Transport of Malic Acid and Other Dicarboxylic Acids in the Yeast
Hansenula anomala

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DL-malic acid-grown cells of the yeast Hansenula anomala formed a saturable transport system that mediated accumulative transport of L-malic acid with the following kinetic parameters at pH 5.0: V_max, 0.20 nmol · s⁻¹ · mg (dry weight)⁻¹; K_m, 0.076 mM L-malate. Uptake of malic acid was accompanied by proton disappearance from the external medium with rates that followed Michaelis-Menten kinetics as a function of malic acid concentration. Fumaric acid, α-ketoglutaric acid, oxaloacetic acid, D-malic acid, and L-malic acid were competitive inhibitors of succinic acid transport, and all induced proton movements that followed Michaelis-Menten kinetics, suggesting that all of these dicarboxylic acids used the same transport system. Maleic acid, malonic acid, oxalic acid, and L-(+)-tartaric acid, as well as other Krebs cycle acids such as citric and isocitric acids, were not accepted by the malate transport system. K_m measurements as a function of pH suggested that the anionic forms of the acids were transported by an accumulative dicarboxylate proton symporter. The accumulation ratio at pH 5.0 was about 40. The malate system was inducible and was subject to glucose repression. Undissociated succinic acid entered the cells slowly by simple diffusion. The permeability of the cells by undissociated acid increased with pH, with the diffusion constant increasing 100-fold between pH 3.0 and 6.0.

Recently, it was shown that the yeast Candida sphaerica transports malate by a proton symport and that the symport also accepts succinate, fumarate, oxaloacetate, and α-ketoglutarate (3). Earlier studies on the transport of malic acid in yeasts have provided evidence that the transport of this acid is carrier mediated in Kluyveromyces lactis (13), Zygossaccharomyces bailii (1), and Schizosaccharomyces pombe (8) and that it is not carrier mediated in Saccharomyces cerevisiae (11). The subject, in addition to its academic interest, has a practical dimension, since L-malic acid and tartaric acid are the principal organic acids in grape must and wine (9) and since a microbiological deacidification process may include the use of yeasts which are able to degrade malic acid during wine fermentation. Hansenula anomala is one of the yeast species which are often found on grapes and in must; it is able to use L-malic acid and other acids of the Krebs cycle as carbon and energy sources.

MATERIALS AND METHODS

Microorganism and growth conditions. H. anomala IGC 4380 was maintained on a medium containing glucose (2%, wt/vol), peptone (1%, wt/vol), yeast extract (0.5%, wt/vol), and agar (2%, wt/vol). For growth under conditions of glucose repression, a mineral medium with vitamins and 0.5% (wt/vol) glucose (12) was used at 25°C with mechanical shaking. Derepressed conditions were obtained by substituting 0.5% (wt/vol) DL-malic acid for glucose in the above medium.

Measurements of uptake rates. Preliminary results showed that malic and succinic acids were accepted by the same carrier in DL-malic acid-grown cells. The uptake rates of dicarboxylic acids were measured by the use of L-[U-14C]malic acid, [2,3,3-13C]succinic acid, or both; for economic reasons, the uptake of undissociated dicarboxylic acid was measured by the use of [2,3,3-13C]succinic acid. Cells were harvested in the mid-exponential phase, centrifuged, washed twice with ice-cold distilled water, and suspended in ice-cold distilled water to a final concentration of about 25 mg (dry weight)/ml. To estimate labeled malic or succinic acid uptake rates, 5-μl amounts of the yeast suspension were mixed in 10-ml conical centrifuge tubes with 35 μl of 0.1 M KH2PO4 buffer at pH values between 3.0 and 5.5. After 2 min of incubation at 25°C in a water bath, the reaction was started by the addition of 10 μl of an aqueous solution of L-[U-14C]malic acid or [2,3-13C]succinic acid at the desired concentration and stopped by dilution with 5 ml of cold water. Sampling times for DL-malic acid-grown cells (active transport of dicarboxylate) were 0, 5, and 10 s. Sampling times for glucose-grown cells (simple diffusion of undissociated acid) were 15 and 30 s. The reaction mixtures were filtered immediately through GF/C filters (Whatman, Inc., Clifton, N.J.), washed on the filters with 10 ml of ice-cold water, and counted in a scintillation fluid that contained 10% (wt/vol) naphthalene, 0.7% (wt/vol) 2,5-diphenyloxazole (PPO), and 0.3% (wt/vol) 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 1,4-dioxane. Radioactivity was measured with a liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

Uptake rates were also calculated from measurements of the proton uptake with a standard pH meter (PHM 62; Radiometer A/S, Copenhagen, Denmark) connected to a flatbed Perkin-Elmer 024 recorder (The Perkin-Elmer Corp., Norwalk, Conn.). The pH electrode was immersed in a water-jacketed chamber provided with magnetic stirring. To the chamber were added 4.5 ml of 10 mM KH2PO4 and 0.5 ml of yeast suspension. The pH was adjusted to the desired value, and a baseline was obtained. The desired amount of dicarboxylic acid (adjusted to the experimental pH value) was added, and the subsequent alkalinization was monitored with the recorder. The initial uptake rate was calculated from the slope of the initial part of the pH trace. Calibration was performed with HCl.

Measurement of the intracellular volume. The intracellular

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volume was measured as previously described (4, 10). A value of 2.98 μl of intracellular water per mg (dry weight) of yeast was obtained for malic acid-grown cells.

**Measurement of succinic acid accumulation.** DL-Malic acid-grown cells (20 μl; 12 mg [dry weight]/ml) were added to 60 μl of 0.1 M KH₂PO₄ buffer (pH 5.0) and also to 60 μl of buffer containing carbonyl cyanide m-chlorophenylhydrazone (CCCP) and were incubated at 25°C with magnetic stirring. The reaction was started by the addition of 20 μl of 5.0 mM [2,3-¹⁴C]succinic acid (about 2,000 cpm/nmol). At appropriate times, 10 μl was taken from the reaction mixture and filtered immediately through Whatman GF/C filters. The filters were washed three times with ice-cold water, and the radioactivity was counted as indicated above. The intracellular concentration of succinic acid was calculated by using the value of the intracellular volume estimated as described above.

**Estimation of amounts of glucose and l-malic acid.** The amount of glucose was estimated by the glucose oxidase method (Test Combination; Boehringer GmbH, Mannheim, Federal Republic of Germany). The amount of l-malic acid was estimated by the enzymatic method previously described (7).

**Calculations of concentrations.** Concentrations of dicarboxylic acids were calculated by the use of the Henderson-Hasselbach equation with the following pK values: succinic acid, pK₁ = 4.18 and pK₂ = 5.56; malic acid, pK₁ = 3.50 and pK₂ = 5.05; fumaric acid, pK₁ = 3.03 and pK₂ = 4.54; oxaloacetic acid, pK₁ = 2.56 and pK₂ = 4.37; α-ketoglutaric acid, pK₁ = 2.47 and pK₂ = 4.68; citric acid, pK₁ = 3.13, pK₂ = 4.76, and pK₃ = 6.40; and isocitric acid, pK₁ = 3.29, pK₂ = 4.71, and pK₃ = 6.40.

**RESULTS AND DISCUSSION**

**Characterization of the malate transport system of H. anomala.** Proton signals were observed when malic or succinic acid was added to a suspension in weak buffer (pH 4.2) of cells that had been grown in DL-malic acid medium. Lineweaver-Burk plots of the initial rates of proton disappearance calculated from the slopes of the proton signals, as well as plots of the initial rates of labeled malic or succinic acid, were linear as a function of the concentration of the acid. Figure 1 shows the results obtained for succinic acid uptake. Similar results were obtained with l-malic acid. This indicates that the uptake mechanism obeyed Michaelis-Menten kinetics and suggests that the transport of either dicarboxylic acid was mediated by a saturable carrier,

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**FIG. 1.** Lineweaver-Burk plots of initial uptake rates of succinic acid and protons by malic acid-grown cells of *H. anomala* IGC 4380 at pH 5.20 as a function of succinate concentration. Symbols: ◦ labeled succinic acid; ○ protons.

**FIG. 2.** Lineweaver-Burk plots of initial uptake rates of labeled malic acid at pH 5.0 as a function of malate concentration. Symbols: □ absence of other dicarboxylic acids; □ presence of 0.9 mM α-ketoglutaric acid; ◦ presence of 0.9 mM 3-malic acid; ○ presence of 0.9 mM fumaric acid; △ presence of 5 mM oxaloacetic acid; □ presence of 0.9 mM L-malic acid.
possibly a proton symporter. Both dicarboxylates were mutual competitive inhibitors (Fig. 2). From the plots of labeled acids, the following kinetic parameters were calculated: for malate, $V_{\text{max}}$ (pH 5.0), $0.2 \pm 0.09$ nmol s$^{-1}$ mg$^{-1}$ (dry weight) of cells$^{-1}$; $K_m$ (pH 5.0), $0.076 \pm 0.006$ mM; for succinate, $V_{\text{max}}$ (pH 5.0), $0.67 \pm 0.1$ nmol s$^{-1}$ mg (dry weight) of cells$^{-1}$; $K_m$ (pH 5.0), $0.038 \pm 0.005$ mM. Results are mean values ± standard deviations of three determinations.

Maleic acid, D-malic acid, fumaric acid, oxaloacetic acid and $\alpha$-ketoglutaric acid were competitive inhibitors of succinic acid transport at pH 5.50 (Fig. 3). This suggests that these acids were transported by the same carrier that transported succinic and malic acid. Indeed, these dicarboxylates induced proton movements that followed Michaelis-Menten kinetics as a function of the concentrations of the acids (Fig. 4). Maleic acid, malonic acid, oxalic acid, and $\alpha$-tartaric acid

\[ \text{TABLE 1. Michaelis constants of succinic acid transport as a function of pH in } H. \text{ anomala IGC 4380 for two hypothetical means of transport} \]

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ (mM) of transport of:</th>
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</thead>
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<tr>
<td></td>
<td>Anions</td>
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<tr>
<td>3.0</td>
<td>0.010</td>
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<tr>
<td>4.0</td>
<td>0.017</td>
</tr>
<tr>
<td>5.5</td>
<td>0.064</td>
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<tr>
<td>6.0</td>
<td>0.046</td>
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$^a$ Facilitated diffusion of undissociated acid.
Symbols: \( V_{\text{max}} \), half-saturation constant \( (K_m) \); \( V \), transport capacity; \( K_n \), transport capacity.

did not act as competitive inhibitors of succinic acid transport, nor did they induce proton movements, indicating that these dicarboxylic acids were not transported by malic acid-grown cells. Citric and isocitric acids also appeared to be accepted by a proton symport (Fig. 5). However, these acids were not competitive inhibitors of succinic transport at pH 5.0 by malic acid-grown cells, suggesting that citric and isocitric acids were transported by a proton symport distinct from the succinic acid carrier. Transport of labeled succinic acid at pH 5.0 was accumulative (Fig. 6). After about 20 min, one-half of the accumulated radioactivity was nonmetabolized succinic acid, since added cold succinic or L-malic acid induced counterflow to this extent (Fig. 6). This result is another indication that malic acid and succinic acid used the same carrier. The accumulation ratio in terms of nonmetabolized succinic acid was about 40. The protonophore CCCP prevented accumulation and induced efflux of accumulated nonmetabolized succinic acid (Fig. 6). The observed accumulation, while consistent with the hypothesis of the existence of a proton symport, does not constitute final proof, since simple or facilitated diffusion of undissociated dicarboxylic acid displays similar accumulation.

As reported earlier for \( C. \) \( \text{sphaerica} \) (3), the rapid efflux of accumulated radioactive succinic acid that was observed in malic acid-grown cells after the addition of CCCP indicates that the dicarboxylic acid carrier of malic acid-grown cells of \( H. \) \( \text{anomala} \) is a proton-dicarboxylate symporter that allows uphill transport and accumulation as a function of pH. To substantiate this hypothesis, estimates of the Michaelis constants of the transport of labeled succinic acid were obtained at several pH values (Table 1). The \( K_m \) values between pH 3.0 and 6.0 varied by a factor of over 15 when calculated as the concentration of undissociated acid and by a factor of less than 6 when expressed as the concentration of succinate ions, suggesting that the negatively charged forms of the acid were transported and that therefore the observed proton movements represented proton symport activity. The capacity of the system, expressed as the maximum transport velocity, was pH dependent (Fig. 7). It increased about twofold between pH 3.0 and 5.50, its optimum pH, and decreased steeply between pH 5.5 and 6.5.

The affinity of the proton malate symport for succinate, expressed as \( K_m \), varied to the same extent. Cells of \( H. \) \( \text{anomala} \) grown in medium with either ethanol (0.5%, vol/vol) or glycerol (0.5% vol/vol) as the carbon source did not transport succinic or L-malic acid, indicating that the dicarboxylic acid carrier was inducible. The growth of the yeast in a medium containing glucose (0.1%, wt/vol) and DL-malic acid (0.5%, wt/vol) was diauxic (Fig. 8). Transport of L-malic acid accompanied by measurable proton movements developed only after the glucose had been consumed, indicating that the system may be subject to glucose repression. As a consequence, the malic acid uptake of \( H. \) \( \text{anomala} \) may be repressed during vinification because of high sugar concentration. At present, we are trying to isolate derepressed mutants of the yeast for the degradation of malic acid during wine fermentation.

**Diffusion of undissociated acid.** Glucose-grown cells lacking the malic acid carrier were still slightly permeable by the acid. Plots of the initial uptake rates of succinic acid as a function of the concentration of undissociated acid were linear (Fig. 9), indicating simple, non-carrier-mediated diffusion.

From the slopes of the linear plots obtained at various pH values, estimates of the diffusion constant were obtained (the diffusion constant as presented in Fig. 9 has the dimen-
sions of volume [microliters], reciprocal time [seconds⁻¹], and reciprocal biomass [milligrams⁻¹]).

The values of the diffusion constants decreased steeply with the extracellular proton concentration from a value of 9 at pH 6.0 to a value of 0.1 at pH 3.0 (Fig. 9B). As a consequence, the passive diffusion of undissociated acid across the plasma membrane of *H. anomala* is subject to opposite pH influences: an increase due to the relative increase of undissociated acid with decreasing pH and a decrease due to decreasing permeability with decreasing pH. Similar behavior was observed earlier with respect to passive proton diffusion across the plasma membrane of *S. cerevisiae* (5), passive diffusion of undissociated lactic acid across the plasma membranes of *Candida utilis* and *S. cerevisiae* (2, 6), and passive diffusion of undissociated malic acid across the plasma membrane of *C. sphaerica* (3).

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LITERATURE CITED


