Biochemical Basis for Glucose-Induced Inhibition of Malolactic Fermentation in Leuconostoc oenos

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The sugar-induced inhibition of malolactic fermentation in cell suspensions of Leuconostoc oenos, recently reclassified as Oenococcus oeni (L. M. T. Dicks, F. Dellaglio, and M. D. Collins, Int. J. Syst. Bacteriol. 45:395–397, 1995) was investigated by in vivo and in vitro nuclear magnetic resonance (NMR) spectroscopy and manometric techniques. At 2 mM, glucose inhibited malolactic fermentation by 50%, and at 5 mM or higher it caused a maximum inhibitory effect of ca. 70%. Galactose, trehalose, maltose, and mannose caused inhibitory effects similar to that observed with glucose, but ribose and 2-deoxyglucose did not affect the rate of malolactic activity. The addition of fructose or citrate completely relieved the glucose-induced inhibition. Glucose was not catabolized by permeabilized cells, and inhibition of malolactic fermentation was not observed under these conditions.31P NMR analysis of perchloric acid extracts of cells obtained during glucose-malate cometabolism showed high intracellular concentrations of glucose-6-phosphate, 6-phosphogluconate, and glycerol-3-phosphate. Glucose-6-phosphate, 6-phosphogluconate, and NAD(P)H inhibited the malolactic activity in permeabilized cells or cell extracts, whereas NADP+ had no inhibitory effect. The purified malolactic enzyme was strongly inhibited by NADH, whereas all the other above-mentioned metabolites exerted no inhibitory effect, showing that NADH was responsible for the inhibition of malolactic activity in vivo. The concentration of NADH required to inhibit the activity of the malolactic enzyme by 50% was ca. 25 μM. The data provide a coherent biochemical basis to understand the glucose-induced inhibition of malolactic fermentation in L. oenos.

Malolactic fermentation is a process that occurs in wine after alcoholic fermentation and consists of the conversion of l-malate to l-lactate and carbon dioxide. As a consequence of this reaction, the total acidity decreases and the organoleptic properties and biological stability of the wine are generally improved (10, 11, 41). Several studies have shown that Leuconostoc oenos is well adapted to high ethanol concentrations and low pH values and is largely responsible for malolactic fermentation in wine (8, 15, 18, 20). Although L. oenos was recently reclassified as Oenococcus oeni (13), the old designation will be used throughout this work. The malate fermentation pathway in L. oenos generates a proton motive force that drives ATP synthesis (9, 29), thus explaining the early report of a pH-independent stimulation of growth by malate (24). Glucose and fructose, the major sugars present in wine, can be utilized by L. oenos as energy sources for growth (41), and another important component in wine, citric acid, also plays an important role in the bioenergetics of this bacterium (26).

The malolactic reaction is catalyzed by the malolactic enzyme, which has been purified from several organisms (2, 4, 22). The malolactic enzymes studied so far are homodimers or tetramers of 60- to 70-kDa subunits, have a Km for malate ranging from 3 to 17 mM, have maximal activity at pH 5.5 to 6.0, and require NAD+ and Mn2+ as cofactors for activity. The sequence of the gene encoding the malolactic enzyme of Lactococcus lactis was elucidated recently and shown to share a high degree of homology to those of malic enzymes from several microorganisms (1, 12). Furthermore, the genes encoding the malolactic enzyme and the malate permease of L. oenos were cloned and characterized and shown to be organized in a cluster (19).

For an improved control of malolactic fermentation in biotechnological applications, it is essential to understand the interactions of malate metabolism with the metabolism of other carbon substrates that are present in wine after alcoholic fermentation, e.g., glucose and other sugars. The relationship between the metabolism of malate and carbohydrates in L. oenos remains controversial. Some studies on malate-carbohydrate cofermentation in L. oenos suggested that the metabolism of malate has a clear effect on the pattern of products derived from sugar metabolism (16), but the opposite has also been reported (30). Several authors have reported that at low pH malate is metabolized at a high rate whereas carbohydrate metabolism proceeds very slowly. The increase in pH induced by the metabolism of malate permits subsequent utilization of carbohydrates, thus explaining the observed malate-induced stimulation of growth (6, 14, 36). Furthermore, sugar-malate cofermentation by L. oenos seems to depend largely on the strain used as well as on the culture conditions, e.g., pH and biomass concentration (for a revision on malolactic fermentation, see reference 16). In previous studies on glucose metabolism by L. oenos, we observed a strong decrease in the rate of malate utilization when glucose was also present in the medium. This surprising result triggered further efforts to understand the biochemical principles underlying this inhibition effect, and here we report on the research work carried out with L. oenos GM. Besides the classical manometric methods, in vivo and in vitro nuclear magnetic resonance (NMR) spectros-
copy analyses were used in this study. A biochemical basis for the sugar-induced inhibition of malolactic activity is presented.

**MATERIALS AND METHODS**

Organisms and growth conditions. *L. oenos* GM, a commercial strain used as a starter culture, was obtained from Microfin De Tecnichs, Sarasota, Fla.; *L. oenos* ITQB M3 and 8A were isolated from Portuguese table wines; the former was supplied by M. V. San Romao (Instituto de Tecnologia Quimica e Biologica, Oeiras, Portugal), and the latter was supplied by A. Mendes Faia (Unidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal). *L. oenos* LIDO04 and LIDO17 were isolated by M. V. San Romao. Cells were routinely grown in FT 80 medium (5) modified by the omission of yeast extract, in 2-liter glass bottles at pH 4.8 and 30°C for 36 h as described previously (17), to the late exponential growth phase (absorbance at 600 nm, 0.8). When appropriate, glucose at a concentration ranging between 5 and 100 mM or other sugars at 45 mM. Specific malolactic activity was expressed as micromoles of CO2 per minute per milligram (dry weight) of cells. For NMR spectroscopy the assay mixtures contained 45 mM L-malate (potassium salt) (pH 6.0), 10 mM glycine and, when appropriate, glucose at a concentration ranging between 5 mM and 100 mM or other sugars at 45 mM. Specific malolactic activity was expressed as micromoles of CO2 per minute per milligram (dry weight) of cells. For NMR experiments, cell suspensions were placed in the NMR tube and the substrates were added via a microsyringe; substrate consumption and product formation were monitored until l-malate was exhausted. Specific malolactic activity was expressed as micromoles of l-lactate per minute per milligram (dry weight) of cells. The concentration of lactate was determined at the end of each experiment by comparison of the resonance intensities due to the methyl group protons before and after the addition of a known amount of lactate. In the experiments where the effect of additional electron acceptors was monitored, the amount of lactate derived from the metabolism of fructose or citrate was estimated by comparison with the acetate/lactate ratio produced by the metabolism of fructose (30) or the citrate-glucose cometabolism (25) and found to be negligible (10-fold lower) compared to the amount of lactate derived from malolactic fermentation.

For determination of malolactic activity in permeabilized cells or cell extracts, the assay mixture contained 45 mM L-malate (potassium salt) (pH 6.0), 100 mM NaCl, 87 mM MnSO4 in 0.1 M potassium phosphate buffer (pH 6.0). The effect of sugars (glucose and fructose), phosphorylated metabolites (glucose-6-phosphate and 6-phosphogluconate), ATP, and cofactors (NADH, NADPH, and NADP) on the malolactic activity was investigated by NMR or volumetric methods.

For the study of the inhibitory effect of several metabolites on the malolactic enzyme, manometric techniques were used. The assay mixtures contained 45 mM l-malate (potassium salt), 87 mM MnSO4, 30 mM NAD+, and a variety of concentrations of NADPH, NADP+, glucose-6-phosphate, or 6-phosphogluconate, in 0.1 M potassium phosphate buffer (pH 6.0).

**RESULTS**

Inhibition of malolactic activity by glucose and other sugars. The malolactic activity assayed by manometric methods in whole cells of *L. oenos* GM at pH 3.5 was 0.50 ± 0.04 μmol of CO2 · min⁻¹ · mg (dry weight) of cells⁻¹ (mean of five independent experiments). When the malolactic activity was measured in the presence of glucose (final concentration, 45 mM),
65 to 70% inhibition was observed. The effect of other sugars on the malolactic activity was also assessed. Galactose, mannose, maltose, and trehalose (all at 45 mM) also inhibited malolactic activity by approximately 60%. On the other hand, inhibition was not found when fructose, ribose, or 2-deoxyglucose was added (Fig. 1). These experiments were performed at pH 3.5 and at 45 mM l-malate, since previous results have shown that 3.5 is the optimal pH for malolactic fermentation in this organism and 45 mM is a saturating concentration of substrate (27).

The effect of glucose concentration (5 µM to 100 mM) on the malolactic activity in L. oenos GM was also examined. The extent of inhibition increased with the concentration of glucose but levelled off at approximately 70% for concentrations above 5 mM (Fig. 2). The concentration of glucose required to cause 50% inhibition of malolactic activity was approximately 2 mM. The inhibition did not depend on the energization status of the cells, since identical inhibitory effects were observed in starved cells that had been kept for 5 h at 25°C (data not shown).

Four other strains of L. oenos (M3, 8A, LOD004, and LOD017) were examined for glucose-induced inhibition of malolactic activity. Glucose at 45 mM inhibited the malolactic activity in the first two organisms, although to a lesser extent than in L. oenos GM (40 and 49%, respectively), whereas no effect was found in strains LOD004 and LOD017.

**Glucose-induced inhibition of malolactic activity in L. oenos GM: effect of additional substrates and gas atmosphere.** The conventional methods based on the determination of released CO₂ (volumetric methods or measurements with a CO₂ electrode) are in principle not fully satisfactory to monitor malolactic fermentation when multiple substrates are involved because CO₂ is also produced from the metabolism of substrates other than malate (e.g., glucose, fructose, or citrate). Furthermore, experiments involving changes in the gas atmosphere are not easily monitored by these methods. ¹H NMR provides...
straightforward means of characterizing the whole metabolic process, since not only lactate but also acetate and other major products of metabolism can be monitored in living cell suspensions. Also, the gas atmosphere in the NMR tube can easily be changed by bubbling the suitable gas without disturbing the signal detection. The time course for the conversion of malate to lactate by a cell suspension of *L. oenos* GM, as monitored by in vivo $^1$H NMR, is shown in Fig. 3. Following the addition of t-malate, consecutive spectra were acquired over a period of 30 min. The intensity of the resonance of the methyl group protons of lactate was plotted as a function of time (inset in Fig. 3). The values for the specific malolactic activity and for the inhibitory effect of glucose were identical to those obtained by volumetric methods. When malate and glucose were cometabolized, the resonance due to the methyl protons of acetate and is therefore very easily detected by $^1$H NMR. The rate of glucose consumption was measured from the decrease in the intensity of the resonance due to C-2 in [2-13C]glucose and was 6.3 nmol $\text{mg}^{-1} \cdot \text{min}^{-1}$ of cells$^{-1}$ when citrate or fructose was supplied in addition to malate.

Intramolecular pools of phosphorylated metabolites. The accumulation of phosphorylated intermediate metabolites of glucose metabolism was investigated by $^{31}$P NMR in perchloric acid extracts. Figure 5 shows the $^{31}$P NMR spectra of perchloric acid extracts obtained during malolactic fermentation in the presence and absence of glucose. In the extract obtained during the cometabolism of malate and glucose, the following phosphorylated compounds accumulated intracellularly at high concentrations: glucose-6-phosphate (5.0 mM), 6-phosphogluconate (1.5 mM), and glycerol-3-phosphate (2.8 mM). A resonance whose chemical shift coincided with that of NADPH was also detected, with intensity corresponding to 0.5 mM, but this assignment is doubtful because NADPH is known to be unstable under the acidic conditions used for the extraction.

**ATP levels in *L. oenos* GM.** ATP levels in cell suspensions metabolizing glucose, glucose plus malate, glucose plus malate plus citrate, or glucose plus malate plus fructose were measured as a function of the time after the addition of the substrates (full data not shown). After a 10-min incubation with glucose alone, the ATP level was very low (0.15 nmol · mg $\text{dry weight}^{-1}$ of cells$^{-1}$), whereas during malate metabolism or glucose-malate cometabolism, the ATP content increased 8- or 15-fold, respectively. When citrate was provided in addition to glucose plus malate, the ATP level was similar to that found during glucose-malate cometabolism (2.2 nmol · mg $\text{dry weight}^{-1}$ of cells$^{-1}$), whereas when fructose was supplied, an increase in the ATP production was measured (3.0 nmol · mg $\text{dry weight}^{-1}$ of cells$^{-1}$).

**NAD(P)H levels in *L. oenos* GM.** Fluorescence measurements in whole cells were performed to evaluate the accumulation of reduced nicotinamide adenine nucleotides (Fig. 6). Following the addition of glucose (45 mM), the level of intracellular NAD(P)H increased rapidly and a steady state was reached within 2 min. The subsequent addition of t-malate (45 mM) had no significant effect on the level of NAD(P)H (trace A), whereas a drastic decrease was observed when citrate was provided (trace B). The addition of fructose instead of citrate caused a similar effect (data not shown).
Effect of phosphorylated metabolites and pyridine nucleotides on malolactic activity. Permeabilized cell suspensions or cell extracts were used in these studies, since whole cells are impermeable to these metabolites. The apparent kinetic parameters of the malolactic enzyme for NAD$^+$ were determined for cell extracts and permeabilized cells in 0.1 M potassium phosphate buffer (pH 6.0) containing 87 mM Mn$^{2+}$. Michaelis-Menten behavior that could be described by a $V_{max}$ of approximately 0.6 μmol of CO$_2$·min$^{-1}$·mg (dry weight) of cells$^{-1}$ and a $K_m$ of 6 μM was found for NAD$^+$. All subsequent assays with permeabilized cells were carried out in the presence of 10 μM NAD$^+$. Glucose utilization and inhibition of malolactic activity were not observed in permeabilized cells of L. oenos GM. The inability of permeabilized cells to utilize glucose was not due to lack of hexokinase activity, since measurements of this enzymatic activity led to a value of 50 μmol·min$^{-1}$·mg of protein$^{-1}$. Moreover, when ATP (5 mM) was provided to permeabilized cells in a medium containing malate plus glucose, the malolactic activity was inhibited by 60%. These results showed that the malolactic activity was inhibited not by glucose itself but by one or more intermediate compounds in the metabolism of glucose.

The phosphorylated metabolites that accumulated in glucose-utilizing cells (glucose-6-phosphate and 6-phosphogluconate), as well as the redox coenzymes NADH, NADPH, and NADP$^+$, were tested for their effect on malolactic activity in permeabilized cells. Glucose 6-phosphate, 6-phosphogluconate, NADH, and NADPH inhibited malolactic activity to a significant extent; in contrast, no inhibition of malolactic activity was caused by NADP$^+$. The inhibitory effects of these metabolites, glucose, and ATP, each at 1 mM, are shown in Fig. 7. It is known that the accumulation of glucose-6-phosphate and 6-phosphogluconate in glucose-metabolizing L. oenos GM cells is a consequence of the difficulty in oxidation of the NAD(P)H pool under anaerobic conditions (38); therefore, tests with the isolated malolactic enzyme were essential to establish which of these metabolites were the genuine inhibitors. Figure 8 shows the results obtained when the activity of pure malolactic enzyme from L. oenos GM was measured in the presence of several metabolites. No inhibition was observed with glucose-6-phosphate, 6-phosphogluconate, and NADPH, all added at a final concentration of 1 mM, whereas NADH caused a strong inhibition. NADH was used at a concentration range from 5 to 200 μM; the concentration required to give 50% inhibition of the malolactic activity was 25 μM.

**DISCUSSION**

The goal of the present work was to elucidate the biochemical basis for the glucose-induced inhibition of malolactic fermentation observed in L. oenos GM. Inhibition of the malolactic activity by glucose in Lactobacillus curvatus was reported previously, but no explanation was given (40). Previous studies concerning the metabolism of glucose by L. oenos have shown that the activity of acetaldehyde dehydrogenase is very low compared to the activity of NAD(P)H-forming enzymes in the early steps of glucose metabolism (see Fig. 9); this prevents efficient NAD(P)H disposal during glycolysis, leading to a high intracellular concentration of NAD(P)H (38). Consequently, glucose-6-phosphate dehydrogenase and 6-phosphogluconate oxidoreductase
dehydrogenase are inhibited, which in turn results in the accumulation of glucose-6-phosphate and 6-phosphogluconate, respectively. The accumulation of phosphorylated intermediates derived from glucose during glucose-malate cofermentation was clearly shown by $^{31}$P NMR analysis of perchloric acid extracts. Our data show that the glucose-induced inhibition of malolactic activity is caused by a direct inhibitory effect of NADH, which is expected to accumulate during glucose catabolism as a result of inefficient NAD(P)H disposal; although the two first steps in glucose metabolism are largely dependent on the cofactor NADP$^+$ (38), the transdehydrogenase activity would lead to an increase in the NADH pool. Evidence for the presence of this activity was provided by the discrepancy between the effects exerted by NADPH on the malolactic activity in assays with permeabilized cells and the isolated malolactic enzyme (compare Fig. 7 and 8): NADPH is unable to inhibit the malolactic activity of the pure enzyme, but in permeabilized cells a significant inhibitory effect is observed which is explained by the conversion of NADPH to NADH due to transdehydrogenase activity. In line with this, the inhibition of malolactic activity caused by glucose-6-phosphate and 6-phosphogluconate in permeabilized cells and cell extracts is most probably due to NADH production derived from the enzymatic conversion of these intermediates by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which are active under these conditions.

Previous studies have reported the inhibition of the malolactic enzyme from L. oenos B211, Lactobacillus plantarum B38, and Leuconostoc mesenteroides B116 and B212 by NADH (2, 34, 35). It has also been reported that the enzyme isolated from L. oenos B211 was inhibited by ATP and fructose-1,6-bisphosphate (2). It is interesting that the malolactic enzyme studied here is strongly inhibited by NADH but not by the related coenzymes NADPH and NADP$^+$; NAD$^+$ is an essential cofactor for the enzyme activity; it is therefore expected that NADH, a closely related compound, may compete efficiently for the binding site of NAD$^+$, thereby causing inhibition. In this context, it is also relevant that the apparent $K_m$ of the enzyme for NAD$^+$ (measured in cell extracts) is of the same order of magnitude as $I_{50}$ determined for NADH. A
detailed characterization of the inhibition by NADH is beyond the scope of this work.

Inhibition of the malolactic activity in whole cells was also observed when other sugars, such as galactose, maltose, man- nose, and trehalose, were supplied. The inhibition caused by these sugars was somewhat lower than that induced by glucose, which suggests that their consumption rate is lower than the rate of glucose metabolism, leading to a lower internal level of NADH. The metabolism of these sugars by lactic acid bacteria also proceeds via glucose-6-phosphate (42), and the rationale for the inhibition of malolactic fermentation by these sugars is therefore similar to that proposed for glucose. Further support for our proposal was provided by the lack of inhibition observed in the experiments in which fructose or ribose was supplied: in fact, ribose enters the heterofermentative pathway at the level of xylulose-5-phosphate and does not have to under- go the oxidative-decarboxylatory steps to become a suitable substrate for xylulose 5-phosphate phosphoketolase. On the other hand, fructose is not inhibitory for malolactic fermenta- tion, since it is partially converted to mannitol via mannitol dehydrogenase (31, 39), thus providing an extra route for the reoxidation of NAD(P)H. Moreover, when citrate was sup- plied, no inhibition of malolactic activity by glucose was de- tected. The direct fluorescence measurements of NAD(P)H reported here fully confirm previous data suggesting that the intracellular pool of NAD(P)H decreased during the cometabolism of citrate and glucose. This is explained by the in- creased conversion of pyruvate to lactate and 2,3-butanediol, with concomitant regeneration of NAD(P)(+) (25). The lack of inhibition observed with the nonmetabolizable glucose analog 2-deoxyglucose is also in full agreement with the proposed explanation for inhibition.

Inhibition of malolactic activity by glucose is not a general observation among L. oenos strains; in fact, it was not observed for L. oenos strains LOD004 and LOD017. This can be due either to increased activity of acetaldehyde dehydrogenase, leading to a more efficient NAD(P)H disposal, or to deficient utilization of glucose by these strains, thereby preventing the accumulation of NADH, or to different inhibitor specificities of the malolactic enzymes from these strains.

Possession of NAD(P)H oxidases appears to be a universal property of lactic acid bacteria (7). These enzymes, which catalyze the reoxidation of NAD(P)H by oxygen as the electron acceptor, were also found in cell extracts of L. oenos (21, 38). Therefore, oxygen was expected to alleviate the inhibition of malolactic fermentation, since accumulation of NADH would decrease because of the supposedly efficient removal by NADH oxidases. However, under the experimental conditions used here, the presence of oxygen did not exert a remarkable effect on the inhibition of malolactic activity; this is probably explained by the high affinity of malolactic enzyme toward NADH. Even in the presence of oxygen, the residual concentration of NADH is likely to remain higher than its Km for the malolactic enzyme.

The results obtained with permeabilized cells support the explanation provided here for the inhibition of malolactic ac- tivity and rule out an important inhibitory effect of glucose at the level of the malate transport; glucose on its own had no inhibitory effect unless ATP was provided, thus allowing glu- cose catabolism and production of NADH. The inhibition of malolactic fermentation by ATP alone is low and probably has no physiological relevance, since high levels of ATP were pro- duced during the cometabolism of glucose and malate and an additional substrate (e.g., fructose or citrate) and yet malolac- tic fermentation was not inhibited. Our explanation for the inhibition of malolactic fermentation by glucose is outlined in Fig. 9. Malolactic fermentation is inhibited by a direct effect of NADH, accumulated during glucose catabolism, on the activity of malolactic enzyme. When additional electron acceptors are provided, the NAD(P)H/NAD(P)(+) ratio decreases, either due to the increased flux from pyruvate to lactate and 2,3-butanediol or due to the reduction of fructose to mannitol, with concomitant draining of the accumulated intermediate compounds in the metabolism of glucose and consequent relief of inhibition.

In addition to the fundamental interest underlying the elu- cidation of sugar-induced inhibition of malolactic fermentation achieved in this work, our results may be relevant to the control of this process in biotechnological applications.

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