Chemiosmotic Energy from Malolactic Fermentation

DONALD J. COX* AND THOMAS HENICK-KLING

Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York 14456

Received 6 March 1989/Accepted 13 July 1989

By using the luciferase-luciferin ATP assay and whole cells of Leuconostoc oenos, we have demonstrated that malolactic fermentation does yield ATP. This energy-yielding mechanism did not occur in a cell extract and was inhibited in the presence of dicyclohexylcarbodiimide or an ionophore such as monensin. A lactate:proton efflux mechanism for this proposed pathway is presented.

During the growth of lactic acid bacteria in wine, l-malic acid is converted to L-lactic acid and CO₂ (15). This reaction is commonly referred to as malolactic fermentation (MLF). Species of Leuconostoc, Lactobacillus, and Pediococcus can carry out MLF, but Leuconostoc oenos is recognized as the bacterium that is most tolerant to the low pH and the alcohol content of wine (2; T. Henick-Kling, Ph.D. thesis, University of Adelaide, Adelaide, Australia, 1986). A reduction in the acidity of wine, caused by the growth of L. oenos, is brought about by a decarboxylating enzyme, the malolactic enzyme (EC 1.1.1.38). In the decarboxylation reaction, no pyruvate intermediate is formed and the reaction is probably catalyzed by a single enzyme which requires NAD and Mn²⁺ for catalytic activity (13). The lack of a pyruvate intermediate prompted researchers to report that MLF serves a non-energy-yielding function (11). However, malic acid did give a slight increase in the cell yield and accelerated the rate of growth; therefore, the researchers who decarboxylation reaction simulated the utilization of carbon sources (11). Henick-Kling (thesis) showed that the catabolism of malate by L. oenos and Lactobacillus plantarum can increase the intracellular pH and the membrane potential. The pathway may conserve some of the energy of the reaction in the form of ATP or an ion gradient. In this study we have investigated the generation of ATP by L. oenos upon the addition of L-malate.

Leuconostoc oenos Lc5b1 was obtained from the Australian Wine Research Institute, Urrbrae, South Australia. L. oenos Lc5b1 was grown in a modified MRS medium containing (per liter) 10 g of glucose, 10 g of fructose, 4 g of d-l-malic acid, 10 g of bacteriological peptone, 8 g of casein hydrolysate, 4 g of yeast extract, 1 ml of Tween 80, 5 g of KH₂PO₄, 0.25 g of MgSO₄, and 0.20 g of MnSO₄·H₂O. The pH of the growth medium was adjusted to 5.5 or 5.5, and the medium was sterilized at 120 °C for 15 min. The cultures were inoculated at 2.0% (vol/vol) with precultures. Cells were harvested at the late exponential phase of growth by centrifugation at 8,000 × g for 15 min and washed in a tartrate-phosphate buffer, pH 3.5 or 5.5 (to match the initial pH of the growth medium), which contained 40 mM tartaric acid, 80 mM KH₂PO₄, 1 mM MgSO₄, and 0.5 mM MnSO₄.

The amount of ATP in cells and cell extracts was measured by using a luciferase assay (8), which was modified slightly. Cells (0.5 ml at 1 to 2 mg of protein per ml) were mixed with 500 µl of 1.2 N HClO₄, allowed to lyse for 2.0 min, and then centrifuged in a Beckman microcentrifuge at 13,000 × g for 2 min. Then, 20 µl of extracted sample was mixed with 2.0 ml of 0.1 M potassium phosphate, pH 7.8, and 50 µl of luciferin-luciferase assay mix at 40 mg/ml (Sigma Chemical Co., St. Louis, Mo.). Luminescence was assayed with a Tracer Delta 300 scintillation counter (Tracor Analytical, Elk Grove, III.). ATP concentrations during malate catabolism were assayed as above except that the cells were incubated in tartrate-phosphate buffer plus 20 mM dL-malate at pH 3.5 or 5.5 for 20 min prior to extraction. Cell extracts were prepared by passing a 20-ml washed-cell suspension, at 1 to 2 mg of protein per ml of cells in tartrate-phosphate buffer, twice through an Aminco model FA-073 French pressure cell at 8.274 MPa (SLM Instruments, Urbana, III.). Whole cells were treated with N,N'-dicyclohexylcarbodi- imide (DCCD; Sigma Chemical Co.) at 10⁻⁴ M and monensin (Sigma Chemical Co.) at 5 × 10⁻⁶ M. Protein concentration determinations were done by the method of Bradford (1). Incubation of cells in tartrate-phosphate buffer of pH 3.5 or 5.5 caused a decrease in ATP content (Fig. 1A and B). ATP concentrations decreased to half the original concentration with a half-time of approximately 20 min. The ATP concentration reached a level of less than 1.0 nmol of ATP per mg of protein within 120 min.

In order to test whether malate is used as an energy

FIG. 1. Effect of malate on decay of the ATP pool in cells of L. oenos in tartrate-phosphate buffer. Cells of L. oenos were incubated in tartrate-phosphate buffer of pH 3.5 (A) and 5.5 (B). Circles represent ATP content during starvation decay; triangles represent malate-dependent ATP production during starvation.

* Corresponding author.
source, 20 mM DL-malate at pH 3.5 and 5.5 was added to cells starved for various times in buffer of pH 3.5 and 5.5, respectively. Figure 1A compares the ATP decay in cells suspended in tartrate-phosphate buffer of pH 3.5 with the ATP content of starved cells which catalyzed 20 mM DL-malate in tartrate-phosphate buffer of the same pH for 2.0 min prior to the ATP assay. The increase in ATP concentration during malate catabolism was generally an order of magnitude greater than starvation levels. The initial concentration of ATP before the addition of malate was less than 1.0 nmol of ATP per mg of protein; after the addition of malate, a maximum response of 30.77 nmol of ATP per mg of protein was measured. The quantity of the ATP response varied depending on the time elapsed between harvesting and measuring the ATP content (starvation time). The largest response was generated for freshly harvested cells which were assayed after harvest, wash, and resuspension procedures at a constant time of 15 min from start of harvest to time of the ATP assay. After prolonged starvation, progressively smaller ATP concentrations were measured upon the addition of DL-malate. This suggests that the amount of ATP produced during l-malate catabolism is directly related to metabolic activity. Cells of L. oenos grown in medium of pH 5.5 showed a similar type of response (Fig. 1B).

Cell extract containing active malolactic enzyme in buffer of pH 6.0 produced no malate-dependent ATP response; however, whole cells of L. oenos suspended in tartrate-phosphate buffer with malate showed a response of 10.0 and 7.94 nmol of ATP per mg of protein, respectively, at pH 3.5 and 5.5 (Table 1). Monensin, an ionophore, inhibited ATP synthesis in whole cells during malate catabolism at either pH 3.5 or pH 5.5 (Table 1). The use of an ATPase inhibitor, DCCD, gave similar results (Table 1).

There have been several attempts to study the energetics of the malolactic decarboxylation reaction (4, 10, 11, 13). These studies used cell extracts and purified enzyme. The results of these experiments suggest that there is no substrate-level phosphorylation associated with MLF. This was confirmed during this investigation by our measurements with cell extract. The measured increase in ATP concentration in cells of L. oenos catalyzing malate shows that whole, intact cells generate the ATP response observed in this study. Apparently, an electrochemical or proton gradient generated across the cell membrane is necessary for ATP production. The inhibition of ATP synthesis by monensin, which dissipates a proton gradient, indirectly shows that this gradient is necessary for the production of ATP in cells of L. oenos catalyzing malate. Since the intact cell is a necessary component of this energy generation scheme, we propose that a chemiosmotic theory for the production of ATP from malate catabolism can be advanced. The chemiosmotic theory as proposed by Mitchell (6, 7) does not provide for end product efflux as a means of creating a proton gradient. Michels et al. (5) proposed that the generation of lactate from glucose catabolism is capable of developing a proton gradient as lactate plus nH⁺ is excreted. MLF is a decarboxylation reaction of l-malate to l-lactate and CO₂. The excreted end products of the malolactic reaction may be capable of generating a proton gradient. We propose that the ability of a cell to expel lactate and CO₂ in symport with n protons (n being between 1 and 2 H⁺) can theoretically generate the proton gradient during the malolactic reaction. The cell membrane is, of course, an integral part of this theory, as is the ATPase. Measurements of the membrane potential in cells of L. oenos have shown that the membrane potential during the catabolism of malate has a value of 258 mV (Henick-Kling, thesis). The measured potential is sufficient to drive the generation of ATP via the ATPase. It was observed in this study that when the ATPase is inhibited with DCCD, or when an ionophore is used, the generation of ATP during malate catabolism ceases. A

![Diagram of ATP-generating chemiosmotic mechanism](https://example.com/diagram.png)
hypothesis that end product efflux and the malolactic enzyme is presented in Fig. 2. The malolactic enzyme may be directly or indirectly (peripheral or soluble) associated with the lactate:H+ symport enzyme. The number of protons excreted as a result of malate catabolism is not yet known; however, previous reports have put this number between 1 and 2 protons for end product efflux for related organisms (3, 9, 14).

A second possibility is that the increase in intracellular pH generated during malolactic decarboxylation may make other cellular reactions more favorable or reduce the need to use ATP to maintain pH homeostasis. Thus, malate catabolism might indirectly contribute to the conservation of available ATP but not be directly involved in its synthesis. However, the quantity of ATP produced during malate catabolism is large enough to support the theory of direct chemiosmotic ATP synthesis.

Further work will survey other lactic acid bacteria containing the malolactic enzyme system for energy production and attempt to define the energy-yielding mechanism of the malolactic decarboxylating reaction.

We thank Peter Hinkle for several helpful discussions and comments during the course of this work.

This work was supported by funds from the New York State Agricultural Experiment Station and U.S. government Hatch funds.

LITERATURE CITED
4. Kunkee, R. E. 1975. A second enzymatic activity for decompo-