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In Vitro Reassembly of the Malolactic Fermentation Pathway of *Leuconostoc oenos* (*Oenococcus oeni*)

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The mechanism of metabolic energy generation by malolactic fermentation was studied with artificial membrane vesicles of *Leuconostoc oenos* (*Oenococcus oeni*). (Note that although *L. oenos* was recently reclassified as *O. oeni* [L. M. T. Dicks, F. Dellaglio, and M. D. Collins, Int. J. Syst. Bacteriol. 45:395–397, 1995], the old designation was kept in the present work.) Purified malolactic enzyme was entrapped in artificial membrane vesicles prepared from *L. oenos* cells able to transport L-malate. We show that the in vitro reconstituted system, including an electrogenic L-malate carrier and the decarboxylating malolactic enzyme, generated a proton motive force that was able to drive intravesicular accumulation of leucine.

Malolactic fermentation is a proton motive force-generating process that occurs in some lactic acid bacteria (3, 4, 6, 7). The pathway includes the uptake of L-malate, the conversion of L-malate to L-lactate plus carbon dioxide, and the excretion of the end products. The decarboxylation of L-malate is catalyzed by a single enzyme, called malolactic enzyme (MLE), which is NAD^+ and Mn^{2+} dependent. The mechanism by which the electrochemical proton gradient (Δp) is generated during malolactic fermentation in *Leuconostoc oenos* (*Oenococcus oeni*) has been inferred from transport studies with membrane vesicles (8). (Note that *L. oenos* was recently reclassified as *O. oeni* [1]; however, the old designation was kept in the present work.) Monoprotonated L-malate (L-malate$^-$) is taken up by an electrogenic uniport in which a net negative charge is moved inwards, thereby generating an electrical potential, $\Delta\psi$ (inside negative relative to outside). Once inside the cell, L-malate is decarboxylated to L-lactic acid plus carbon dioxide in a reaction that requires one proton (2, 4, 6, 7). This alkalinization of the cytoplasm results in the creation of a pH gradient (ΔpH) that, together with the $\Delta\psi$, forms the proton motive force across the cytoplasmic membrane. It seems most likely that L-lactic acid and CO_2 leave the cell as neutral species. If this scheme is correct, it should be possible to reconstitute the malolactic fermentation pathway by trapping MLE in artificial membrane vesicles bearing the L-malate uniporter. The addition of L-malate should lead to the generation of a Δp across the membrane and the accumulation of amino acids through Δp-driven transport systems.

Bacterial cultures were grown as described earlier (8). At the end-log phase of growth, cells were harvested by centrifugation at 3,500 × *g* (10 min, 4°C) and suspended in 50 mM potassium phosphate (pH 6.0). Membrane vesicles were prepared from *L. oenos* Lo 84.13 MLE(−) (mutant lacking MLE) as described previously (8). MLE was purified from *L. oenos* GM, a commercial strain from Microlife Technics. For the enzyme purification, cells were disrupted by passage of the suspension through a French press at 20,000 lb/in^2. The cell extract was separated from the bacterial debris by centrifugation at 70,400 × *g* (30 min, 10°C), and the supernatant containing the cytoplasmic proteins was made 10 U/ml with DNAse I. The proteins were fractionated by ammonium sulfate precipitation at concentrations of 35% (wt/vol) and 80% (wt/vol). The pellet of the second precipitation step was dialyzed against 100 mM potassium phosphate (pH 6) and concentrated by ultrafiltration through a Centricon-30 filter (Amicon). This extract was layered on a DEAE-Sepharose CL-6B column equilibrated with the same buffer and eluted with a linear gradient of phosphate buffer (100 to 400 mM, 120 ml/h). The fractions with malolactic activity were pooled (peak fractions around 180 mM potassium phosphate), dialyzed against 10 mM potassium phosphate (pH 6), adjusted to 1 M with $(NH_4)_2SO_4$, and applied to a Phenyl-Sepharose CL-4B column equilibrated with 10 mM potassium phosphate (pH 6) plus 1 M ammonium sulfate. The elution was carried out with a linear gradient, decreasing from 1 to 0 M ammonium sulfate at a rate of 40 ml/h. The fractions with malolactic activity were pooled (peak at 0.44 M ammonium sulfate), concentrated, and stored at −20°C. All steps were performed at 4°C. At this point, the enzyme was purified 15-fold and had a specific activity of 32.1 μmol of CO_2/min/mg of protein (Table 1). Malolactic activity was determined by manometric measurement with a Warburg apparatus (9) of the CO_2 produced. The initial velocity determination was made with 50 mM potassium phosphate (pH 6) in the presence of 50 mM L-malate, 50 μM NAD^+, and 80 μM $MnSO_4$. The production of CO_2 was linear with time throughout the experiment. The MLE preparation did not contain any L-malate or L-lactate dehydrogenase activity. Denaturing gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel

TABLE 1. Purification of MLE from *L. oenos* GM

Purification step	Amt of total protein (mg)	Sp act (μmol/min/mg of protein)	Purification factor
Cell extract	1,040	2.1	1
Ammonium sulfate (35–80%)	715	3.2	1.5
DEAE-Sepharose	48.3	9.0	4.2
Phenyl-Sepharose	12.1	32.1	15.3

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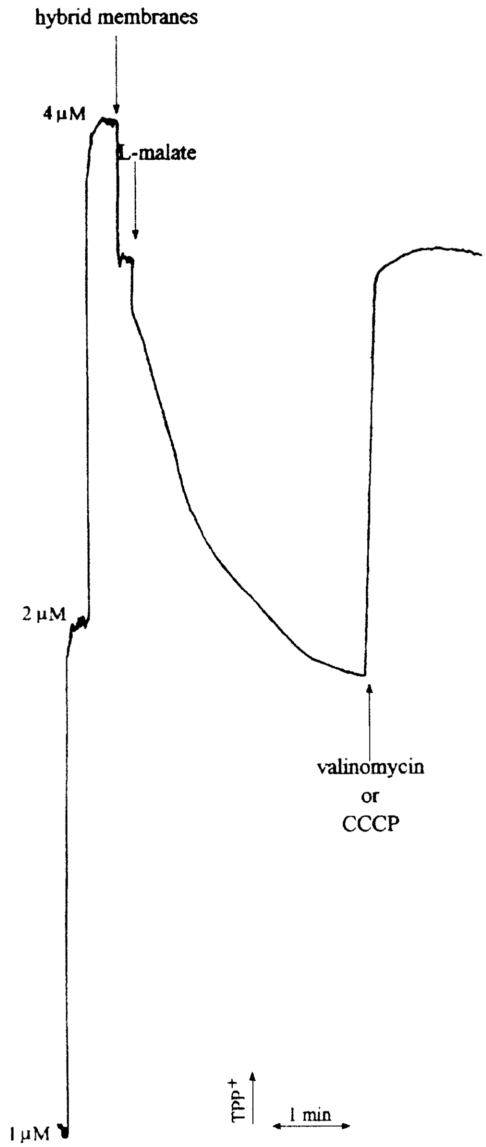

FIG. 1. Membrane potential generation by malolactic fermentation in MLE-containing hybrid membrane vesicles from *L. oenos*. The trace represents the TPP^+ electrode response. Vesicles were suspended to 0.12 mg of protein per ml in 50 mM potassium phosphate (pH 5) plus 4 μM TPP^+ as indicated. The arrows indicate the addition of 20 mM L-malate or 5 μM CCCP.

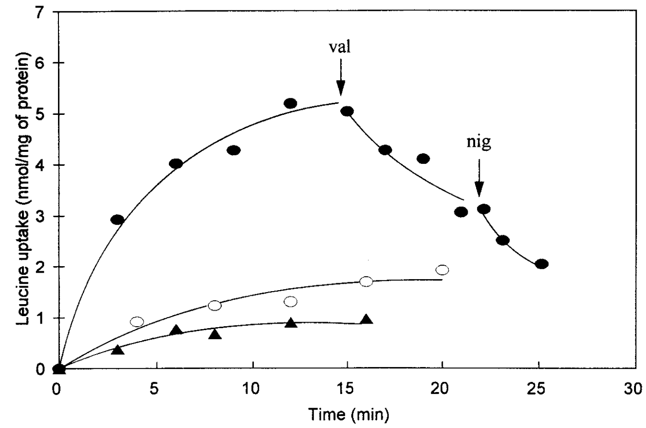

FIG. 2. Uptake of 22 μM L-[^{14}C]leucine by hybrid membrane vesicles of *L. oenos* containing MLE. The uptake was stopped at different time intervals by the addition of 2 ml of ice-cold 100 mM LiCl, and the membranes were collected on 0.45-μm-pore-size cellulose nitrate filters. Filters were washed with 2 ml of LiCl and placed into 2 ml of scintillation liquid, and the radioactivity was counted. Transport in the presence (●) and absence (○) of 20 mM L-malate is shown. The arrows indicate the addition of 1 μM valinomycin (val) or 0.5 μM nigericin (nig). Transport in hybrid membranes devoid of malolactic activity in the presence of L-malate (▲) is also shown. The uptake was assayed at pH 5 and 25°C. The final protein concentration was 0.14 mg/ml.

electrophoresis) of the purified enzyme preparation revealed a polypeptide with an apparent molecular mass of 60 kDa.

To entrap MLE in the hybrid vesicles, *L. oenos* MLE(−) membrane vesicles and liposomes prepared from acetone-ether-washed *Escherichia coli* lipids were mixed at a protein/lipid ratio of 1:10 (wt/wt) in the presence of 50 μM NAD^+–80 μM $MnSO_4$ plus MLE (1 mg/ml, final concentration), unless specified otherwise in the figure legends. The membranes were fused by freezing in liquid nitrogen and slow thawing at room temperature. Subsequently, the thawed suspension was extruded 11 times through 200-nm-pore-size polycarbonate filters (Avestin). The external MLE was removed by passage of the suspension over a Sepharose CL-6B column (135 by 5 mm) and was eluted at normal pressure with 50 mM potassium phosphate (pH 6.0). The fractions with the hybrid vesicles, identified by turbidity, were concentrated by centrifugation (250,000 × *g*, 15 min, 4°C) and suspended in 50 mM potassium phosphate (pH 5.0). Both the extrusion and the gel filtration were performed at 4°C.

To study the metabolic energy conservation by the malolactic fermentation pathway, L-malate (potassium salt) was added to the membranes, and the membrane potential was assessed from the external concentration of the lipophilic tetraphenylphosphonium ion (TPP^+), with a specific electrode as described previously (8). The $\Delta\psi$ values were not calculated because the internal volume of the enzyme-containing vesicles was not measured. Immediately upon the addition of L-malate to the hybrid membranes containing MLE, TPP^+ was taken up, indicating that a membrane potential (inside negative) was built up (Fig. 1). Addition of the potassium ionophore valinomycin or the protonophore CCCP dissipated the potential. Uptake of TPP^+ was not observed when vesicles without MLE were used in a similar experiment (results not shown).

In *L. oenos*, leucine is transported by a leucine/H^+ symporter (8). Since a $\Delta\psi$ is formed upon addition of L-malate to the hybrid membranes containing MLE, it should be possible to accumulate leucine in response to the in vitro malolactic fermentation. L-Malate was added to a final concentration of 20 mM, and after 4 min of incubation, the leucine transport reaction was started by the addition of L-[^{14}C]leucine. Indeed, leucine was taken up in response to L-malate fermentation, and the amino acid was accumulated against the concentration gradient (Fig. 2). Accumulation of leucine was abolished upon dissipation of the membrane potential and the pH gradient by the ionophores valinomycin and nigericin.

Figure 3 shows the uptake of leucine in response to malolactic fermentation at pH 4, 5, and 6. The optimum pH of MLE is around 5.5 (data not shown). Our data show that leucine uptake in response to malolactic fermentation in the hybrid membranes is highest at pH 5, which may not only reflect the optimal activity of MLE but may also be determined in part, by the activity of the leucine carrier.

In conclusion, in this study we demonstrate, for the first time, that it is possible to generate a Δp in vitro by the action of an electrogenic uniporter in combination with scalar proton

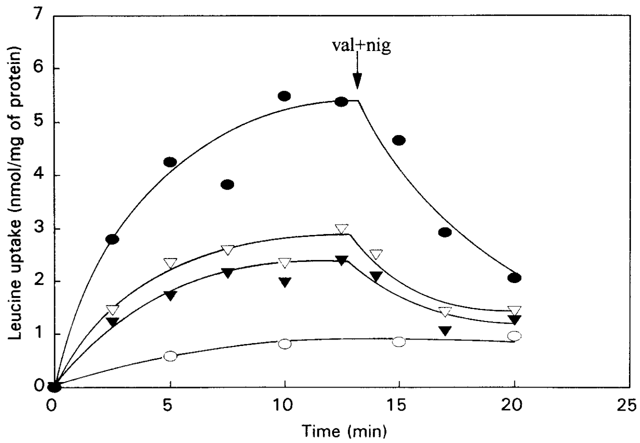

FIG. 3. Effect of pH on leucine uptake in response to malolactic fermentation in artificial membranes. Uptake of 17.5 μM L-[^{14}C]leucine by hybrid membrane vesicles of *L. oenos* fused in the presence of 0.6 mg of MLE per ml was determined. Uptake in the presence of 20 mM L-malate at pH 4 (▼), 5 (●), and 6 (▽) and uptake without L-malate (○) are shown. The L-malate used was at the same pH as the buffer. The arrow indicates the addition of 1 μM valinomycin plus 0.5 μM nigericin (val + nig). The uptake was assayed at 25°C. The final protein concentrations varied between 0.11 and 0.16 mg/ml.

consumption by an L-malate decarboxylation reaction in the intravesicular compartment. These results are entirely consistent with previous in vivo studies and establish the minimal requirements for metabolic energy generation in this type of metabolic pathway (see references 2 and 5 for other examples of decarboxylation-driven metabolic energy generation).

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