Distinct Forms of Lactate Dehydrogenase Purified from Ethanol- and Lactate-producing Cells of *Clostridium thermohydrosulfuricum*

By MARJA TURUNEN,* ELKE PARKKINEN, JOHN LONDESBOROUGH
AND MATTI KORHOLA

Research Laboratories of the Finnish State Alcohol Company, Alko Ltd, POB 350,
SF-00101 Helsinki, Finland

(Received 16 February 1987; revised 1 May 1987)

Thermostable lactate dehydrogenases (EC 1.1.1.27) were purified to homogeneity from *Clostridium thermohydrosulfuricum* cells grown on starch and producing mainly ethanol (LDH_E) and from cells grown on sucrose and producing mainly lactic acid (LDH_L), and were found to be distinct isoenzymes. The two enzymes both had native M_r values close to 145×10^3 , but slightly different subunits with M_r values about 37×10^3 . LDH_L dissociated into subunits more readily. The isoelectric points were 5.0 for LDH_L and 5.2 for LDH_E. The catalytic activity of LDH_E had an almost absolute requirement for fructose 1,6-bisphosphate (FBP) at all temperatures (22-fold activation with $K_{1/2}$ 12 μ M-FBP at 65 °C, pH 6.0). LDH_L was activated by FBP only at temperatures over 40 °C (5-fold activation with $K_{1/2}$ 80 μ M-FBP at 65 °C, pH 6.0). For both enzymes the optimum temperature for pyruvate reduction in the presence of 1 mM-FBP was 70 °C and the pH optimum at 65 °C was sharp and at 5.5–6.0. FBP lowered the apparent K_m of LDH_L for pyruvate. At 50 μ M-FBP both enzymes showed a positive co-operative dependence on NADH.

INTRODUCTION

Clostridium thermohydrosulfuricum is one of the thermophilic saccharolytic species that are of considerable interest for industrial ethanol production. It ferments starch, cellobiose, sucrose and a wide variety of hexoses and pentoses yielding ethanol, carbon dioxide, lactate, acetate and hydrogen. The production of ethanol in *C. thermohydrosulfuricum* is influenced by substrate type, substrate concentration and pH (Carreira *et al.*, 1983; Parkkinen & Korhola, 1984). *C. thermohydrosulfuricum* catabolizes glucose via the Embden–Meyerhof–Parnas pathway (Zeikus *et al.*, 1981), and contains both NAD⁺- and NADP⁺-dependent alcohol dehydrogenase (ADH) activities (Lamed & Zeikus, 1981) and FBP-activated LDH (Hyun *et al.*, 1985; Germain *et al.*, 1986). The regulation of these catabolic pathways at the enzyme level is poorly understood.

In the present work we studied the activities of LDH and ADH during glucose, sucrose and starch fermentations to investigate further why sucrose fermentations yield sometimes ethanol and sometimes lactic acid as the main product. We found that ethanol-producing and lactate-producing cells contained distinct isoenzymes of LDH. Both isoenzymes were purified to homogeneity and some of their kinetic characteristics were examined.

METHODS

Bacterial strain. *Clostridium thermohydrosulfuricum* strain E-101-69 was obtained from F. Hollaus (Sugar Research Institute, Fuchsenbigl, Austria).

Cultivation conditions. Cells were grown in a complex medium containing (g l⁻¹) tryptone (Difco, 10), meat extract (Oxoid, 5), yeast extract (Difco, 5), K₂HPO₄ (1), FeSO₄·7H₂O (0.02), MgSO₄·7H₂O (0.1) and either

Abbreviations: ADH, alcohol dehydrogenase; FBP, fructose 1,6-bisphosphate; LDH, lactate dehydrogenase.

glucose, sucrose or starch (30). The starch was from Merck (starch soluble, GR 1252). The inocula were grown in 100 ml of medium containing glucose (10 g l^{-1}) in 100 ml flasks enclosed in anaerobic jars at 68°C . The fermentations were done in fermentors with working volumes in 10 l (Bioengineering) or 1.5 l (Biostat M, B. Braun). The medium was inoculated with 5% (v/v) of 12 h (late exponential phase) flask culture. Fermentor cultures were maintained with constant stirring (100 r.p.m.) and continuous gassing with oxygen-free nitrogen ($1\text{ l l}^{-1}\text{ h}^{-1}$) at 68°C . The pH was kept at the desired value, usually 6.8, with 5 M-KOH. Growth was monitored by using a Klett-Summerson colorimeter.

Measurement of substrates and products. Glucose, sucrose, L-lactic acid and acetic acid were determined enzymically with Boehringer Test Combination kits after centrifugation of the media at 10000 g for 10 min. Starch and ethanol were determined as described by Parkkinen (1986).

Preparation of cell-free extracts. Cells were harvested from 1 to 2 l of culture medium by centrifugation at 8000 g for 10 min at 4°C , washed once with 25 mM-Tris/HCl (pH 7.4) containing 2 mM-dithiothreitol and 0.1 mM-EDTA or with 50 mM-imidazole/HCl (pH 6.1) containing 0.1 mM-EDTA (IE buffer, used for enzyme purification), suspended in the same buffer [$2\text{ ml (g wet wt cells)}^{-1}$] and stored at -20°C . The cells were ruptured by two passes through an X-press (Biox) at -25°C and then centrifuged at 25000 g for 45 min at 4°C . Protein was estimated by the method of Bradford (1976) with bovine serum albumin as standard.

Enzyme purification. Steps were done at 0 to 10°C unless otherwise stated. Yields (U) and specific activities [$\text{U (mg protein)}^{-1}$] measured at 40°C are shown for preparations of LDH_L from 6.6 g wet wt cells from a sucrose fermentation that produced $16\text{ g lactic acid l}^{-1}$ and $0.6\text{ g ethanol l}^{-1}$, and of LDH_E from 25 g wet wt cells from a starch fermentation that produced $0.6\text{ g lactic acid l}^{-1}$ and $10\text{ g ethanol l}^{-1}$.

Streptomycin sulphate precipitation. The cell-free extracts (LDH_L $4100\text{ U at }34\text{ U mg}^{-1}$; LDH_E $1600\text{ U at }2.8\text{ U mg}^{-1}$) were mixed with 1 ml streptomycin sulphate (25 mg ml^{-1} in IE buffer) per 100 mg protein, and after 30 min centrifuged at 17000 g for 30 min. Streptomycin sulphate was removed from the supernatant by ultrafiltration using an Amicon PM 10 membrane. (LDH_L $3100\text{ U at }39\text{ U mg}^{-1}$; LDH_E $1800\text{ U at }4.0\text{ U mg}^{-1}$).

Affinity chromatography. The supernatant (about 13 ml) was applied to a Blue Sepharose CL-6B column ($1.5\text{ cm} \times 30\text{ cm}$) equilibrated with IE buffer. The column was washed with IE buffer and then developed with a linear gradient containing 0 to 5 mM-NADH in 100 ml IE buffer at a flow of 4.6 ml h^{-1} . Pooled active fractions were concentrated to about 2 ml and the buffer was changed to 25 mM-bis-Tris/HCl (pH 6.0) by using a Centricon microconcentrator (cutoff 30000). (LDH_L $1700\text{ U at }560\text{ U mg}^{-1}$; LDH_E $850\text{ U at }200\text{ U mg}^{-1}$).

Chromatofocusing. This was done at about 23°C on a Mono P HR 5/20 FPLC column (Pharmacia) equilibrated with 25 mM-bis-Tris/HCl (pH 6.0). Proteins were eluted at 30 ml h^{-1} with a buffer (adjusted to pH 3.3 with HCl) containing in 100 ml 5 ml Polybuffer 74 and 1 ml Pharmalyte 2.5-5 (Pharmacia). The most active fractions were pooled. For LDH_E a second run on Mono P was done with a pH gradient of 5.6-3.9. (LDH_L $1010\text{ U at }590\text{ U mg}^{-1}$; LDH_E $240\text{ U at }420\text{ U mg}^{-1}$).

Gel filtration. The pooled fractions were transferred to 20 mM-sodium phosphate (pH 6.8) containing 180 mM-NaCl by using a Centricon-30 microconcentrator and $200\text{ }\mu\text{l}$ portions were run through a calibrated Superose 12 HR 10/30 FPLC column at about 23°C and 30 ml h^{-1} . (LDH_L $620\text{ U at }520\text{ U mg}^{-1}$; LDH_E $180\text{ U at }700\text{ U mg}^{-1}$).

Enzyme assays. Standard enzyme activities were measured at 40°C by the spectrophotometric methods used by Lamed & Zeikus (1980) with slight modifications. The assays were done in the presence of air with the exception of NAD^+ -dependent ADH activity which was determined anaerobically (reaction mixtures were flushed with nitrogen before starting the reaction). NADH oxidase activity was negligible with the amounts of crude extract used. Assay mixtures for ADH (EC 1.1.1.1 and EC 1.1.1.2) contained in 3 ml: 0.1 M-Tris/HCl (pH 7.8), 2 mM-dithiothreitol, 0.25 mM-NAD(P)H and 25 mM-acetaldehyde. Standard assay mixture for NAD^+ -dependent LDH (EC 1.1.1.27) contained in 3 ml: 0.1 M-imidazole/HCl (pH 6.4 at 40°C), 0.25 mM-NADH, 10 mM-sodium pyruvate and, when indicated, 1 mM-FBP. The reaction was started by adding pyruvate. The assay mixture for the oxidation of lactate contained in 3 ml: 100 mM-imidazole/HCl (pH 8.0), 0.75 mM- NAD^+ , 100 mM-lithium L-lactate or lithium D-lactate with or without 1 mM-FBP. One unit (U) of each enzyme catalyses the oxidation of $1\text{ }\mu\text{mol NAD(P)H min}^{-1}$.

Electrophoresis and gel staining. Discontinuous polyacrylamide gel electrophoresis of native proteins was done at 10°C using the buffer system described by Davis (1964) with 12.5% (w/v) polyacrylamide in the separation gel. After electrophoresis NAD^+ -dependent LDH activity was located by modifications of methods described by Garvie (1969). In the direct staining method the gel was incubated for 15 min at 50°C in a solution containing 50 mg NAD^+ , 10 mg nitro blue tetrazolium, 2 mg phenazine methosulphate and 2 mmol lithium D-lactate or 4 mmol sodium DL-lactate in 100 ml 0.1 M-Tris/HCl (pH 8.3). In the indirect method the gel was placed in a solution containing 100 mg NADH, 1 mmol pyruvate and (where indicated) 0.1 mmol FBP in 100 ml 0.1 M-imidazole/HCl (pH 6.2) for 15 min at 50°C . The reaction mixture was removed and the gel stained in a solution containing 50 mg nitro blue tetrazolium and 4 mg phenazine methosulphate in 100 ml 0.1 M-imidazole/HCl (pH 6.2).

Gel electrophoresis of native proteins and M_r standards (Pharmacia) on 4–30% (w/v) polyacrylamide gradients was for 2600 volt hours at 10 °C on PAA 4/30 gels (Pharmacia) in 0.09 M-Tris, 0.08 M-boric acid, 0.0025 M- Na_2EDTA (pH 8.4). The gels were fixed with 10% (w/v) sulphosalicylic acid and stained with Coomassie blue.

PAGE in the presence of SDS was done by the method of Laemmli (1970) with a linear gradient from 13% to 7% (w/v) acrylamide in the separator gel and a 3.75% (w/v) stacking gel. Gels were fixed with 50% (w/v) trichloroacetic acid and stained with Coomassie blue.

Isoelectric focusing. Isoelectric points were determined by isoelectric focusing on ready made Ampholine thin-layer gels (LKB, pH range 4.0–6.5). Protein pI standards (Pharmacia) included soybean trypsin inhibitor (4.55) and β -lactoglobulin A (5.20). The gels were run, fixed and stained according to the manufacturer's instructions.

RESULTS

Fermentations and enzymes involved in pyruvate catabolism

The mass ratio of the main fermentation products ethanol:L-lactate:acetate was always close to 10:1:1 after growth on 3% (w/v) glucose and 10:0.1:1 after growth on 3% (w/v) soluble starch at pH 6.8. However, after growth on 3% (w/v) sucrose this ratio varied between 10:0.5:0.5 and 0.1:10:0.1 (data not shown). The causes of this variability during sucrose fermentations are still unclear, though some factors have been identified (Parkkinen & Turunen, 1986). Extensive lactic acid production was followed by significant cell lysis.

Fig. 1 shows the product formation kinetics and the enzyme levels during fermentations with different carbon sources. The activity of LDH in samples from glucose and starch fermentations was increased about 10-fold by addition of 1 mM-FBP to the reaction mixture. LDH activity was very high at the end of exponential growth on sucrose at pH 7.2, and in this case added FBP had little effect on the LDH activity measured at 40 °C. The activity of NADP⁺-linked ADH was usually about 10-fold that of NAD⁺-linked ADH. The activity of NADP⁺-linked ADH was markedly decreased during sucrose fermentation when lactic acid was the main product.

Fig. 1. Activities of LDH and ADH and kinetics of product formation during fermentations with different carbon sources. Fermentations containing 10.13% glucose pH 6.8 (A), 3% starch pH 6.8 (B), 3% sucrose pH 6.8 (C) or 3% sucrose pH 7.2 (D) were done as described in Methods. ●, L-Lactate; ○, acetate; △, ethanol; ◆, carbon source; ◇, growth; ▼, LDH assayed at 40 °C with 1 mM-FBP; ▽, LDH assayed at 40 °C without added FBP; □, NADP⁺-linked ADH; ■, NAD⁺-linked ADH. Samples for enzyme assays were taken at the points (I, II, III and IV) shown by arrows.

Fig. 2. Distribution of LDH activity after discontinuous PAGE of cell-free extracts. Lanes 1, 3, 5 and 7, extracts of lactate-producing cells grown on sucrose (DI in Fig. 1) (1.1 μ g, 1.1 μ g, 1.1 μ g and 33.6 μ g protein respectively); lanes 2, 4 and 6, extracts of ethanol-producing cells grown on starch (BII in Fig. 1) (15.3 μ g, 7.7 μ g and 15.3 μ g protein respectively); lane 8, extract of lactate- and ethanol-producing cells (CII in Fig. 1) (2.8 μ g protein). Before electrophoresis the samples were dialysed against 25 mM-Tris/HCl (pH 7.4) containing 2 mM-dithiothreitol and 0.1 mM-EDTA. After electrophoresis the gel was cut into strips which were then stained for LDH activity as described in Methods. Lanes 1 to 6 and 8 were stained by the indirect method, lanes 1, 2 and 8 with FBP, lanes 3 and 4 without FBP and lanes 5 and 6 without FBP and without pyruvate. Lane 7 was stained by the direct method with sodium DL-lactate as substrate and without FBP. The origin is at the top.

After electrophoresis of dialysed cell extracts LDH activity was detected in two different areas (Fig. 2). A slow-moving broad band (c, Fig. 2) was observed only when FBP was present in the staining mixture. This band was not detected in the extract of cells grown on sucrose and producing mainly lactic acid. A faster-moving and sharper band (a, Fig. 2) that did not require FBP was detected only in extracts of lactate-producing cells. This band could also be detected by direct staining with DL-lactate (Fig. 2) or with L-lactate but not with D-lactate (not shown). Both LDH bands were observed during electrophoresis of extracts of sucrose-grown cells that produced ethanol and lactic acid in nearly equal amounts (lane 8, Fig. 2). The sharp bands (b and d, Fig. 2) that also appeared in the absence of pyruvate may be NADH oxidases.

Enzyme purification

These results suggested that *C. thermohydrosulfuricum* might contain two different LDH enzymes: LDH_E during fermentations producing ethanol, and LDH_L during sucrose fermentations in which lactate was the main product. To test this hypothesis, LDH was purified both from cells producing mainly ethanol and from cells producing mainly lactic acid (corresponding to fermentations B and D respectively in Fig. 1).

Fig. 3. SDS-PAGE of the purified LDH preparations. Lane 1, 7.1 μg LDH_E; lane 2, 1.2 μg LDH_E; lane 3, 1.2 μg LDH_E and 2.1 μg LDH_L; lane 4, 2.1 μg LDH_L; lane 5, 12.6 μg LDH_L. Electrophoresis and gel staining were done as described in Methods. M_r standards are indicated on the left.

Representative purifications are described in Methods. Preparation of homogeneous enzyme required a 15-fold purification from the lactate-producing cells but 250-fold purification from ethanol-producing cells. However, LDH activity behaved similarly in both purifications. It eluted from the Blue Sepharose at about 0.5 mM-NADH. In chromatofocusing, LDH_L eluted at a slightly lower pH (activity peak at pH 4.4) than LDH_E (activity peak at pH 4.7). The maximum specific activities at 40 °C agreed within 15% but LDH_L then lost some activity in the final gel-filtration.

Physical properties of the purified enzymes

Both enzymes exhibited native M_r values (at room temperature, pH 6.8) of 145×10^3 during the gel-filtration step of the purification. Both migrated as single polypeptides with M_r values of about 37×10^3 in SDS-PAGE (Fig. 3). This indicates that both enzymes are tetramers. LDH_L moved slightly slower either because it is bigger (by about 2×10^3) or because of some other difference in primary structure. A mixture of the purified enzymes could be resolved by isoelectric focusing: LDH_L focused at pH 5.0 and LDH_E as a single band at pH 5.2 (not shown). This agrees with the behaviour during chromatofocusing. When the native purified enzymes were subjected to gel electrophoresis in the same conditions as used for crude extracts, LDH_E

Fig 4. Effect of temperature on the activities of the LDH enzymes (A, LDH_E; B, LDH_L) in the presence and absence of 1 mM-FBP. Reactions were started by addition of the purified enzymes (5–20 μ l) to assay mixtures at the indicated temperatures with (●) and without (○) 1 mM-FBP.

travelled as a slow broad band corresponding to band (c) in Fig. 2 and LDH_L as a faster sharper band corresponding to band (a) in Fig. 2 (data not shown).

These large mobility differences suggested that although the two enzymes have similar pI and M_r values at room temperature and pH 6.8, their M_r values must differ under the conditions of the discontinuous electrophoresis, where the pH reaches 9.5 (Ornstein, 1964) during operation. Indeed, electrophoresis at pH 8.4 through polyacrylamide gradients showed that LDH_L dissociates, probably to a monomer (observed $M_r < 67 \times 10^3$), whereas LDH_E migrated as at least three separate bands with apparent M_r values between 110 and 130×10^3 (not shown).

General catalytic properties of the purified enzymes

The optimum temperature for pyruvate reduction in the presence of FBP was 70 °C for both enzymes (Fig. 4). The data in Fig. 4 are linear initial reaction rates, so the decreases in rate above 70 °C appear to reflect real decreases in catalytic efficiency (rather than denaturation of the enzyme during measurement). LDH_E was highly activated by FBP at all temperatures (from 7-fold at 30 °C to 47-fold at 70 °C). LDH_L was activated by FBP only above 40 °C (8-fold at 70 °C). Because NADH became unstable at and above 70 °C subsequent kinetic experiments were done at 65 °C, this being as close as was practical to the optimum growth temperature of *C. thermohydrosulfuricum*. No measurable decrease in absorbance at 340 nm was observed when NADPH was substituted for NADH at 65 °C. The pH dependence was determined in 100 mM-sodium acetate/acetic acid (pH 4.0–6.0) and imidazole/HCl (pH 6.0–8.0) buffers at 65 °C in the presence of 1 mM-FBP (data not shown). The pH optimum of both enzymes was at pH 5.5–6.0 and activity decreased rapidly on either side of the optimum. No activity was detectable at pH 8 or above.

Although the oxidation of L-lactate could be demonstrated by the gel staining method with LDH_L, the reaction was too slow to be detected with either enzyme by spectrophotometric assay at 340 nm (50 or 65 °C).

Inorganic phosphate (pH 6.0 at 65 °C) had opposite effects on the two enzymes. It strongly activated LDH_E in the absence of FBP (activations of 2-, 8- and 15-fold at 25, 100 and 200 mM-sodium phosphate), but had no effect in the presence of 1 mM-FBP. However, at concentrations up to 80 mM it increasingly inhibited LDH_L both in the presence (80% inhibition) and absence (55% inhibition) of FBP; between 80 mM and 200 mM the activity was partially restored. Similar concentrations of NaCl caused inhibition (reaching about 40% at 250 mM-NaCl) of both enzymes in both the presence and absence of FBP, suggesting that the activations by sodium phosphate are specific effects.

Dependence of catalytic activity on FBP and substrate concentrations

At pH 6.0 and 65 °C and high pyruvate (10 mM) and NADH (0.25 mM) concentrations the rate of LDH_E activity was increased 22-fold with a half-maximum effect at 12 μ M-FBP, whereas

Fig. 5

Fig. 6

Fig. 5. Dependence of catalytic activity on NADH at 65 °C, pH 6.0. Activities of LDH_E (open symbols) and LDH_L (closed symbols) were measured as described in Methods at 10 mM-pyruvate and either 1 mM (○, ●) or 0.05 mM (□, ■) FBP. The NADH concentration was varied between 20 and 250 μM (NADH concentration can be obtained by joining the markers on the curved scale to the origin with a straight edge). For each enzyme the initial velocity (*v*) was set to be 100% at 250 μM-NADH and 1 mM-FBP.

Fig. 6. Dependence of catalytic activity on pyruvate at 65 °C, pH 6.0. Activities of LDH_E (open symbols) and LDH_L (closed symbols) were measured as described in Methods at 250 μM-NADH and either 1 mM (○, ●) or 0.05 mM (□, ■) FBP. The pyruvate concentration was varied between 0.15 and 30 mM (the curved pyruvate scale is read as described in Fig. 5). For each enzyme the initial velocity (*v*) was set to be 100% at 10 mM-pyruvate and 1 mM-FBP.

LDH_L was insensitive up to 10 μM-FBP and then showed a 4-fold increase in rate with a half-maximum effect at 80 μM-FBP (not shown). Experiments with crude extracts of starch-grown cells at 0.25 mM-NADH and 40 °C showed that the FBP requirement of LDH_E was greatly dependent on both pH and pyruvate concentration. In these conditions, the concentrations of FBP needed for half-maximum stimulation at 10 mM- and 1 mM-pyruvate, respectively, were 3 μM and 20 μM at pH 6.0 but 40 μM and 260 μM at pH 7.0.

NADH dependence at 10 mM-pyruvate is shown in Fig 5. At a saturating FBP concentration (1 mM) the enzymes had similar apparent *K_m* values (LDH_E 80 μM; LDH_L 130 μM). At a lower FBP concentration (0.05 mM) both enzymes showed a positively co-operative dependence on NADH, but this co-operativity was strong for LDH_E and weak for LDH_L. *V_{max}* values could not be accurately estimated because of problems in measuring rates above 0.4 mM-NADH, but reasonable extrapolations (broken lines in Fig. 5) suggest that decreasing the FBP concentration also decreased the apparent *V_{max}* values.

Pyruvate dependence at 0.25 mM-NADH is shown in Fig 6. At 1 mM-FBP the enzymes had similar *K_m* values (LDH_E 1.5 mM; LDH_L 1.3 mM). Decreasing the FBP concentration to 0.05 mM increased the apparent *K_m* for LDH_L by 10-fold to 12 mM with little or no decrease in *V_{max}*. For LDH_E the apparent *K_m* for pyruvate at 0.05 mM-FBP was still similar (2 mM) to that observed at 1 mM-FBP, and *V_{max}* was only slightly smaller. No evidence for co-operative dependence on pyruvate was found for either enzyme. However, concentrations of pyruvate between 10 and 30 mM caused a marked substrate inhibition of LDH_E but not LDH_L.

The following sugar phosphates were tested with pure enzymes at 65 °C for the ability to substitute for FBP: mannose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, glucose 1,6-bisphosphate, fructose 1-phosphate and fructose 6-phosphate. Only glucose 1,6-bisphosphate activated LDH_E; at 1 mM it gave 55% of the activity obtained with FBP. Neither glucose 1,6-bisphosphate nor the other sugar phosphates tested had any effect on the LDH_L activity.

DISCUSSION

Lactic acid production by *C. thermohydrosulfuricum* increases at the expense of ethanol production when the initial concentration of glucose or starch is increased (Parkkinen, 1986; unpublished observations cited by Germain *et al.*, 1986). So far, however, we have found yields of lactic acid to approach the theoretical maximum only in certain fermentations with sucrose as carbon source. Several authors (e.g., Hyun *et al.*, 1985; Germain *et al.*, 1986) have investigated the enzymes catabolizing pyruvate in *C. thermohydrosulfuricum* with a view to understanding how the balance between ethanol and lactic acid is controlled. Our results (Fig. 1) are consistent with the notion that the production of lactic acid rather than ethanol in some sucrose fermentations is caused by a large increase in the amount of LDH in the cells. We also found that NADP⁺-dependent ADH levels were drastically lower in cells producing lactic acid than in cells producing ethanol. The metabolic roles of the NAD⁺- and NADP⁺-linked ADHs in *C. thermohydrosulfuricum* are not understood. The NADP⁺-linked ADH is reversible (Lamed & Zeikus, 1981) and so might operate *in vivo* to produce ethanol provided that NADPH can be regenerated. In this case, the smaller amount of this enzyme in cells producing lactate may also have contributed to their decreased formation of ethanol. Unlike Hyun *et al.* (1985), we were unable to detect NADP⁺-dependent LDH in *C. thermohydrosulfuricum*, at least not in aerobic assay conditions. The cause of this discrepancy is not known. Hyun *et al.* (1985) used a different bacterial strain (39E) and anaerobic assay conditions.

Our results suggest that distinct isoenzymes of LDH, LDH_E and LDH_L respectively, are present in cells producing mainly ethanol or mainly lactic acid. They may be products of different genes or be related by post-translational modifications, such as proteolysis or phosphorylation. LDH_E dissociates less readily than LDH_L into subunits (see Results), which may be connected with the differences in catalytic properties, as seems to be the case with LDH from *Streptococcus uberis* (Williams & Andrews, 1986). *C. thermohydrosulfuricum* is a thermophilic bacterium able to grow at temperatures between 40 and 78 °C, with an optimum at 68 °C (Wiegel *et al.*, 1979). Fig. 4 emphasizes the importance of measuring the catalytic properties of its enzymes at physiological temperatures. At 40 °C only LDH_E was activated by FBP. However, both isoenzymes showed a true temperature optimum at about 70 °C in the presence of 1 mM-FBP. The degree of activation (47-fold for LDH_E and 8-fold for LDH_L at 10 mM-pyruvate and 0.25 mM-NADH) is much higher than that (1.4-fold at 8 mM-pyruvate and 0.1 mM-NADH) reported by Germain *et al.* (1986) at an unknown temperature and in 0.1 M-phosphate, pH 6.9. The use of phosphate may have contributed to the low degree of activation observed by these authors, because at 65 °C 0.1 M-phosphate inhibited LDH_L, activated LDH_E and decreased the response of both enzymes to FBP (see Results). The ability of phosphate to inhibit LDH_L and to mimic the activation of LDH_E by FBP is interesting, because high yields of lactic acid are never obtained in fermentations of sucrose in media containing 0.1 M-phosphate (Parkkinen & Turunen, 1986). Which isoenzyme is present in these cells and the intracellular concentration of phosphate are not known. Intracellular phosphate can reach 75 mM in *Streptococcus faecalis* (Harold & Spitz, 1975). The strong activation of LDH_E by phosphate is unusual (see Garvie, 1980). More commonly the FBP-dependent enzymes such as that from *Lactobacillus casei* (Holland & Pritchard, 1975; Gordon & Doelle, 1976) are inhibited.

In agreement with studies on crude enzyme by Germain *et al.* (1986), we found that increasing the concentration of FBP decreased the K_m for pyruvate, greatly for LDH_L and slightly for LDH_E (Fig. 6), and increased the affinity of both isozymes for NADH. However, the dependence on NADH became positively co-operative at the lower FBP concentration. In common with the most FBP-dependent LDHs (Garvie, 1980) both isoenzymes had a high specificity for FBP. However, glucose 1,6-bisphosphate activated LDH_E, as has also been shown for the enzyme from *Streptococcus cremoris* (Thomas, 1975).

The physiological significance of the effects of FBP is difficult to assess. Our results imply that LDH_E and LDH_L would both be saturated at the intracellular concentrations of FBP [1.7 to 13.4 µmol (g dry wt)⁻¹] reported by Germain *et al.* (1986). We have found somewhat smaller FBP concentrations [between 2 and <0.04 µmol (g fresh wt)⁻¹] but they did not correlate with lactic acid production (M. Turunen, unpublished results). Also, at least at 40 °C the

concentration of FBP required for half-maximum activation was higher at pH 7.0 than pH 6.0 and higher at 1 mM-pyruvate than at 10 mM-pyruvate. Clearly, accurate information about intracellular pH and metabolite concentrations is needed before the kinetic data for LDH can be fully interpreted.

REFERENCES

- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.
- CARREIRA, L. H., WIEGEL, J. & LJUNGDAHL, L. G. (1983). Production of ethanol from biopolymers by anaerobic, thermophilic and extreme thermophilic bacteria: I. regulation of carbohydrate utilization in mutants of *Thermoanaerobium ethanolicus*. In *Biotechnology and Bioengineering Symposium 13*, pp. 183–191. Edited by C. D. Scott. New York: John Wiley.
- DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.
- GARVIE, E. I. (1969). Lactic dehydrogenases of strains of the genus *Leuconostoc*. *Journal of General Microbiology* **58**, 85–94.
- GARVIE, E. I. (1980). Bacterial lactate dehydrogenases. *Microbiological Reviews* **44**, 106–139.
- GERMAIN, P., TOUKOUREOU, F. & DONADUZZI, L. (1986). Ethanol production by anaerobic thermophilic bacteria: regulation of lactate dehydrogenase activity in *Clostridium thermohydrosulfuricum*. *Applied Microbiology and Biotechnology* **24**, 300–305.
- GORDON, G. L. & DOELLE, H. W. (1976). Purification, properties and immunological relationship of L(+)-lactate dehydrogenase from *Lactobacillus casei*. *European Journal of Biochemistry* **67**, 543–555.
- HAROLD, F. M. & SPITZ, E. (1975). Accumulation of arsenate, phosphate, and aspartate by *Streptococcus faecalis*. *Journal of Bacteriology* **122**, 266–277.
- HOLLAND, R. & PRITCHARD, G. G. (1975). Regulation of the L-lactate dehydrogenase from *Lactobacillus casei* by fructose 1,6-diphosphate and metal ions. *Journal of Bacteriology* **121**, 777–784.
- HYUN, H. H., SHEN, G.-J. & ZEIKUS, J. G. (1985). Differential amylosaccharide metabolism of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum*. *Journal of Bacteriology* **164**, 1153–1161.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAMED, R. & ZEIKUS, J. G. (1980). Glucose fermentation pathway of *Thermoanaerobium brockii*. *Journal of Bacteriology* **141**, 1251–1257.
- LAMED, R. J. & ZEIKUS, J. G. (1981). Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. *Biochemical Journal* **195**, 183–190.
- ORNSTEIN, L. (1964). Disc electrophoresis. I. Background and theory. *Annals of the New York Academy of Sciences* **121**, 321–349.
- PARKKINEN, E. (1986). Conversion of starch into ethanol by *Clostridium thermohydrosulfuricum*. *Applied Microbiology and Biotechnology* **25**, 213–219.
- PARKKINEN, E. & KORHOLA, M. (1984). Starch fermentation by *Clostridium thermohydrosulfuricum*. In *Third European Congress on Biotechnology*, vol. II, pp. 223–230. Weinheim: Verlag Chemie.
- PARKKINEN, E. & TURUNEN, M. (1986). Fermentation of sucrose by *Clostridium thermohydrosulfuricum*. In *Abstracts of the 14th International Congress of Microbiology ('Microbe 86')* (September 1986, Manchester, UK), p. 204.
- THOMAS, T. D. (1975). Tagatose 1,6-diphosphate activation of lactate dehydrogenase from *Streptococcus cremoris*. *Biochemical and Biophysical Research Communications* **63**, 1035–1042.
- WIEGEL, J., LJUNGDAHL, L. G. & RAWSON, J. R. (1979). Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. *Journal of Bacteriology* **139**, 800–810.
- WILLIAMS, R. A. & ANDREWS, P. (1986). Purification of the fructose 1,6-bisphosphate-dependent lactate dehydrogenase from *Streptococcus uberis* and an investigation of its existence in different forms. *Biochemical Journal* **236**, 721–727.
- ZEIKUS, J. G., BEN-BASSAT, A. & LAMED, R. J. (1981). Thermophilic ethanol fermentations. In *Trends in the Biology of Fermentations in Fuels and Chemicals*, pp. 441–461. Edited by A. Hollander. New York: Plenum Press.