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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. ^{31}P 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 K_m for malate ranging from 3 to 17 mM, have maximal activity at pH 5.5 to 6.0, and require NAD^+ and Mn^{2+} 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-

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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 Microlife Technics, Sarasota, Fla.; *L. oenos* ITQB M3 and 8A were isolated from Portuguese table wines; the former was supplied by M. V. San Romão (Instituto de Tecnologia Química e Biológica, Oeiras, Portugal), and the latter was supplied by A. Mendes Faia (Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal). *L. oenos* LOD004 and LOD017 were isolated by M. V. San Romáo. *L. oenos* GM, ITQB M3, and 8A were stored at −80°C in 0.1 M phosphate buffer (pH 5.5) containing 50% (vol/vol) glycerol, and the other strains were kept in the freeze-dried form. The cells were routinely grown in FT 80 medium (5) modified by the omission of Tween 80. Glucose, fructose, malate (5 g · liter^{-1}), and citrate (1 g · liter^{-1}) were used as carbon sources. The solutions of glucose and fructose were autoclaved separately and added prior to inoculation of the culture. Cells were grown without shaking 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).

Sample preparation for in vivo ^{1}H and ^{13}C NMR experiments. The cells were harvested by centrifugation (2,000 × *g* for 15 min at 4°C), washed with 0.1 M glycine buffer (pH 3.5), and transferred to a 10-mm-diameter NMR tube containing 0.1 M glycine buffer (pH 3.5). ^2H_2O was added to a final concentration of 10% (vol/vol) to provide a lock signal. Approximately 12 and 55 mg (dry wt) of cells were used for ^{1}H- and ^{13}C-NMR measurements, respectively. Efficient mixing of the cell suspensions and supply of N_2 and O_2 were achieved by using an air lift system in the NMR tube (32). For dry weight determinations, the cell suspensions were filtered through 0.2-μm-pore-size membranes and dried to constant weight at 100°C.

Cell permeabilization. The cells were harvested by centrifugation and washed twice with 2 mM potassium phosphate buffer (pH 6.0). The cell pellet was suspended to a final concentration of 20 to 30 mg (dry wt) of cells · ml^{-1} in the same buffer containing 0.5 mg of lysozyme · ml^{-1}, and the cell suspension was incubated at 37°C for 1 h. After incubation, the cells were cooled on ice, Triton X-100 at a final concentration of 0.03% (vol/vol) was added, and the cells were used immediately in experiments monitored by either manometric or NMR methods.

Preparation of cell extracts. The cells were disrupted with a Braun Lab-U ultrasonicator with a 0.9-cm-diameter tip by applying low power three times (6 min each, duty cycle, 40% of full power). Cell debris was removed by centrifugation (30,000 × *g* for 20 min at 4°C), and the cell extracts were used immediately for determination of malolactic activity. Hexokinase activity in cell extracts was measured as described previously (33), and the protein concentration was determined by the method of Bradford (3).

Purification of the malolactic enzyme. The malolactic enzyme from *L. oenos* GM was purified as described previously (28). The final preparation was judged to be pure by the observation of a single band in sodium dodecyl sulfate-gel electrophoresis.

Determination of malolactic activity. For experiments where malolactic activity was monitored in whole cells by manometric techniques, the cells were harvested as described above and resuspended in 0.1 M glycine buffer (pH 3.5) to a final concentration of 20 to 25 mg (dry wt) of cells · ml^{-1}. The malolactic activity was determined at 30°C by measuring the CO_2 released by using a Warburg apparatus and the volumetric method described previously (37). The assay mixtures contained, in 0.1 M glycine buffer (pH 3.5), 45 mM L-malate and, when appropriate, glucose at a concentration ranging between 5 μM and 100 mM or other sugars at 45 mM. Specific malolactic activity was expressed as micromoles of CO_2 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 from the metabolism of fructose (39) or the citrate-glucose cometabolism (25) and found to be negligible (100-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), 10 μM NAD^+, and 87 μM $MnSO_4$ 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, $NADP^+$, and NADPH) on 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 μM $MnSO_4$, 50 μM NAD^+, and a variety of concentrations of NAD(P)H, $NADP^+$, glucose-6-phosphate, or 6-phosphogluconate, in 0.1 M potassium phosphate buffer (pH 6.0).

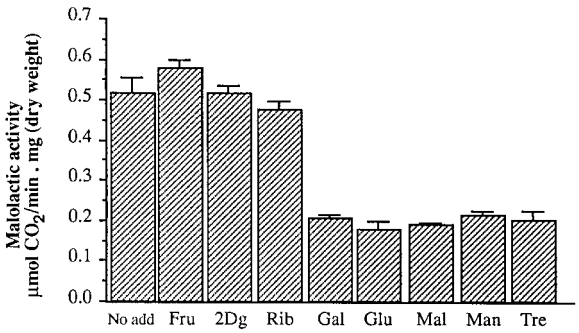

FIG. 1. Effect of sugars on malolactic activity in intact cells of *L. oenos* GM as monitored by manometric methods. The experiments were performed in 0.1 M glycine buffer (pH 3.5) supplemented with 45 mM L-malate and 45 mM each sugar at 30°C. No add, no additions; Fru, fructose; 2Dg, 2-deoxyglucose; Rib, ribose; Gal, galactose; Glu, glucose; Mal, maltose; Man, mannose; Tre, trehalose. The results are the mean for five independent experiments.

All experiments were performed at 30°C.

NMR spectroscopy. ^{1}H NMR spectra (about 2 min each) were recorded in a Bruker AMX300 spectrometer with a 10-mm broad-band probe head. The following acquisition parameters were used: spectral width, 6 kHz; pulse width, 15 μs (corresponding to a flip angle of 45°); data size, 32K; repetition delay, 5.7 s; delay for water presaturation, 3 s. ^{13}C NMR spectra were recorded in a Bruker AMX500 spectrometer operating at 125.77 MHz with a 10-mm broad-band probe head. Spectra (4.4 min each) were consecutively acquired after the addition of the substrates. The acquisition parameters were as follows: spectral width, 38 kHz; data size, 16K; repetition delay, 1 s; pulse width, 10 μs (corresponding to a flip angle of 60°). ^{31}P NMR spectra were obtained with a Bruker AMX500 spectrometer operating at 202.45 MHz, as described by Veiga-da-Cunha et al. (38). The ^{1}H chemical shifts were relative to 3-(trimethylsilyl)propanesulfonic acid (sodium salt), and the ^{13}C chemical shifts were referenced against external methanol designated at 49.3 ppm. All spectra were run at a probe head temperature of 30°C.

Preparation of perchloric acid extracts and quantification of phosphorylated metabolites. Cells were grown and harvested by centrifugation as described above and resuspended to a final volume of 60 ml in 0.1 M glycine buffer (pH 3.5). The cell suspensions were transferred to a test tube placed on ice and deaerated by bubbling N_2 for approximately 10 min; the tube was placed in a water bath at 30°C, and the substrates were added. The cell suspensions were kept under a stream of N_2 during substrate utilization. After 10 min, the metabolism was stopped by the addition of 14% (vol/vol) perchloric acid containing 9 mM EDTA. Identification and quantification of phosphorylated compounds in perchloric acid extracts (pH 8.0) were performed by ^{31}P NMR as described by Veiga-da-Cunha et al. (38).

ATP determination. The cells were harvested as described for the preparation of perchloric acid extracts. The cell suspensions (3.5 mg [dry weight] of cells) were incubated at 30°C, the substrates were added, and samples were taken at different times. The metabolism was stopped by extraction with cold perchloric acid. Following neutralization of the samples, the ATP concentration was determined by the luciferin-luciferase assay (23).

Fluorescence measurements of NAD(P)H. The cells were harvested as described above and resuspended in 0.1 M glycine buffer (pH 3.5) to a final concentration of 20 mg (dry weight) of cells · ml^{-1}. The levels of intracellular reduced nicotinamide nucleotides were monitored with a fluorimeter at excitation and emission wavelengths of 350 and 440 nm, respectively (1a). The experiments were carried out in a thermostatted cuvette at 30°C with stirring, and substrates (glucose, malate, citrate, or fructose) were added at a final concentration of 45 mM. Two independent cell batches were examined, and measurements were done in duplicate for each one.

Chemicals. [2-^{13}C]glucose (99% enriched), glucose-6-phosphate, and 6-phosphogluconate were purchased from Sigma Chemical Co., St. Louis, Mo. The pyridine nucleotides were from Boehringer Mannheim Inc. All other chemicals were of reagent grade.

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 CO_2 · 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),

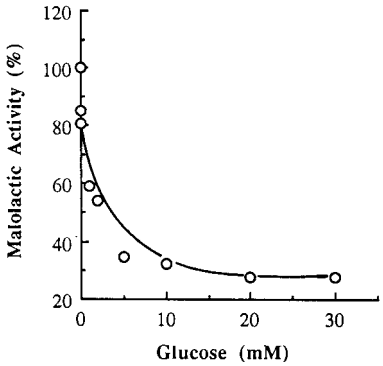

FIG. 2. Effect of glucose concentration on the inhibition of malolactic activity in whole cells of *L. oenos* GM, monitored by manometric methods. The assay conditions used were the same as those described in the legend to Fig. 1, but variable concentrations of glucose were added.

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 CO_2 (volumetric methods or measurements with a CO_2 electrode) are in principle not fully satisfactory to monitor malolactic fermentation when multiple substrates are involved because CO_2 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. ^{1}H NMR provides

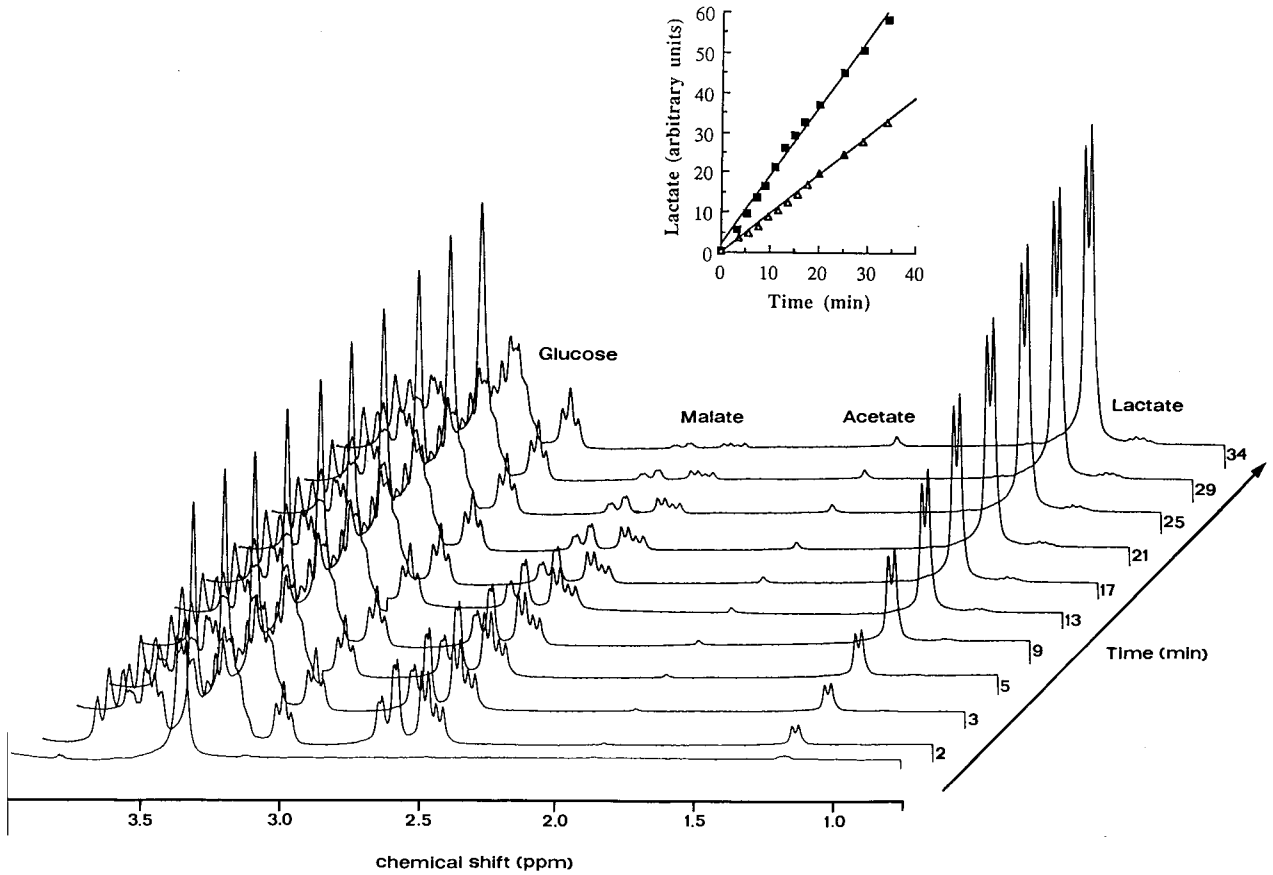

FIG. 3. Time course for the consumption of malate plus glucose by a cell suspension of *L. oenos* GM under anaerobic conditions, as monitored by in vivo ^{1}H NMR. The experiments were performed in 0.1 M glycine buffer (pH 3.5) supplemented with 45 mM L-malate (potassium salt) and 45 mM glucose at 30°C. The inset shows a plot of the intensity of the resonance due to the methyl group of lactate as a function of time both in the presence (△) and in the absence (■) of glucose.

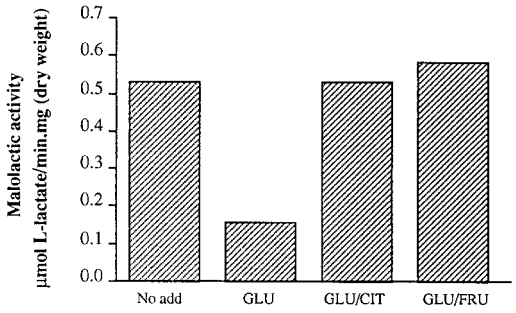

FIG. 4. Effect of fructose or citrate on the glucose-induced inhibition of malolactic activity. Malolactic activity was measured by in vivo ^{1}H NMR under the conditions described in the legend to Fig. 3, with 45 mM fructose or citrate. No add, no additional substrate added; GLU, glucose; GLU/CIT, glucose plus citrate; GLU/FRU, glucose plus fructose. The results are the mean for three independent experiments.

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 L-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 was detected; in fact, 6.4 nmol of acetate $\cdot$ min^{-1} $\cdot$ mg (dry weight) of $cells^{-1}$ was produced. The formation of acetate showed that glucose was consumed to some extent, despite the low pH used in these experiments (pH 3.5). Similar experiments performed under an oxygen atmosphere showed that the inhibitory effect of glucose on the malolactic activity was only 6 to 10% lower than under anaerobic conditions (data not shown).

The effect of citrate or fructose on the glucose-induced inhibition of malolactic activity was also monitored by in vivo ^{1}H NMR of whole cells of *L. oenos* GM, and the results are shown in Fig. 4. The presence of citrate or fructose (final concentration, 45 mM) totally relieved the inhibition of malolactic activity caused by glucose. Experiments with isotopically enriched [^{13}C]glucose coupled to in vivo ^{13}C NMR analysis were performed to check whether the lack of inhibition in the presence of citrate or fructose was due to failure of the cells to metabolize glucose under these conditions. Glucose labeled on C-2 was chosen as the substrate since the label is expected to end in the methyl group of acetate and is therefore very easily detected by ^{13}C NMR. The rate of glucose consumption was measured from the decrease in the intensity of the resonance due to C-2 in [2-^{13}C]glucose and was 6.3 nmol $\cdot$ min^{-1} $\cdot$ mg (dry weight) of $cells^{-1}$ in the presence of malate and 14.4 or 17.2 nmol $\cdot$ min^{-1} $\cdot$ mg (dry weight) of $cells^{-1}$ when citrate or fructose, respectively, was supplied in addition to malate.

Intracellular 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 $\cdot$ mg [dry weight] 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 $\cdot$ mg [dry weight] of $cells^{-1}$), whereas when fructose was supplied, an increase in the ATP production was measured (3.0 nmol $\cdot$ mg [dry weight] 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 L-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).

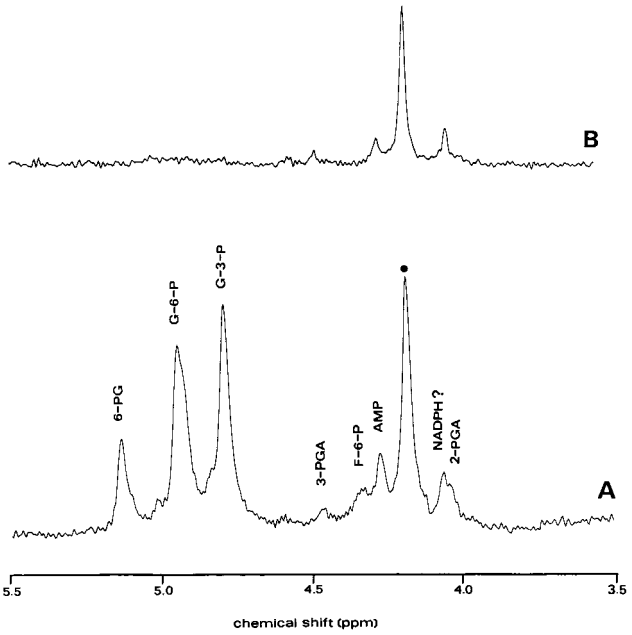

FIG. 5. ^{31}P NMR spectra of perchloric acid extracts (final pH adjusted to 8.0) of cell suspensions of *L. oenos* GM metabolizing malate in the presence (A) and absence (B) of glucose. Chemical shifts (in ppm) of the identified resonances: 6-phosphogluconate (6-PG), 5.13; glucose-6-phosphate (G-6-P), 4.90; glycerol-3-phosphate (G-3-P), 4.79; 3-phosphoglycerate (3-PGA), 4.47 ppm; fructose-6-phosphate (F-6-P), 4.35 ppm; AMP, 4.27 ppm; 2-phosphoglycerate (2-PGA), 4.00; NADPH (tentative), 4.06. ●, unassigned resonance.

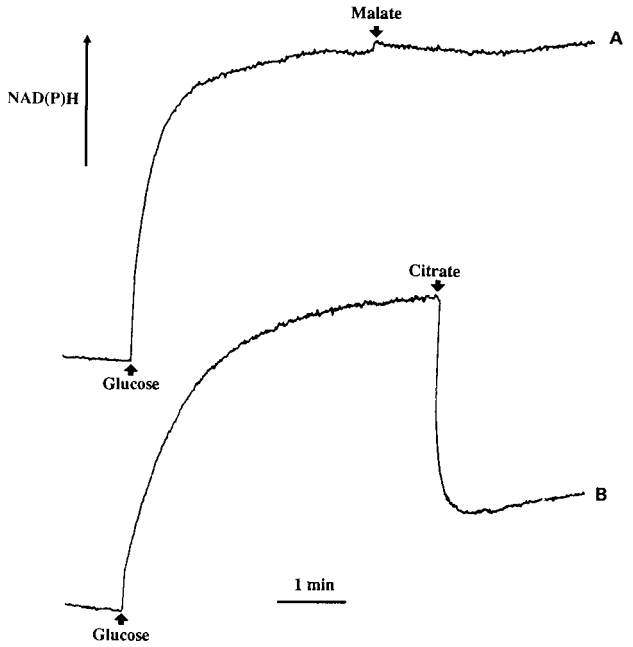

FIG. 6. Time course for the change in the fluorescence of a cell suspension of *L. oenos* GM upon excitation at 350 nm [maximum absorption of NAD(P)H]. The experiments were performed in 0.1 M glycine buffer (pH 3.5) at 30°C. Traces A and B show the effect of malate or citrate, respectively, on the intracellular level of NAD(P)H. All substrates were added at a final concentration of 45 mM. The traces shown represent typical results.

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 μM Mn^{2+}. Michaelis-Menten behavior that could be described by a V_{max} of approximately 0.6 μmol of $CO_2 \cdot min^{-1} \cdot mg$ (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 $\mu mol \cdot min^{-1} \cdot mg$ 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.

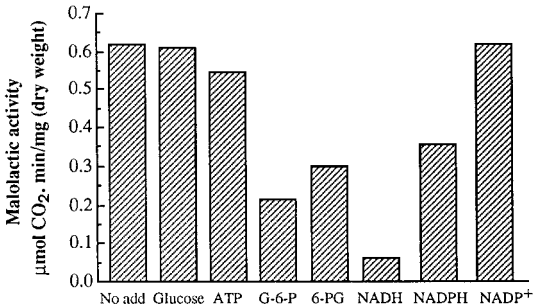

FIG. 7. Effect of several compounds on the malolactic activity measured by ^1H NMR spectroscopy in permeabilized cells of *L. oenos* GM, under anaerobic conditions. The experiments were performed in 0.1 M potassium phosphate buffer (pH 6.0) containing 87 μM $MnSO_4$, 10 μM NAD^+, and 45 mM L-malate (potassium salt). Each compound was added at a final concentration of 1 mM. No add, no additions; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate.

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

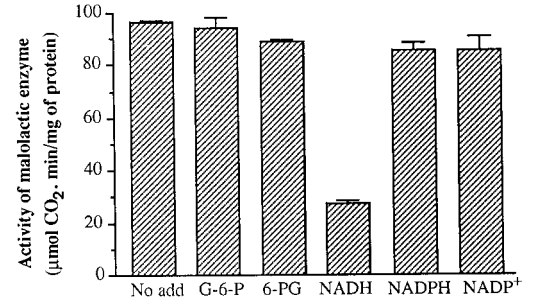

FIG. 8. Effect of several metabolites on the activity of the malolactic enzyme isolated from *L. oenos* GM. The experimental conditions are similar to those described in the legend to Fig. 6, except for the concentration of NAD^+, which was increased to 50 μM. Glucose-6-phosphate, 6-phosphogluconate, NADPH, and $NADP^+$ were each added at a final concentration of 1 mM. The concentration of NADH was 200 μM. The results are the mean for at least three independent determinations.

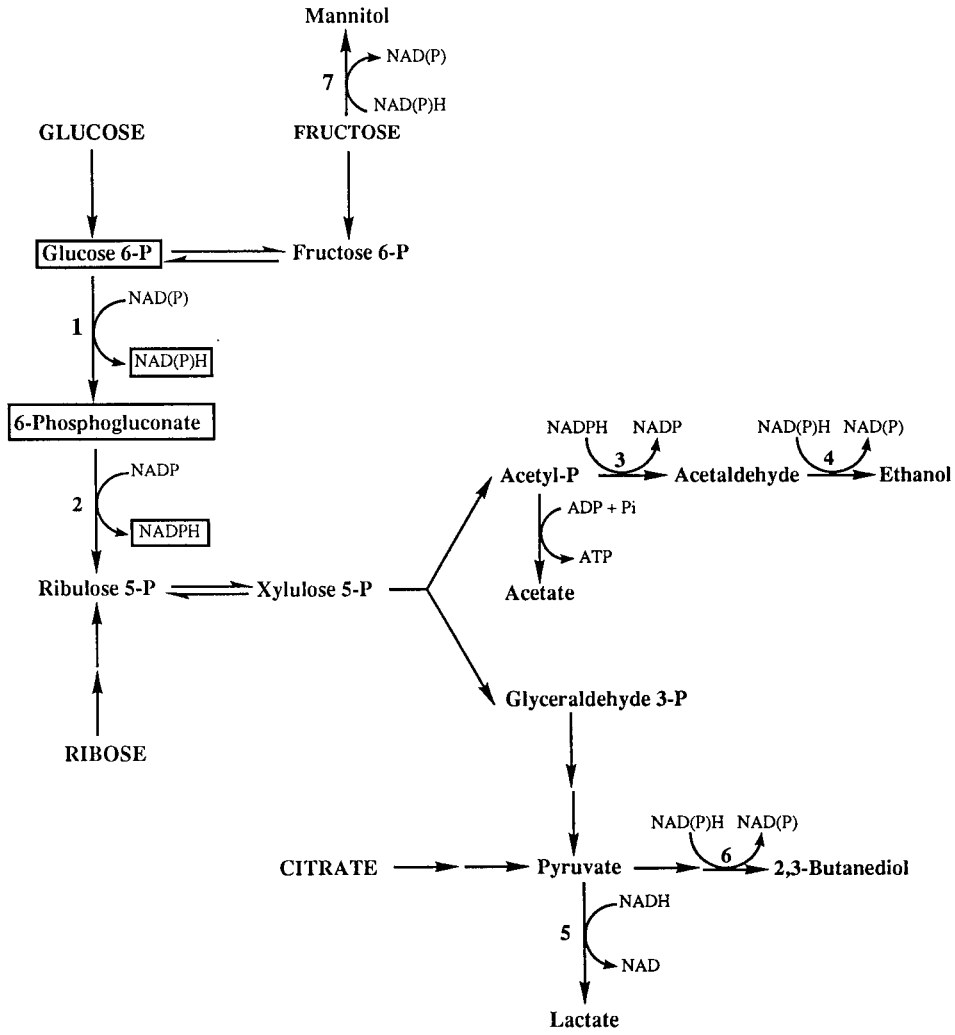

FIG. 9. Proposed model for the sugar-induced inhibition of malolactic fermentation in *L. oenos*. The basis for the relief of inhibition by the metabolism of fructose or citrate is outlined. Inside boxes are the metabolites that accumulate intracellularly during glucose metabolism. The reactions are catalyzed by the following enzymes: 1, glucose-6-phosphate dehydrogenase; 2, 6-phosphogluconate dehydrogenase; 3, acetaldehyde dehydrogenase; 4, alcohol dehydrogenase; 5, lactate dehydrogenase; 6, 2,3-butanediol dehydrogenase; 7, mannitol dehydrogenase.

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, mannose, 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 undergo the oxidative-decarboxylative steps to become a suitable substrate for xylulose 5-phosphate phosphoketolase. On the other hand, fructose is not inhibitory for malolactic fermentation, 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 supplied, no inhibition of malolactic activity by glucose was detected. 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 increased 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 using 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 K_m for the malolactic enzyme.

The results obtained with permeabilized cells support the explanation provided here for the inhibition of malolactic activity 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 glucose 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 produced during the cometabolism of glucose and malate and an additional substrate (e.g., fructose or citrate) and yet malolactic 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 elucidation 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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