

The Proton Motive Force Generated in *Leuconostoc oenos* by L-Malate Fermentation

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In cells of *Leuconostoc oenos*, the fermentation of L-malic acid generates both a transmembrane pH gradient, inside alkaline, and an electrical potential gradient, inside negative. In resting cells, the proton motive force ranged from -170 mV to -88 mV between pH 3.1 and 5.6 in the presence of L-malate. Membrane potentials were calculated by using a model for probe binding that accounted for the different binding constants at the different pH values at the two faces of the membrane. The $\Delta\psi$ generated by the transport of monovalent malate, H-malate⁻, controlled the rate of fermentation. The fermentation rate significantly increased under conditions of decreased $\Delta\psi$, i.e., upon addition of the ionophore valinomycin in the presence of KCl, whereas in a buffer depleted of potassium, the addition of valinomycin resulted in a hyperpolarization of the cell membrane and a reduction of the rate of fermentation. At the steady state, the chemical gradient for H-malate⁻ was of the same magnitude as $\Delta\psi$. Synthesis of ATP was observed in cells performing malolactic fermentation.

Lactic acid bacteria are strictly fermentative and, with the exception of a few streptococci (22), lack electron transfer chains. Therefore, in these bacteria, generation of a proton motive force (expressed as Δp) can be achieved only by proton translocation via the membrane-bound F_0F_1 H⁺-ATPase driven by the hydrolysis of ATP or by some other chemiosmotic processes. Michels et al. (15) proposed that Δp can be formed by carrier-mediated excretion of fermentation end products in symport with protons; indeed, this was demonstrated in cells of *Lactococcus lactis* subsp. *cremoris* (18, 29) and *Enterococcus faecalis* (26) and in membrane vesicles of *Escherichia coli* (27). In addition, two other chemiosmotic mechanisms for proton motive force generation were described in lactic acid bacteria: electrogenic precursor-product exchange (1, 6, 16, 19, 20) and electrogenic uniport (17, 20, 21, 24) in combination with the metabolic breakdown of the substrate inside the cell. Examples of the former are decarboxylation of oxalate in *Oxalobacter formigenes* (1), L-malate in *L. lactis* (20), and histidine in *Lactobacillus buchneri* (16). Examples of the anion uniport mechanism are the decarboxylation of L-malate in *Leuconostoc oenos* (24) and *Lactobacillus plantarum* (17) and citrate metabolism in *L. oenos* (21). In *L. lactis*, the transporter responsible for the exchange of malate (precursor) and lactate (product) in the malate decarboxylation pathway (malolactic fermentation) was shown to be able, at least in vitro, to catalyze electrogenic monoanionic H-malate⁻ uniport (or malate²⁻/H⁺ symport) (20). For the same process in *L. plantarum*, a variable stoichiometry for L-malate/proton symport which depends on the external L-malate concentration was described (17). In this model, the ratio of H-malate⁻ to proton transported increased with increasing external concentrations of L-malate. Only at concentrations higher than 5 mM, the transport proceeded by a low-affinity uniport which was able to generate Δp . For *L. oenos*, three different models for

L-malate uptake and Δp generation, all based on L-malate uniport, have been proposed (12, 24, 30). Loubiere et al. suggested a system similar to that of *L. plantarum*, in which the combined action of H-malate⁻/H⁺ symport and H-malate⁻ uniport depends on the external malate concentration (12). Other authors proposed simultaneous low-affinity H-malate⁻ uniport and passive diffusion of nondissociated L-malate, the relative contribution being dependent on the external pH (30). In a previous report on studies in membrane vesicles (24), we proposed a model showing H-malate⁻ uniport in the pH range 3 to 5.6 and at low concentrations of L-malate. The driving force for L-malate uptake would be the H-malate⁻ concentration gradient. A second component that became manifest only at L-malate concentrations above 1 mM was observed and could occur by passive diffusion or transport with a very low affinity (apparent K_m of >10 mM). It was not possible to distinguish between these two possibilities from the kinetic data.

The relation between the magnitude of the components of the proton motive force and the steady-state accumulation ratio of a solute provides information about the driving force for secondary transport. In *Leuconostoc* species or, more generally, in heterofermentative lactic acid bacteria, little has been reported about the dependence of the Δp or the intracellular pH on external pH. Although it was shown that the presence of a proton motive force is not essential for growth under certain conditions (5), the Δp becomes important as the external pH decreases and the concentration of essential nutrients that are transported by secondary transport systems becomes low. In lactococci, higher values of Δp were found with decreasing pH values (8).

In the present work, we investigated the magnitude and the composition of the Δp , as a function of external pH, generated in *L. oenos* fermenting L-malate. Furthermore, the transport mechanism studied in membrane vesicles (24) was confirmed to operate in cells. Moreover, the results presented suggest that both low- and high-affinity transport components found in membrane vesicles are electrogenic.

The species *L. oenos* was recently reclassified as *Oenococcus*

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TABLE 1. Internal pH, total concentrations, and chemical gradients of protonation states of malic and lactic acids during malolactic fermentation (pK_as of 3.4 and 5.1 for malic acid and pK_a of 3.8 for lactic acid)^a

pH _{out}	pH _{in}	Total concn (mM)			Chemical gradient ^b (mV)				
		L-Lactate _{out}	L-Lactate _{in}	L-Malate _{in}	H ₂ -Malate	H-Malate ⁻	Malate ²⁻	H-Lactate	Lactate ⁻
3.0	5.8	4.2	248	49	-169 (0.04, 29)	-12 (8.1, 13)	148 (41, 0.13)	-9 (2.5, 3.5)	150 (246, 0.7)
3.5	5.8	4.3	248	49	-172 (0.02, 20)	-31 (6.7, 22)	111 (42, 0.6)	-9 (2.0, 2.9)	132 (246, 1.4)
4.1	6.0	3.9	205	52	-160 (0.02, 7.9)	-46 (5.8, 35)	66 (46, 3.5)	-2 (1.2, 1.3)	112 (204, 2.6)
4.6	6.1	3.0	163	47	-159 (5 × 10 ⁻³ , 2.3)	-57 (3.5, 32)	38 (44, 10)	12 (0.7, 0.4)	106 (162, 2.6)
5.2	6.3	2.7	120	47	-113 (5 × 10 ⁻³ , 0.4)	-50 (1.8, 19)	15 (44, 24)	32 (0.4, 0.1)	98 (120, 2.6)
5.6	6.3	2.5	103	62	-75 (3 × 10 ⁻³ , 0.1)	-31 (3.7, 12)	11 (58, 39)	52 (0.3, 0.04)	96 (103, 2.5)

^a Samples were withdrawn 12 min after addition of 50 mM L-malate and analyzed as described in Materials and Methods.

^b The concentrations on both sides of the membrane (in, out) are presented in parentheses.

oeni (4). Since in this study a commercial strain was used, it was considered convenient to keep the old designation.

MATERIALS AND METHODS

Strain and growth conditions. The commercial strain *L. oenos* GM (MicroLife Technics, Sarasota, Fla.) was used. After recovery from the lyophilized form, the cells were resuspended in sterile 4 mM potassium phosphate solution (pH 4.8) with 30% (vol/vol) glycerol, kept frozen at -80°C, and used as inocula. Cultures were grown in a modified F.T.80 medium (pH 4.8) (3) containing 5 g of DL-malic acid per liter but lacking Tween 80. Glucose (2 g/liter) and fructose (8 g/liter) were autoclaved separately and added to the medium just before inoculation. Cells were grown in batch cultures at 30°C, harvested at the end of the exponential phase of growth, washed, and kept on ice until use.

Malolactic fermentation activity. Malolactic activity was determined by the manometric method of Warburg (31) for measurement CO₂ production. The fermentation was started by the addition of 50 mM L-malate (potassium salt) to cell suspensions in 50 mM K-morpholinethanesulfonic acid (MES)-50 mM glycylglycine at the selected pH. The measurements were performed at 25°C. Malolactic activity was also measured by the alkalization of extracellular medium, monitored with a pH meter connected to a recorder. The fermentation was started by the addition of the cells to 5 mM K-MES-10 mM L-malate. To calibrate the signal, 10 mM HCl was used. L-Lactate produced was enzymatically determined, and a relationship with proton consumption was established at pH 3, 4, and 5.

Measurement of intracellular pH and intracellular concentrations of malic and lactic acids. Intracellular pH was determined from the distribution of [¹⁴C]benzoic acid (23), using the silicone oil centrifugation technique (28). Cells (0.5 mg [dry weight]/ml) were incubated in K-MES-glycylglycine buffer at pH 3.1 to 5.6 for 12 min at 25°C in the presence of 50 mM L-malate and 1 μCi of [¹⁴C]benzoic acid per ml. Samples of 500 μl were centrifuged through 300 μl of silicone oil (density = 1.02 g/cm³) at 16,000 × g for 10 min at room temperature. Radioactivity in the supernatant (20 μl) and pellets was determined by liquid scintillation counting. The pellets were recovered by cutting the tubes after they were frozen in liquid nitrogen, and the internal concentration of benzoic acid was calculated by using the value for the internal water volume calculated as described below. For the internal concentrations of malic and lactic acids, the same technique was used, but the tubes contained 300 μl of silicone oil on top of 100 μl of 22% (vol/vol) perchloric acid. Supernatants and perchloric acid fractions were analyzed enzymatically for their content of L-malic and L-lactic acid with L-malate and L-lactate dehydrogenases, respectively. Corrections were performed for extracellular adhering water in the pellet fraction, calculated as indicated below.

Taking into account the intracellular and extracellular pH, the concentrations of the several chemical species of malic and lactic acids involved were calculated, assuming the same dissociation constants inside and outside the cell. Chemical potentials were calculated for the species, applying the Nernst equation. A negative value indicates that the driving force is inward (25).

Internal volume. Intracellular volume was measured as described by Rottenberg (23). For probing the extracellular volume (*V*₀), [¹⁴C]polyethylene glycol (PEG) 4000 was used, since unrealistically low values for *V*₀ were obtained with taurine, the probe commonly used to determine internal volumes in lactic acid bacteria (8). Cells were incubated with 25 μCi of [³H]H₂O, 0.25 μCi of [¹⁴C]PEG 4000, and 5 mg of nonlabeled PEG 4000 per ml to prevent adsorption of the probe to the cells. Subsequently, cells were centrifuged through silicone oil as described above. From the data, internal water volumes of 2.19 ± 0.12 and 1.82 ± 0.27 μl/mg (dry weight) for the external water, equivalent to 45% of total water in the pellet, were calculated.

Membrane potential measurements. To determine the membrane potential, the distribution of the lipophilic tetraphenylphosphonium ion (TPP⁺) across the membrane was evaluated by monitoring the external concentration with a selective electrode for TPP⁺ (7). The uptake of TPP⁺ was measured at 25°C in a 1-ml vessel containing approximately 1 mg of cells per ml and 4 μM TPP⁺. Nonspe-

cific binding of the probe was estimated after the cells were deenergized by incubation with 2% toluene for 1 h at 30°C. Treated cells were unable to build up a membrane potential in the presence of L-malate. The distribution of the binding sites among the cellular components was estimated as described elsewhere (10). Binding constants have been calculated for TPP⁺ binding to the membrane and to cytoplasmic components. To correct Δψ values for the nonspecific binding of TPP⁺, Lolkema et al. (11) derived an equation that assumes the simultaneous binding of the probe to several cellular components. In the present work, that equation was modified in the following way: (i) the term representing binding to the outer membrane of gram-negative bacteria was omitted and (ii) the term representing the binding to the cytoplasmic membrane was split into separate terms for the binding to the inner and the outer leaflets of the membrane because of a strong pH dependence of nonspecific binding (see results). In the resulting equation,

$$\Delta\psi = Z \log \left\{ \frac{\frac{C_o}{C_e} - 1 + x \left(1 - \frac{1}{2} f_{cm} K_{cm}^{out} \right)}{x \left(1 + K_i + \frac{1}{2} f_{cm} K_{cm}^{in} \right)} \right\} \quad (1)$$

*C*_o and *C*_e represent the probe concentrations in the absence and presence of the cells, respectively; *f*_{cm}*K*_{cm}^{out} and *f*_{cm}*K*_{cm}ⁱⁿ are the constants for binding to the cytoplasmic membrane at the external and internal pHs, respectively; *K*_i is the constant for binding to intracellular components; and *x* is the fractional internal volume of the cells. The numerator in the equation accounts for the binding to the external face of the membrane, whereas the term 1 + *K*_i + 1/2*f*_{cm}*K*_{cm}ⁱⁿ accounts for the binding to the internal components and to the internal face of the membrane.

ATP. L-Malate was added to cells suspended in K-MES-glycylglycine buffer and incubated at 25°C. Samples (0.5 ml) were withdrawn, immediately diluted into 0.5 ml of 14% (vol/vol) perchloric acid-9 mM EDTA, and kept on ice for 20 min. Cell debris and the precipitate were removed by centrifugation at 19,000 × g for 5 min at 0°C, and 20 μl of supernatant was neutralized by dilution into 2 ml of 100 mM phosphate buffer (pH 7.8) and analyzed for ATP content by the luciferase-luciferase method (14), using an enzymatic kit from Lumac. The luminescence reaction was carried out at room temperature in a Biocounter M2500 photometer, also from Lumac.

Protein. Protein was quantified by the method of Lowry et al. (13), using bovine serum albumin as a standard.

Materials. L-Lactate and L-malate dehydrogenases were obtained from Boehringer Mannheim. [¹⁴C]benzoic acid (22 mCi/mmol) was from NEN, and [³H]H₂O and PEG 4000 (60 mCi/mmol) were obtained from Amersham. The silicone oil, at a density of 1.02 g/cm³, was prepared by mixing 3:2 (vol/vol) Dow Corning 556 and Dow Corning 550. All other chemicals were of reagent grade and commercially available.

RESULTS

Intracellular concentration of the chemical species involved in malic acid fermentation. When malic acid was added to a buffered cell suspension, a transient situation occurred during which the internal concentrations of malic and lactic acids and pH increased. After about 12 min, a steady state was reached in the cytoplasm. Thereafter, the external concentrations of the organic acids kept changing at a slow rate (depending on the cell density and pH). The internal pH and total concentrations on each side of the membrane were measured at steady state in the pH range from 3.0 to 5.6. The internal concentrations of the chemical species involved were calculated as indicated in

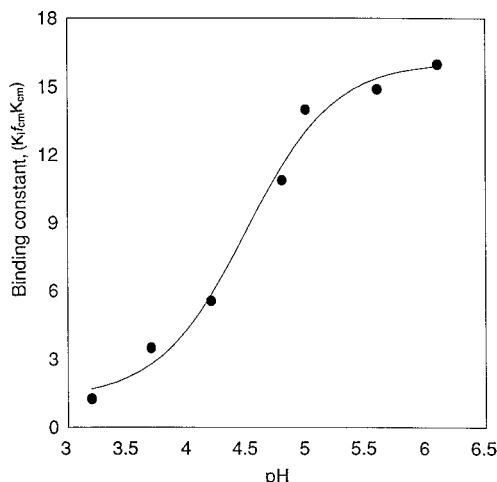


FIG. 1. Effect of pH on the binding constant of TPP⁺ to cells of *L. oenos*. Constants were determined in deenergized cells. The TPP⁺ electrode was calibrated at each pH assayed.

Materials and Methods, taking into account the values of internal pH and assuming that the pK_as of the acids were the same on both sides of the membrane. Table 1 summarizes these data and the gradients for the species that follow from the concentrations. Malate fermentation brought about a significant alkalization of the cytoplasm: e.g., at an external pH of 3.0, the internal pH changed from 5.0 to 5.8 after addition of malate. Over the pH range analyzed, the intracellular pH was relatively constant, approximately 6. Consequently, the pH gradient (ΔpH) varied with external pH and decreased from -159 mV at pH 3.1 to -41 mV at pH 5.6. The total internal concentration of malate was about the same as in the external buffer at all pH values assayed (50 mM). This corresponds to an inwardly directed H-malate⁻ chemical gradient with a maximum at pH 4 to 5. At these pH values, the external concentration of H-malate⁻ is maximal.

Calculation of $\Delta\psi$ from TPP⁺ distribution in *L. oenos*. Calculations of $\Delta\psi$ that do not account for the binding of probes like TPP⁺ to cell components result in overestimates of $\Delta\psi$ values (8, 10, 11, 32). Measurement of the binding of TPP⁺ to deenergized cells of *L. oenos* ($\Delta\psi = 0$) was proportional to the free concentration of TPP⁺ up to 80 μM (data not shown), as was shown previously for *Bacillus subtilis* (32) and *Rhodospseudomonas sphaeroides* (10). The binding constants estimated from this slope showed a strong dependence on pH (Fig. 1), decreasing sigmoidally from pH 6.2 to pH 3.2, at which binding of TPP⁺ to the cells was almost negligible. Since pH_{in} and pH_{out} are different in energized cells, appropriately different corrections must be made for the nonspecific TPP⁺ binding to the two sides of the membrane in order to obtain a more accurate $\Delta\psi$ value. We calculated that the membrane was responsible for 63% of the total binding, and 37% was attributed to the intracellular fraction. The possible binding to the cell wall, in particular to teichoic acids, was not taken into consideration in this model. The equation used in our calculations (see equation 1) was

$$\Delta\psi = Z \log \left\{ \frac{\frac{C_o}{C_e} - 1 + x \left(1 - \frac{1}{2} f_{\text{cm}} K_{\text{cm}}^{\text{out}} \right)}{10.8x} \right\} \quad (2)$$

in which the magnitude of $f_{\text{cm}} K_{\text{cm}}^{\text{out}}$ depends on the external pH as depicted in Fig. 1. The importance of the different correc-

tions for the calculated $\Delta\psi$ values is shown in Fig. 2. Especially at pH below 5, the pH-dependent correction for binding described above was important and resulted in much lower $\Delta\psi$ values.

Cells incubated at pH 3.1 in the presence of 50 mM malate had a very low $\Delta\psi$ of approximately -11 mV. The membrane potential increased up to -47 mV at pH 5.6. The membrane potential was low over the pH range tested, and in the physiological pH range (pH < 4), the proton motive force consisted mainly of a pH gradient.

Figure 3 summarizes the results calculated for the proton motive force based on the values of $\Delta\psi$ and ΔpH .

Driving forces involved in malolactic fermentation. The driving force and the contribution of ΔpH and $\Delta\psi$ depend on the mechanism of transport. For a weak acid, the ΔpH contributes indirectly to the concentration gradient of each species as they dissociate inside the cell, where the pH is more alkaline, and contributes directly when there is symport of a solute with protons. For each of the possibilities of malic and lactic acid translocation in malolactic fermentation, the driving force involved when the flux reaches a steady state was calculated on the basis of internal and external concentrations (data from Table 1) and ΔpH and $\Delta\psi$ (Fig. 3). For considering ΔpH and $\Delta\psi$ as positive and negative, the recommendations of Silverstein et al. (25) were followed. For lactate, either the efflux of lactate-H or the symport of lactate⁻/H⁺ is conceivable, and the two possibilities cannot be distinguished (Fig. 4): for pH below 4.5, the driving force for lactic acid diffusion composed of its own concentration gradient, $\Delta\mu_{\text{Hlac}}/F$, as well as the driving force for the symport, $\Delta\mu_{\text{lac}}/F - Z\Delta\text{pH}$, were zero. Both lactic acid and lactate⁻ distributions were in equilibrium with the pH gradient, although for both mechanisms an apparent outwardly directed driving force was present at higher pH (a negative value for the driving force means that it is oriented inward). In the case of malate, three malate translocation mechanisms were considered: diffusion of H₂-malate, uniport of H-malate⁻, and uniport of malate²⁻. The calculated driving force for H₂-malate diffusion ($\Delta\mu_{\text{H}_2\text{mal}}/F$) was negative at all pH values (Fig. 4), pulling this species inward. For H-malate⁻

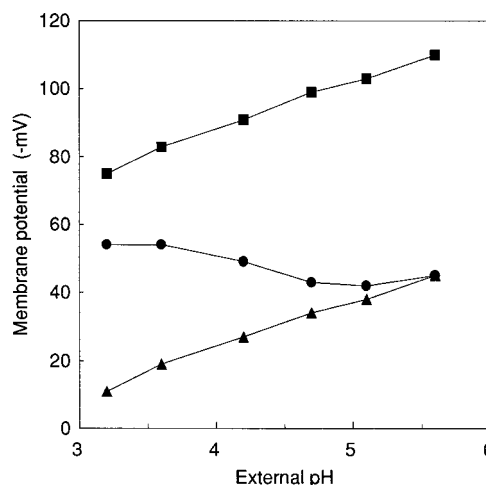


FIG. 2. Comparison of the different corrections in the evaluation of $\Delta\psi$ values. Shown are values calculated by applying the Nernst equation to the distribution ratio of TPP⁺ inside and outside the cells (■), values corrected for the concentration-dependent binding of TPP⁺ by using equation 2 but not taking into account the different intra- and extracellular pH (●), and values corrected with equation 2 with different constants for the intra- and extracellular binding (▲).

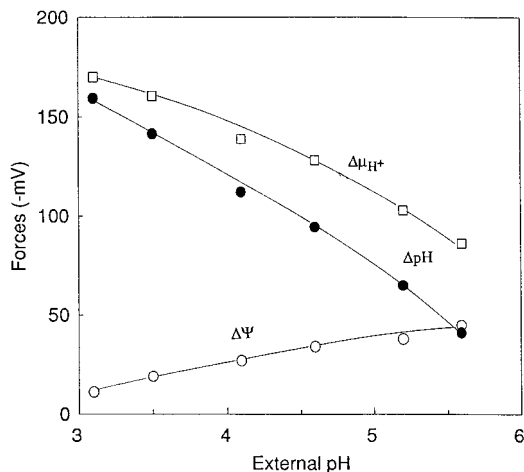


FIG. 3. Composition and magnitude of the proton motive force in *L. oenos* as a function of pH. Cells were suspended in 50 mM K-MES-50 mM glycylglycine at each pH. Experiments were performed at 25°C. \square , $\Delta\mu_{H^+}$; \bullet , ΔpH ; \circ , $\Delta\Psi$. Proton motive force was calculated by adding $\Delta\psi$ and $Z\Delta pH$ (\square).

uniport (as well as for the symport of $\text{malate}^{2-}/\text{H}^+$), the total driving force was zero, meaning that $\Delta\mu_{\text{Hmal}^-}$ was approximately at equilibrium with $\Delta\psi$. The driving force calculated for the hypothesis of the uniport of malate^{2-} was largely positive (Fig. 4), which makes this mechanism improbable.

Inhibition of malolactic fermentation by a membrane potential. The effect of membrane potential on malolactic activity can be observed by manipulating $\Delta\psi$ with valinomycin. This ionophore allows potassium ions to cross the membrane freely. Addition of 2 μM valinomycin to cells performing malolactic fermentation in the presence of 100 mM K^+ at pH 3.5 stimulated malolactic activity (Fig. 5A). Under these conditions, the membrane potential was dissipated (Fig. 5B, curve A). As $\Delta\psi$ at this pH was low, its dissipation caused a small stimulation of the activity. In contrast, if the cells were hyperpolarized by adding valinomycin to a cell suspension depleted of external K^+ (Fig. 5B, curve B), malolactic activity was strongly inhibited (Fig. 5A). In the absence of valinomycin, malolactic activity was significantly lower in the absence of K^+ when $\Delta\psi$ was higher. In a parallel experiment in which malolactic activity was monitored by measuring the external pH, the addition of 2 μM valinomycin to the cells accelerated the rate of external alkalization during malate fermentation 40% at pH 3 and 120% at pH 5, at which the $\Delta\psi$ is higher (data not shown). These results strongly suggest that the $\Delta\psi$ generated controls the rate of malolactic fermentation.

ATP synthesis as a consequence of the fermentation of malate. The highest rates of ATP synthesis were seen at pH 3.5 when Δp was also highest, and both declined with increasing pH (Fig. 6). This observation suggests a correlation between the proton motive force and ATP synthesis. Confirmation was obtained from experiments with valinomycin and nigericin, in which dissipation of Δp completely inhibited ATP synthesis. Moreover, cells which were pretreated with *N,N'*-dicyclohexylcarbodiimide (DCCD) to inhibit the F_1F_0 ATPase could not generate ATP but were still able to generate proton motive force, showing that the ATPase under normal conditions functions in the synthesis mode.

DISCUSSION

Malolactic fermentation generates a proton motive force in *L. oenos*. Δp values of the order of -170 to -88 mV were

measured in nongrowing cells metabolizing malate, which is in agreement with a Δp of -110 mV reported for this organism during growth on malate-glucose medium at pH 5 (12). Other malate-fermenting bacteria, such as *L. lactis*, developed higher Δp (20) and maintained an internal pH that, at pH 5, was about 1.4 pH units higher than those measured for *L. oenos*. For pH values between 3 and 5, the magnitude and composition of Δp were comparable to those found in acidophilic bacteria (8). The mechanism by which the electrochemical proton gradient is generated during malolactic fermentation in *L. oenos* was proposed previously on the basis of transport studies in membrane vesicles (24). H-malate⁻ is taken up by an electrogenic uniport mechanism. A net negative charge is moved inward, and thus an electrical gradient (of normal polarity) is built up across the cell membrane. In the same work, a second component of malate transport became manifest at high malate concentrations (>1 mM), but it could not be characterized from the kinetic data. The mechanism could be either simple passive diffusion of protonated malate or carrier-mediated malate transport of low affinity. The results in this work provide evidence in favor of both components being electrogenic. A reduction in $\Delta\psi$ (facilitating the uptake of H-malate⁻) stimulated fermentation, whereas hyperpolarization of the membrane was inhibitory (Fig. 5). Passive diffusion neither generates nor is affected by a membrane potential. Furthermore, if there was significant passive diffusion, the fermentation would not be limited by the transport as shown, since the H_2 -malate concentration was not equilibrated at both sides of the membrane and the calculated driving force for its diffusion was highly negative (Fig. 4). In these conditions, and if H_2 -malate were permeative, diffusion would have proceeded.

Although a mechanism cannot be established just by analyzing the driving forces, these forces do have an indicative value. The calculated driving forces for H-malate⁻ uniport and for $\text{malate}^{2-}/\text{H}^+$ symport under steady-state conditions were approximately zero, indicating that one of these mechanisms may operate in malolactic fermentation. The possibility of malate^{2-} , instead of H-malate⁻, being the species transported by the uniporter, already rejected on the basis of the kinetic results (24), could also be ruled out by the large positive (oriented

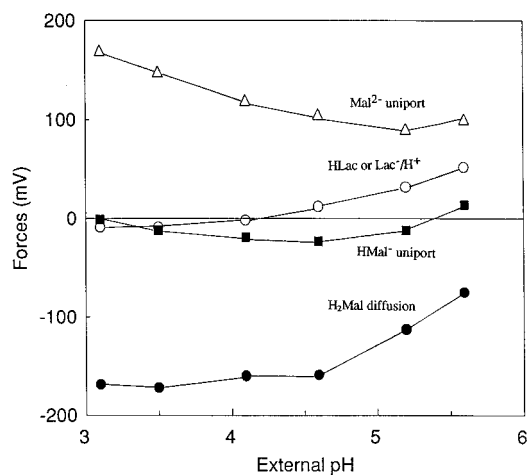


FIG. 4. Calculated driving forces for the different transport mechanisms putatively involved in malolactic fermentation. The driving forces were calculated from data in Table 1 and Fig. 3 and are composed of the following: just the chemical gradient for the cases of H_2 -malate and H-lactate diffusion, $\Delta\mu_{(\text{Hmal}^-)}/F - \Delta\psi$ for the uniport of H-malate⁻, $\Delta\mu_{(\text{mal}^{2-})}/F - 2\Delta\psi$ for the uniport of malate^{2-} , and $\Delta\mu_{(\text{lac}^-)}/F - Z\Delta pH$ for the symport of lactate⁻/H⁺. By definition, a positive value for the driving force means that it is directed outward.

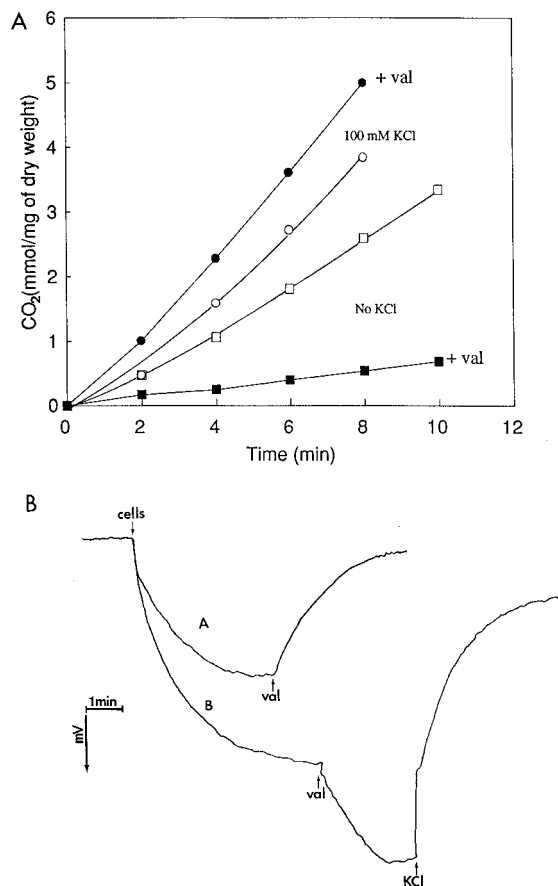


FIG. 5. (A) Effect of the membrane potential on malolactic activity in *L. oenos*. Shown are CO₂ production after the addition of 50 mM L-malate to cells incubated at pH 3.5 with 100 mM KCl (●, ○) or 100 mM NaCl (■, □) and with (●, ■) or without (○, □) 2 μM valinomycin (val). (B) Effect of valinomycin on Δψ generated by malolactic fermentation. Plotted lines represent the external concentration of TPP⁺, obtained when cells were added to buffer containing 50 mM L-malate at pH 3.5 in the presence (curve A) or absence (curve B) of KCl. Arrows indicate the addition of valinomycin (val) or 25 mM KCl.

outward) value for the driving force. Once inside the cell, malate is immediately decarboxylated to lactate plus carbon dioxide, and both leave the cell in electroneutral processes. At the pH values found in the cytoplasm, little CO₂ combines with water and probably CO₂ diffuses passively through the membrane as a dissolved gas. It is not possible to determine, on the basis only of driving forces, whether lactate extrusion is carrier mediated or which mechanism is involved. Surprisingly, for pH above 4.5, the driving force for lactate was not zero at steady state but it was directed outward, suggesting a kinetic barrier for lactate efflux.

The buildup of the membrane potential (negative inside) precluded L-malate uptake (Fig. 5), supporting the concept that a negative charge is moved into the cell. These observations and the fact that malate (total) did not accumulate suggest that the membrane transport is a bottleneck in malolactic activity. Malolactic activity decreased when the ATPase was inhibited with DCCD (results not shown), since the ATPase allows the entrance of protons into the cell and a consequent decrease in Δψ and ΔpH occurs. Because the electrogenic process is slowed by the generation of Δψ, malolactic fermentation is favored at the pH at which Δψ is lower and the external H-malate⁻ concentration is maximal. Indeed, the

highest rates were determined at pH 3.5, at which both requirements are satisfied. Malolactic fermentation is important as part of the regulatory mechanism of pH homeostasis in *L. oenos*, especially at very acidic pH values. At these pH values, cellular functions are inhibited and ATP synthesis by substrate-level phosphorylation would not proceed as efficiently. Malolactic fermentation is used to generate the proton motive force necessary for the uptake of different nutrients, to synthesize ATP, and to keep suitable internal conditions for enzymatic activity and growth under conditions of acidic pH as in wine.

In this study, we tried to detect and assess the phenomenon of alkalization that occurs during malolactic fermentation. Our approach was based on the assumption that the alkalization (or consumption of free protons) can be analyzed just taking into account equations of acid-base equilibria. In fact, proton consumption in the conversion of an acid (malic acid) with two pK_as into another acid with only one pK_a (lactic acid) at a certain pH can be calculated. This equation can be used to predict how many protons are consumed per malate converted into lactate. Experimentally, H⁺ consumption was monitored with a pH electrode during malolactic fermentation. The calculated ratio H⁺ consumed/lactate produced in a medium at pH 3 was 0.15; it increased sharply until pH 3.5, reaching a value of 0.25, and then remained almost constant to pH 4.7. From this pH to 6, the ratio increased again abruptly until it reached 1. These calculations confirmed what was measured within this pH range when a pH electrode was used to monitor the external alkalization during malate fermentation: alkalization was more effective at a higher pH. Analysis of the internal pH is not so straightforward. In fact, not only the decarboxylation reaction together with different dissociation constants of malic and lactic acid but also the different accumulation levels of the organic acids and the proton movements via ATPase or by diffusion contribute to alkalization. Macroscopically, the intracellular decarboxylation results in a global increase of the pH of the culture, which follows from the new acid-base equilibrium generated upon the consumption of malic acid and production of lactic acid and CO₂.

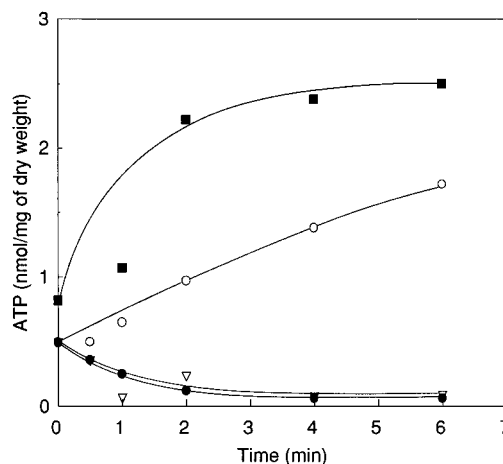


FIG. 6. Synthesis of ATP during malolactic fermentation. L-malate (50 mM) was added to cells incubated at pH 3.5 (■) or pH 5 (○), 2 μM valinomycin and 2 μM nigericin were added at time zero at pH 5 (●), and cells were pretreated with 0.1 mM DCCD at pH 5 (▽).

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