correct functioning of the system and indicating that this amino acid is taken up in symport with a proton (Fig. 3B). In contrast, citrate uptake was inhibited under energized conditions (Fig. 3A). To establish which component of the PMF was counteracting transport, the effect of the ionophores valinomycin and nigericin was studied. In the presence of valinomycin, when the PMF consists solely of a pH gradient, citrate accumulated in the vesicles (Fig. 3A). In contrast, citrate uptake was completely blocked in the presence of nigericin, when the PMF consists solely of a membrane potential (Fig. 3A). These results fit with citrate uptake's involving transport of net negative charge into the vesicles.

TABLE 1. Intracellular pH of *L. oenos* in the presence and absence of citrate[a]

Time (min)	Untreated cells				DCCD-treated cells			
	Without citrate		With citrate		Without citrate		With citrate	
	pH_{in}	ΔpH	pH_{in}	ΔpH	pH_{in}	ΔpH	pH_{in}	ΔpH
3	5.3	1.3	6.0	2.0	4.9	0.9	5.6	1.6
10	5.1	1.1	5.9	1.9	4.8	0.8	5.9	1.9

[a] The internal pH (pH_{in}) of resting cells of *L. oenos* in the presence or absence of 1 mM citrate was measured at an external pH of 4.

Experiments with artificially imposed membrane potentials supported this conclusion. Membrane vesicles were fused to liposomes without the PMF-generating system and equilibrated with either K^+ or Na^+ buffer. When no $\Delta\Psi$ was imposed, uptake of citrate was negligible (Fig. 4). Dilution of K^+-equilibrated membranes into Na^+ buffer in the presence of valinomycin results in a potassium diffusion potential, inside negative, and no citrate uptake (Fig. 4). However, citrate accumulation was observed upon dilution of Na^+-equilibrated membranes into K^+ buffer, resulting in an inverted membrane potential (Fig. 4).

Citrate was also taken up at a high rate in response to a sudden lowering of the external pH by the addition of an aliquot of sulfuric acid (pH jump). The maximal accumulation level was reached within 1 min (Fig. 5A). In addition to a pH gradient, the pH jump results in the generation of a proton diffusion potential (inside positive), which was monitored by the distribution of the permeant anion thiocyanate (Fig. 5B). The development of the proton diffusion potential is a consequence of the higher permeability of protons than of the sulfate and phosphate anions (14). In the presence of valinomycin, the diffusion potential was significantly reduced (Fig. 5B), as was the accumulation of citrate (Fig. 5A). Apparently, in addition to the pH gradient, the inside-positive membrane potential contributed to the driving force of citrate uptake, consistent with the transport of a negatively charged substrate.

Kinetic analysis and specificity of the citrate uptake system. The pH jump technique was used to kinetically characterize citrate uptake at several external pHs. Uptake was linear in the first 3 s in the range from 5 to 250 μM citrate. The kinetic parameters apparent affinity constant (K_m^{app}) and maximal rate (V_{max}) are summarized in Table 2. The V_{max} and the K_m^{app} for total citrate did not change dramatically over the pH range studied (3.5 to 5.5). The K_m^{app} for total citrate was found to be about 50 μM. The fraction of the different protonated species that make up total citrate at a given pH can be

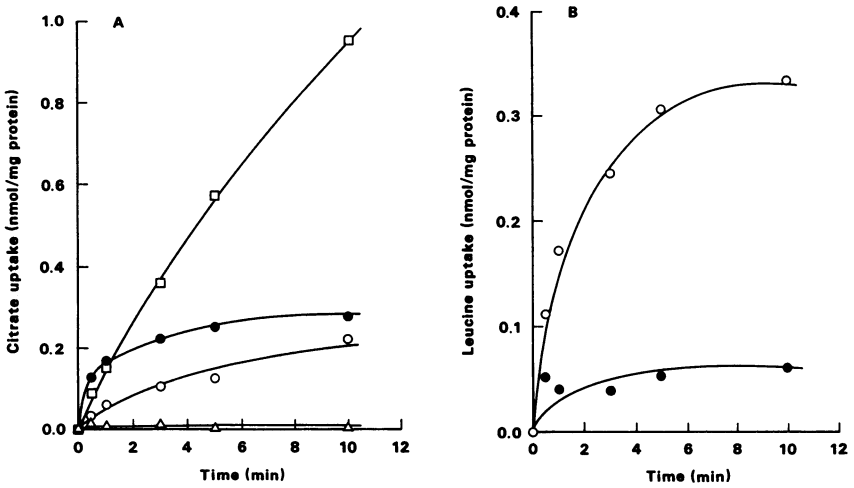

FIG. 3. PMF-driven citrate uptake. Uptake of 5 μM [^{14}C]citrate (A) and 1.6 μM [^{14}C]leucine (B) by membrane vesicles of *L. oenos* fused with proteoliposomes containing cytochrome *c* oxidase in 50 mM potassium phosphate, pH 5. Uptake was assayed in the presence (○) or absence (●) of the electron donor system ascorbate-TMPD-cytochrome *c*. The experiment with the energized membranes was repeated in the presence of the ionophores valinomycin (□) and nigericin (△), added at final concentrations of 1 and 0.5 μM, respectively.

calculated by using the Henderson-Hasselbach equation. Assuming that one particular protonated species is the actual substrate of the carrier, the affinity constants for that species can be calculated by correcting the K_m^{app} for total citrate by the fraction of that species present at every pH. The results of the calculations for each species at the experimental pHs are listed in Table 2. The species-specific K_m for the monovalent anionic species (H_2cit^-) was rather constant over the pH range tested, whereas the affinity constants for the other species varied significantly (30- to 3,000-fold) in a systematic manner.

A slow release of labeled citrate from preloaded hybrid membranes was observed in the presence of a 100-fold excess of unlabeled citrate (Fig. 6A), demonstrating exchange of internal and external citrate. A number of potential substrates for the permease were tested for their ability to induce efflux of labeled citrate in a similar way. Control experiments showed that at the concentration used, these acids did not affect PMF-driven uptake of leucine (data not shown). Neither acetate, L-malate, L-lactate, D-lactate, pyruvate, oxaloacetate, α-ketoglutarate, nor succinate induced any exit or had any inhibitory effect on further uptake of citrate, showing that the permease is highly specific for citrate. These results demonstrate that the citrate transport system of *L. oenos* catalyzes homologous citrate-citrate exchange but not heterologous exchange with any of the acids tested. The rate of homologous exchange increased up to external concentrations of about 2 mM, as shown in Fig. 6B. The rate of citrate efflux from preloaded membranes induced by collapsing the pH gradient with 0.5 μM nigericin is much faster than the maximal rate of exchange (Fig. 6B).

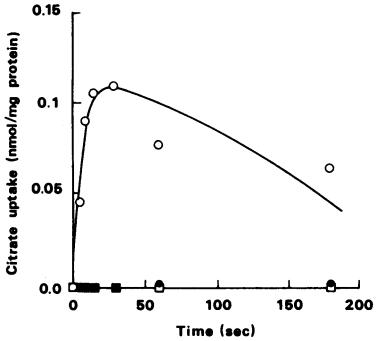

FIG. 4. Electrogenic uptake of citrate. Potassium diffusion potentials of opposite polarity were imposed across the membrane of membrane vesicles of *L. oenos* fused with liposomes. The procedures and buffers are described in Materials and Methods. The conditions were as follows: no ΔΨ (●), ΔΨ inside negative (□), and ΔΨ inside positive (○).

DISCUSSION

The mechanism of citrate uptake in *L. oenos* cannot be studied in whole cells because of rapid citrate metabolism. Therefore, membrane vesicles with a right-side-out orientation were prepared by the osmotic lysis procedure. The membrane preparation is devoid of citrate metabolism, as evidenced by the lack of release of $^{14}CO_2$ from [^{14}C]citrate (not shown). In order to be able to generate a PMF, the vesicles were fused with proteoliposomes containing cytochrome *c* oxidase. This study demonstrates that these hybrid membranes are an adequate system to study secondary solute transport in *L. oenos*.

The following observations support the conclusion that citrate uptake in *L. oenos* involves transport of net negative charge. (i) A PMF generated by cytochrome *c* oxidase results in the accumulation of citrate only when the PMF is composed solely of a ΔpH. (ii) A PMF generated by cytochrome *c* oxidase consisting solely of a ΔΨ inhibits the uptake of citrate to levels below equilibration. (iii) An artificially imposed inverted membrane potential (inside positive) results in the accumulation of citrate. Finally, (iv) citrate accumulation following a pH jump is much higher when, in addition to the pH gradient, a proton diffusion potential is allowed to develop. Transport of net negative charge is achieved when one of the deprotonated species of citrate, i.e., H_2cit^-, $Hcit^{2-}$, or cit^{3-}, is transported by the carrier. The kinetic analysis of the uptake reaction showed that the affinity constant for H_2cit^- in the pH range from 3.5 to 5.5 was rather constant, whereas the affinity constants calculated for the other species varied considerably and in a systematic manner. The maximal rate in the same pH

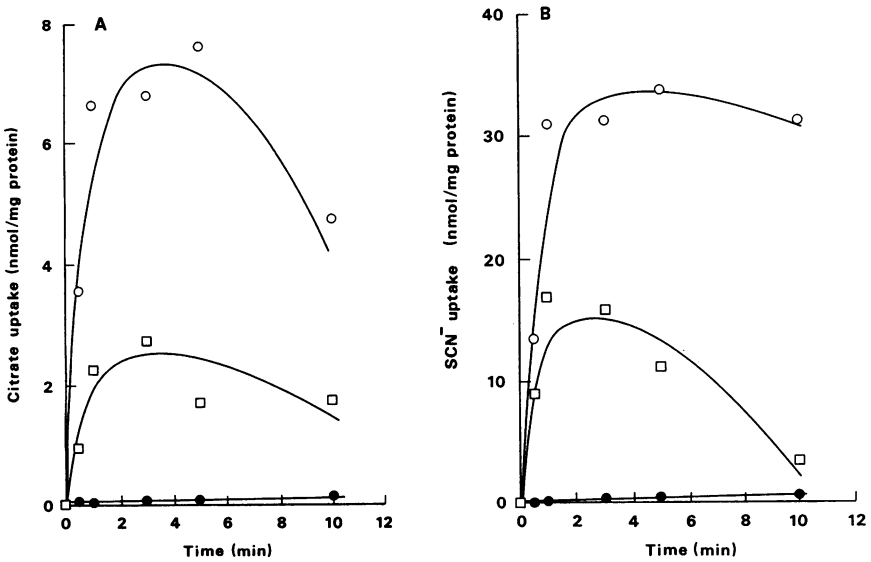

FIG. 5. pH jump-driven citrate uptake. Membrane vesicles fused with liposomes were equilibrated with 5 μM [1,5-^{14}C]citrate (A) or 20 μM [^{14}C]thiocyanate (B) in 50 mM potassium phosphate, pH 6.5. Uptake was measured upon addition of sulfuric acid (0.5 N), which lowered the external pH to 5, in either the absence (○) or the presence (□) of 1 μM valinomycin and when no acid was added (●).

range did not change significantly. Assuming that the pH profile of the activity of the carrier itself does not lead to major changes in the affinity constant for its substrate, the analysis suggests that H_2cit^- is the actual substrate of the carrier (25, 26). This conclusion would be in line with the low pHs found in wine, the natural habitat of *L. oenos*, at which H_2cit^- is the predominant species present. From these experiments, we propose that transport of citrate in membrane vesicles of *L. oenos* occurs via a uniport mechanism, with the monovalent anionic state of citrate as the transported particle. Accumulation of citrate in a vesicular system is driven by a pH gradient, inside alkaline, because of trapping of the transported particle (H_2cit^-) inside by deprotonation caused by the higher internal pH.

Citrate utilization by resting cells of *L. oenos* generates a PMF, composed of both a membrane potential and a pH gradient. The mechanism of citrate uptake involving transport of negatively charged H_2cit^- explains the generation of the membrane potential and, indirectly, the generation of the pH gradient (see below). Figure 7 shows the pathway for citrate breakdown by *L. oenos* in the absence of any other carbon and energy source. At low pHs, citrate is converted to acetate, acetoin, and carbon dioxide (22a). No ATP is formed by substrate-level phosphorylation, and none of the enzymes in the pathway is known to be involved in chemiosmotic energy conservation. The characteristics of PMF generation by citrate utilization are consistent with a mechanism other than ATP-driven proton pumping by the membrane-bound F_0F_1 ATPase. A membrane potential was built up immediately upon addition of citrate to a cell suspension of *L. oenos* (Fig. 1). Inhibition of F_0F_1 ATPase by treating the cells with DCCD (23) resulted in a higher membrane potential (Fig. 1) and an increasing pH gradient (Table 1), indicating that the PMF did not follow from ATP hydrolysis. Instead, the data suggest that the citrate-induced PMF in untreated cells is used to generate ATP via F_0F_1 ATPase.

TABLE 2. Effect of external pH on the kinetic parameters of citrate uptake[a]

pH	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)				
		Total citrate	H_3cit	H_2cit^-	$Hcit^{2-}$	cit^{3-}
3.5	237	77	21	53	2.7	0.0033
4	187	51	5	40	6.3	0.025
4.5	167	52	1.3	34	17	0.21
5	203	40	0.19	15	24	0.95
5.5	151	96	0.06	14	72	9.1

[a] Hybrid membranes were incubated with [1,5-^{14}C]citrate at concentrations of between 5 and 250 μM. Artificial transmembrane pH gradients were imposed by the pH jump technique. The amount of sulfuric acid to be added to give the desired external pH in the presence of each concentration of citrate was carefully determined prior to the experiment. The initial rate of citrate uptake was determined from the amount of label accumulated during the first 3 s. The three pKs of citrate used to calculate the species-specific K_m values were 3.1, 4.8, and 6.4.

The overall reaction for citrate breakdown by resting cells of *L. oenos* (Fig. 7) is 2 $H_3cit \rightarrow$ 2 HAc + acetoin + 4 CO_2, in which H_3cit and HAc represent citric acid and acetic acid, respectively. The pathway is pH neutral when all reactants are either fully protonated or fully deprotonated. However, at intermediate pHs, the reaction consumes protons because of pK differences between the substrate citrate and the products acetate and carbonate. The net alkalinization of the pathway at pH 5 is demonstrated by the continuous rise in pH of the medium when citrate is added to resting cells of *L. oenos* (Fig. 2). The development of a pH gradient across the membrane relies on the actual substrate and product species that are transported into and out of the cells. Citrate enters the cell as H_2cit^-, and the products will leave the cell in their uncharged protonated states (Fig. 8). Inside the cell, 1 proton per citrate is necessary to convert H_2cit^- into the protonated products (see equation above). Outside the cell, the pH is affected by redistribution of citrate over the different states of protonation to form H_2cit^- and the dissociation of the excreted products. At pH 5, this results in a calculated proton production of 0.078 H^+ per citrate molecule. The overall effect is the generation of a pH gradient across the membrane, inside alkaline. The pH

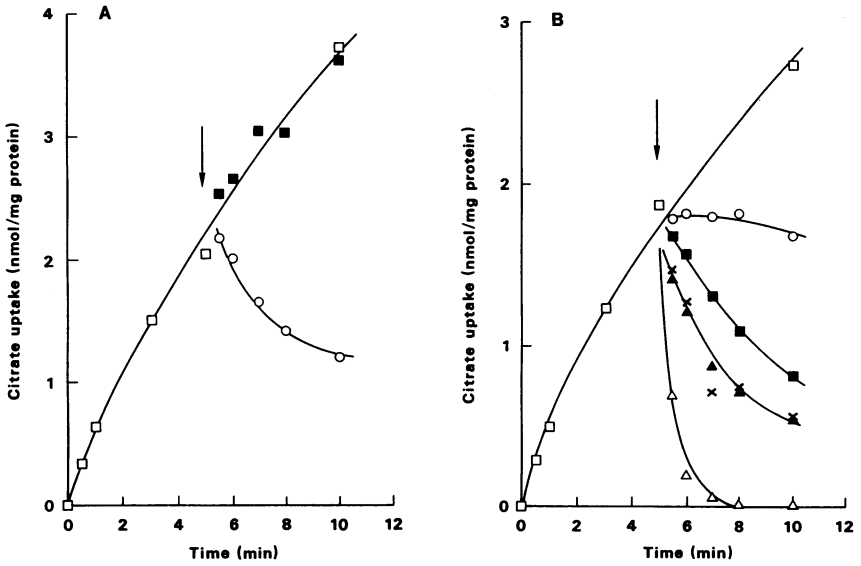

FIG. 6. Exchange and efflux activity of the citrate transport system. Hybrid membranes were allowed to accumulate [1,5-^{14}C]citrate in the presence of the electron donor system ascorbate-TMPD-cytochrome *c* and 1 μM valinomycin (□). (A) At the time indicated by the arrow, a 100-fold excess of unlabeled citrate (○), or of each of the following acids: acetate, L-malate, L-lactate, D-lactate, pyruvate, oxaloacetate, α-ketoglutarate, and succinate (■) was added. Only one symbol was plotted for all of these acids because the results were similar. (B) At the time indicated by the arrow, 0.5 μM nigericin (△) or unlabeled citrate at final concentrations of 0.1 mM (○), 0.5 mM (■), 2 mM (▲), and 10 mM (×) was added.

gradient builds up during the lag time observed before the continuous alkalinization of the external medium shown in Fig. 2. The pH gradient reaches a maximal value because of the influx of protons in response to the PMF generated (e.g., via F_0F_1 ATPase). As a result, in the steady state, the net alkalinization takes place in the external medium.

The mechanism of citrate transport in *L. oenos* described above is different from that of the H^+- and Na^+-dependent citrate transport systems of *Klebsiella pneumoniae*, which transport the divalent anionic form of citrate, $HCit^{2-}$, in symport with three cations, 3 H^+ or 1 H^+ and 2 Na^+, respectively (10, 26). The mechanism also differs from the citrate carrier (CitP) of the related organisms *Lactococcus lactis* and *Leuconostoc lactis*, for which the mechanism was proposed to be a PMF-driven proton symporter (5). At variance with this proposal is a report showing the generation of a membrane potential upon the addition of citrate to *Lactococcus lactis*, as we observed with *L. oenos* (7). The authors suggest that the mechanism of citrate transport in *Lactococcus lactis* is similar to the electrogenic malate-lactate exchange which has been described for malolactic fermentation in lactococci (20). A model in which divalent citrate is exchanged with any of the monovalent products of the metabolism of citrate, e.g., acetate or pyruvate, was postulated. In a vesicular system, the malate transporter of *Lactococcus lactis* catalyzes two reactions, uniport of negatively charged deprotonated malate and exchange of the same species with lactic acid. Under physiological conditions in whole cells, the exchange mode is favored (20). This prompted us to look for exchange activity catalyzed by the citrate transporter of *L. oenos* with any of its metabolic products. Only homologous citrate-citrate exchange could be demonstrated, and no heterologous exchange with any of the potential products, e.g., acetate, pyruvate, oxaloacetate, and many other possible candidates was found (Fig. 6). Therefore, no indication is found for a precursor-product exchange mechanism, as suggested for malolactic fermentation in *Lactococcus lactis*. Further studies

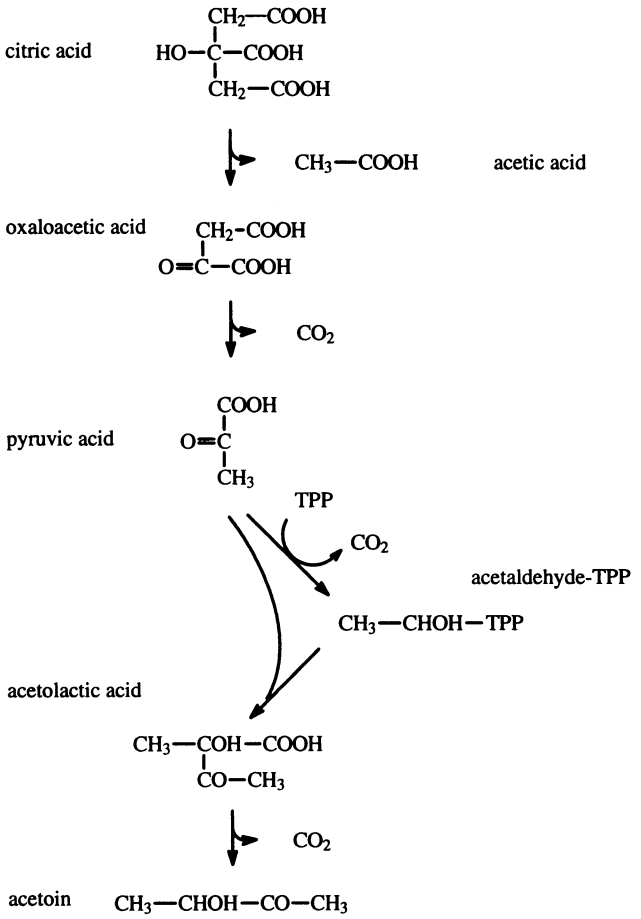

FIG. 7. Pathway for citrate breakdown by *L. oenos* in the absence of any other carbon and energy source.

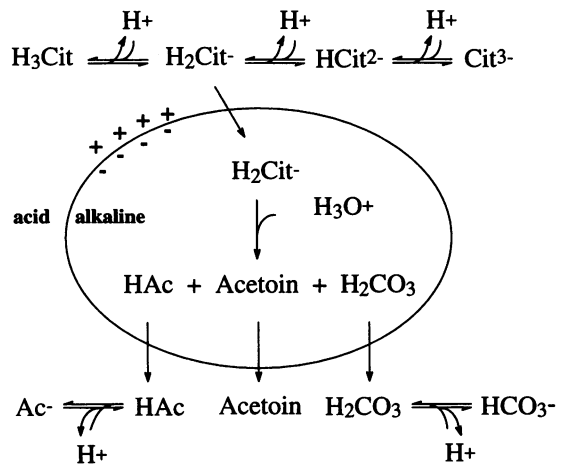

FIG. 8. Proposed model for PMF generation by citrate metabolism in *L. oenos*. H_3cit, citric acid; HAc, acetic acid.

on the lactococcal citrate transport system are required to elucidate the apparent mechanistic discrepancies.

Our model for PMF generation by citrate utilization in *L. oenos* is depicted in Fig. 8. The membrane potential is generated at the level of citrate uptake into the cell. Conservation of metabolic energy by a mechanism involving uniport of a negatively charged substrate operating under physiological conditions has not been described before. Proton-consuming reactions in the catabolism of citrate are responsible for the generation of a pH gradient across the membrane. Under physiological conditions, the driving force for citrate uptake is the inwardly directed citrate concentration gradient across the membrane that is maintained by a fast rate of citrate utilization.

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