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Energetic aspects of malate and lactate fermentation by *Acetobacterium malicum*

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1. SUMMARY

Acetobacterium malicum ferments L-malate and L-lactate to acetate (and CO_2) as sole fermentation product. Molar growth yields were 14.5 g and 6.8 g dry cell matter, respectively. With a different *Acetobacterium* sp. strain, similar results were obtained. No oxaloacetate-decarboxylating enzyme activity was detected in cell-free extracts. L-Malate was oxidized by an unusual NAD-dependent, oxygen-sensitive malic enzyme. No oxaloacetate decarboxylase or malate dehydrogenase activity was found. L-Lactate was oxidized to pyruvate by an NAD(P)-independent enzyme which was partly membrane-associated. Pyruvate conversion to acetate proceeded via pyruvate synthase, phosphate acetyltransferase, and acetate kinase. A dichlorophenol indophenol-dependent NADH dehydrogenase was found at high activity. Cytochromes or quinones were not detected. These results, seen against the background of thermodynamical considerations, provide evidence that the difference in growth yields between malate and lactate utilization is due to the difference of redox potentials at which electrons are released during oxidative pyruvate formation from either substrate, and that *A. malicum* forms ATP by electron transport phosphorylation between NAD and a carrier with a redox potential close to that of quinones.

2. INTRODUCTION

Homoacetogenic bacteria are characterized by their ability to use CO_2 as electron acceptor and to form acetate as reduced fermentation product [1]. Most strains metabolize a broad range of organic and inorganic substrates in energy metabolism [1,2]. Only very few homoacetogens, e.g. *Acetobacterium malicum* [3], can utilize both malate and lactate as substrates, and ferment them to acetate, according to the following equa-

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tions (calculations of free energies according to [4]):

$$2\ ^{-}OOCCH_2CHOHCOO^{-} + H^{+} \rightarrow 3\ CH_3COO^{-} + 2\ CO_2$$

$\Delta G_0' = -83.4$ kJ per mol malate

$$2\ CH_3CHOHCOO^{-} \rightarrow 3\ CH_3COO^{-} + H^{+}$$

$\Delta G_0' = -56.3$ kJ per mol lactate

In the original publication, molar cell yields of 9.9 and 4.4 g per mol malate or lactate, respectively, were reported [3]. Obviously, the energetic difference between both substrates (−27 kJ per mol) was exploited somehow by these bacteria. The present study was undertaken to elucidate whether a membrane-bound ion-pumping decarboxylase enzyme or another means of energy conservation was present in this bacterium.

3. MATERIALS AND METHODS

Acetobacterium malicum DSM 4132 [3] and *Acetobacterium* sp. strain AmMan1 [5] were obtained as pure cultures from the respective authors. Cultures were grown in defined bicarbonate-buffered, sulfide-reduced mineral medium [6] containing trace element solution SL 10 [7] and 7 vitamins [8]. Low-sodium media (< 0.1 mM Na^+) were prepared with potassium salts instead of sodium salts, and were reduced with cystein · HCl. Growth was followed by direct insertion of 22-ml screw-cap culture tubes into a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, New York, NY). Cell densities were calibrated to dry cell matter contents by direct gravimetric cell mass determination. L-Lactate and L-malate were quantified enzymatically with glutamate pyruvate aminotransferase and L-malate dehydrogenase plus glutamate oxaloacetate aminotransferase plus L-lactate dehydrogenase [9]. Acetate was determined by gas–liquid chromatography [10], protein by a micro-biuret assay [11]. Cytochromes were checked for in redox difference spectra of crude cell extracts. Quinone extraction and identification was carried out according to [12].

Cell-free extracts for enzyme assays were prepared by either French press or lysozyme or cellosyl treatment of malate- or lactate-grown cells of *A. malicum* harvested and washed under strictly anoxic conditions. For details, see reference [13]. Cytoplasm and membrane fractions were separated by ultracentrifugation (45 min at 120 000 × *g*). Malate dehydrogenase, NAD-dependent lactate dehydrogenase, phosphate acetyltransferase, and acetate kinase were assayed after Bergmeyer [9], oxaloacetate decarboxylase after Dimroth [14], malate lactate transhydroxylase after Allen [15], malic enzyme after Stams et al. [16], NAD-independent lactate dehydrogenase after Molinari and Lara [17], pyruvate synthase after Odom and Peck [18], and NADH dehydrogenase after Kremer et al. [19].

All chemicals used were of reagent grade quality and obtained from Merck (Darmstadt), Sigma (München), Boehringer (Mannheim), or Fluka (Neu Ulm), F.R.G.

4. RESULTS

Among numerous *Acetobacterium* strains tested, only *Acetobacterium malicum* and *Acetobacterium* sp. strain AmMan1 exhibited reproducible growth both on L-malate and L-lactate. The D-enantiomers were not utilized. Doubling times were 3.3–4.5 h with L-malate and 9.3–11.6 h with L-lactate, and were comparable with both strains. Cell yields were determined with the pure L-enantiomers (Table 1), and were 14.4–14.6 or 6.6–7.0 g per mol malate or lactate, respectively, with *A. malicum* (9.2–10.3 g and 5.1–5.6 g with strain AmMan1; results not shown). Obviously, the difference in energy conservation with the pure enantiomers was even higher than originally reported from experiments with racemic substrate preparations. The presence or absence of sodium ions in the medium had no influence on these yields with either substrate, as similar growth experiments after 12 subsequent transfers in low-sodium (< 0.1 mM Na^+) medium revealed (results not shown).

Table 1

Stoichiometry of substrate conversion and growth yields of *Acetobacterium malicum* with L-malate and L-lactate

Substrate	Amount consumed (mM)	Cell mass formed [a] (mg l^{-1})	Substrate assim. [b] (mM)	Acetate formed (mM)	Electron balance (%)	Growth yield (g mol^{-1})
L-Malate	4.95	71.1	0.98	6.1	102	14.4
	7.80	113.6	1.6	10.3	108	14.6
	8.84	129.5	1.8	11.9	110	14.6
L-Lactate	9.3	61.4	0.85	13.1	103	6.6
	12.6	87.6	1.2	19.4	113	7.0
	15.5	101.5	1.4	23.0	108	6.6

[a] Cell dry matter formed was calculated via optical density using an experimentally determined conversion factor (0.1 OD_{578} = 29.5 mg l^{-1}).

[b] Substrate assimilated was calculated according to the formulas:

17 malate^{2-} + 34 H^+ → 12 $\langle C_4H_7O_3\rangle$ + 20 CO_2 + 9 H_2O

17 lactate$^-$ + 17 H^+ → 12 $\langle C_4H_7O_3\rangle$ + 3 CO_2 + 9 H_2O

thus, 13.7 μmol of substrate was assimilated into 1 mg of cell material.

No activity of any of the following enzymes was detected in cell-free extracts of *A. malicum*: NAD(P)-dependent malate dehydrogenase, NAD(P)-dependent lactate dehydrogenase, NADP-dependent malic enzyme, oxaloacetate decarboxylase, malate lactate transhydrogenase. Instead, in malate-grown cells we found an unusual NAD-dependent malic enzyme activity together with benzyl viologen-dependent pyruvate synthase, phosphate acetyltransferase, acetate kinase, and NADH dehydrogenase (Table 2).

Table 2

Enzyme activities detected in crude cell-free extracts of *Acetobacterium malicum*

Enzyme	E.C. number	Specific activity (μmol min^{-1} g $protein^{-1}$)
Malic enzyme, NAD-dependent	1.1.1.39?	80– 230
L-lactate dehydrogenase, NAD-independent	?	800–1200
Pyruvate synthase	1.2.7.1	40– 330
Phosphate acetyltransferase	2.3.1.8	1000–1200
Acetate kinase	2.7.2.1	1100–2000
NADH dehydrogenase, DCPIP-dependent	1.6.99.4?	930–2100

Substrate utilization activity was calculated to be 350 μmol min^{-1} g $protein^{-1}$ for cells growing with L-malate, and 290 μmol min^{-1} g $protein^{-1}$ for cells growing with L-lactate.

The malic enzyme found was very oxygen-sensitive (96% activity loss during 30 min exposure to air), reacted only inefficiently with NADP (< 10% of NAD-dependent activity) and could be measured optimally in 125 mM Tris · HCl buffer, pH 7.8–8.4, in the presence of 2.5 mM dithioerythritol. Higher buffer concentrations, lower or higher pH, omission of dithioerythritol, or use of different buffer systems (tricine, triethanolamine, diethanolamine, 3-morpholinopropanesulfonate) resulted in measurable activities 60–97% lower than under optimal conditions. 10 mM $MnCl_2$ was required for optimal activity (100%) as well; $MgCl_2$ (39%), KCl (10%) or NaCl (9%) could replace $MnCl_2$ at identical concentration only to a limited extent (8% activity without additions). Optimal activity was observed if the cell extract was preincubated with NAD (5 mM) at least 40 min before the reaction was started by malate addition; the measurable activity depended directly on the preincubation time (Fig. 1a). The apparent K_M of this enzyme for L-malate was 6.5 mM (Fig. 1b). The enzyme was found entirely in the cytoplasmic fraction.

Lactate-grown cells of *A. malicum* contained also an NAD-independent lactate dehydrogenase which could be measured with dichlorophenol indophenol. This enzyme was moderately oxygen-sensitive (30% activity loss during 30 min

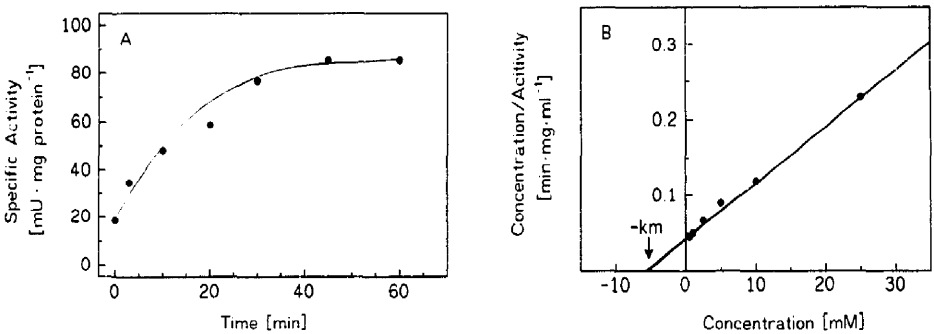

Fig. 1. A. Influence of preincubation time of crude cell extract with 5 mM NAD before reaction start on measurable malic enzyme activity of *Acetobacterium malicum*. B. Determination of apparent K_M for L-malate of malic enzyme in crude cell extract of *A. malicum*. Values were plotted after Hanes.

under air) and turned out to be membrane-associated (up to 20% in the membrane fraction after cell disintegration with the French press). Neither cytochromes nor quinones (ubiquinone or menaquinone) could be detected in extracts of this bacterium.

5. DISCUSSION

The results presented in this paper confirm an earlier observation [3] that *Acetobacterium malicum* conserves considerably more energy from malate than from lactate fermentation, as cell yield determinations revealed. It was assumed, therefore, that these yield differences were accomplished in oxaloacetate decarboxylation to pyruvate, e.g. through a sodium-pumping oxaloacetate decarboxylase enzyme [20,21].

Assay of enzymes in crude extracts of malate as well as lactate-grown cells of *A. malicum* revealed that there was neither malate dehydrogenase nor oxaloacetate decarboxylase activity present. Also, the presence or absence of sodium ions had no influence on energy conservation with either substrate, unlike hydrogen oxidation by homoacetogens [22,23] in which sodium ions are obviously involved. Rather, L-malate was oxidized and decarboxylated in one step to pyruvate, with NAD as electron acceptor. This 'malic enzyme' was similar to other bacterial malic enzymes [24] in that its pH optimum is on the alkaline side, oxaloacetate is not decarboxylated, and the enzyme is activated by divalent cations. The new enzyme is unusual and different from others described so far by its specificity for NAD rather than for NADP, its high sensitivity towards oxygen, and its activation by preincubation with NAD. The apparent K_M for L-malate is relatively high (6.5 mM), but in the same range as e.g. with the recently described malic enzyme of *Desulfovibrio* strains [19].

These results provide evidence that the difference in conservation of metabolic energy from malate versus lactate in *A. malicum* has to be attributed to the difference of redox potentials at which electrons are released in substrate oxidation to pyruvate: whereas electrons of lactate oxidation are released at a relatively high redox potential ($E_0' = -190$ mV) in a reaction not coupled to NAD reduction, electrons of malate oxidation through malic enzyme arise at a considerably lower potential ($E_0' = -331$ mV) which allows stoichiometric reduction of NAD (-320 mV). Direct coupling of the oxidation with the decarboxylation reaction shifts the redox potential to such an extent that the released electrons can give rise to considerably more energy conservation in electron transport phosphorylation than with the lactate electrons. We do not know what the physiological acceptor of the lactate dehydrogenase electrons is; neither cytochromes nor quinones could be detected by standard assay procedures in the present study, or have been

reported in earlier publications on *Acetobacterium* spp. [1]. The redox potential difference between the two dehydrogenation reactions discussed here (−141 mV) reflects a difference of free energy change between both substrates of 27 kJ per mol (see above) which gives rise to a comparably high cell yield difference of 5–8 g per mol between both substrates. This interpretation of our data provides a further example on how decarboxylation energy can be conserved in bacterial energy metabolism.

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