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CHARACTERIZATION OF THE FRUCTOSE 1,6-BISPHOSPHATE-ACTIVATED, L(+)-LACTATE DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS*

FRANK O. BRYANT*

Department of Biochemistry, University of Georgia, Athens, GA 30602

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The L(+)-lactate dehydrogenase from *Thermoanaerobacter ethanolicus* wt was purified to a final specific activity of 598 μmol pyruvate reduced per min per mg of protein. The specific activity of the pure enzyme with L(+)-lactate was 0.79 units per mg of protein. The M_r of the native enzyme was 134,000 containing a single subunit type of M_r 33,500 indicating an apparent tetrameric structure. The L(+)-lactate dehydrogenase was activated by fructose 1,6-bisphosphate in a cooperative manner affecting V_{max} and K_m values. The activity of the enzyme was also effected by pH, pyruvate and NADH. The K_m for NADH at pH 6.0 was 0.05 mM and the V_{max} for pyruvate reduction at pH 6.0 was 1082 units per mg in the presence of 1 mM fructose 1,6-bisphosphate. The enzyme was inhibited by NADPH, displaying an uncompetitive pattern. This pattern indicated that NADPH was a negative modifier of the enzyme. The role of L(+)-lactate dehydrogenase in controlling the end products of fermentation is discussed.

KEY WORDS: *Thermoanaerobacter ethanolicus*, L(+)-lactate dehydrogenase, fructose 1,6-bisphosphate, NADH, NADPH.

INTRODUCTION

A prospect for the development of alternate energy and chemical feedstock resources is the conversion of biomass to ethanol.[1,2] Ethanol as a fuel, is highly combustible and adaptable to use in motorized vehicles as exemplified by gasohol. Ethanol is non-polluting compared to petroleum fuels which generate particulate matter, ozone, sulfuroxides, nitrogenoxides, carbon monoxide, CO_2 and uncombusted hydrocarbons.[3]

In the recent past, several anaerobic, thermophilic bacteria were isolated that ferment biomass to ethanol or other useful chemicals offering significant advantages for industrial fermentation processes.[4] *Thermoanaerobacter ethanolicus* (ATCC 31550) is a thermophilic, nonsporulating, anaerobic bacterium which ferments hexoses, pentoses, disaccharides, starch, pectin, pullulan and pyruvate.[5–7] The major fermentation products are ethanol and CO_2. Minor products are lactate, acetate and H_2. The bacterium grows well at substrate concentrations in excess of 1% (w/v), but rarely ferments more substrate than the amount that yields an ethanol concentration of 80 mM.[8] At substrate concentrations in excess of 1% a small shift in the fermentation is observed;[9] lactate and acetate production increase and ethanol production decreases. These observations indicate the presence of a mechanism regulating fermentation by *T. ethanolicus*.

*Current address: TA1 c/o USEPA, College Station Rd., Athens, GA 30613, USA.

To obtain an understanding of the mechanisms that control product formation, study of the enzymes involved in the production of ethanol, acetate and lactate was undertaken. The purification of two NADP-dependent alcohol dehydrogenases from *T. ethanolicus* was reported.[10] Both enzymes catalyze the reduction of acetaldehyde to ethanol. However, one enzyme is only active with primary alcohols while the other is a secondary alcohol dehydrogenase displaying little activity with primary alcohols. Other proteins isolated from *T. ethanolicus* are an ATP-dependent acetate kinase, a rubredoxin and two ferredoxins.[6] We report here the purification and properties of the NADH-linked, fructose 1,6-bisophosphate [Fru(1,6)P_2]-activated L(+)-lactate dehydrogenase (L(+)-LDH) that catalyzes the reduction of pyruvate to L(+)-lactate.

MATERIALS AND METHODS

Chemicals and Reagents

Pyruvate, Fru(1,6)P_2, NAD, NADP, ATP, ADP, GTP and GDP were obtained from Sigma Chemical Co. (St. Louis, Mo). Diethylaminoethyl Cellulose (DE 23) was obtained from Whatman Ltd. (Maidstone, Kent, England). Matrex Gel Blue A was obtained from Amicon Corp. (Lexington, MA). Hydroxyapatite was obtained from Bio-Rad Laboratories (Richmond, CA). Ultrogel AcA 34 was obtained from LKB (Broma, Sweden). All other chemicals were reagent grade.

Growth of the Bacterium

Thermoanaerobacter ethanolicus wt (JW 200 wt) was used throughout this study. The bacterium was cultivated as previously described[6] under strict anaerobic conditions in a 500 liter New Brunswick fermentor. The medium was constantly stirred and continuous N_2 gassing was maintained during growth. The temperature of the vessel was held at 50°C. Cells were harvested in late logarithmic growth phase and collected with a Sharpless centrifuge. In excess of 1 Kg of the bacterial cells were obtained. The cells were stored in −20°C until used.

Enzyme Assay

Lactate dehydrogenase was assayed spectrophotometrically at 50°C by following the oxidation of NADH by pyruvate at 340 nm (extinction = 6.22 mM^{-1} cm^{-1}). The assay mixture contained pyruvate, 2 mM; NADH, 0.2 mM; Fru(1,6)P_2, 1 mM; 2(*N*-morpholino)ethanesulfonic acid (MES)-KOH, 100 mM pH 6.0. It should be noted that the concentration of pyruvate and NADH in the assay were lower than required for zero-order kinetics since both NADH and pyruvate were inhibitory at high concentrations. Cuvettes containing 1 ml of assay mixture were brought to the assay temperature and then the reaction was started by the addition of enzyme. The reduction of NAD by lactate was assayed using a mixture containing L(+)-lactate, 1 M; NAD^+, 1.25 mM and 100 mM Tris buffer pH 9.5. One unit is defined as the amount of enzyme that oxidizes or reduces one μmol per min of NADH or NAD^+, respectively. Specific activity is units per mg of protein. Protein was estimated using the Rose Bengal dye method of Elliot and Brewer[11] with bovine serum albumin as the standard.

Gel Electrophoresis

Analytical polyacrylamide disc gel electrophoresis at pH 8.9 was performed as described by Brewer and Ashworth.[12] Protein staining was done with 0.4% Coomassie Brilliant Blue G-250 in 3.5% (w/v) perchloric acid. Activity staining was done essentially as described by Hensel *et al.*[13] as follows. When the reaction was in the direction of pyruvate to lactate, the gels were first incubated in 10 ml of 100 mM MES-KOH pH 6.0 containing pyruvate, 15 mg; NADH, 1.5 mg and Fru(1,6)P_2, 4.0 mg for 30 min. They were then transferred to 10 ml of a solution of 100 mM Tris-HCl pH 9.0 containing nitro blue tetrazolium (NBT), 15 mg and phenazine methosulfate (PMS), 1 mg and incubated for 1 hour. To determine the oxidation of lactate to pyruvate, the gels were incubated for about 6 h in 10 ml of Tris buffer pH 9.5 containing NAD^+, 10 mg; L(+)- or D(−)-lactate, 500 mg; NBT, 10 mg and PMS, 1 mg. All gels were then rinsed and stored in distilled water.

Molecular Weight Determination

A column (1.5 cm × 75 cm) containing Ultrogel AcA 34 equilibrated with 100 mM Tris-HCl pH 7.6 and 2 mM dithiothreritol (DTT) was used to estimate molecular weight.[14] SDS gel electrophoresis was used to estimate subunit molecular weight.[15]

Purification of Lactate Dehydrogenase

The purification of lactate dehydrogenase from *T. ethanolicus* is summaried in Table I.

(*i*) *Cell extract* Frozen cells (60 g wet weight) were suspended in 180 ml of 20 mM Tris-HCl, pH 7.6 and passed through a French pressure cell at 12,000 psi. The suspension was centrifuged at 60,000 × g for 60 min at 15°C.

(*ii*) *Heat treatment* The supernatant was collected and placed in a 70°C water bath for 1 h. The heated extract was centrifuged for 30 min at 15,000 × g at 15°C. The enzyme activity was recovered in the supernatant.

(*iii*) *Matrex Gel Blue A affinity chromatography* A column (1.8 cm × 14 cm) of Matrex Gel Blue A was equilibrated with five volumes of 10 mM Tris-HCl, pH 7.6 containing 2 mM DTT. The supernatant solution from the previous step was applied to the column. The enzyme was eluted with the equilibrating buffer.

TABLE I
Purification of L(+)-lactate dehydrogenase from *T. ethanolicus*

	Step	Protein (mg)	Units	Sp. Act.
(I)	Crude Extract	2319	3845	1.45
(II)	Heat Treatment	1343	3811	3.58
(III)	Matrex Gel Blue A	222	2024	9.15
(IV)	Hydroxyapatite	26.6	1481	55.7
(V)	Ultrogel AcA 34	7.8	1133	145.4
(VI)	Matrex Gel Blue A	2.4	739	312.5
(VII)	DEAE 32 Cellulose	0.6	359	598.1

Units are μmoles of NADH oxidized per min. Sp. Act. is units per mg of protein.

Fractions of 5 ml were collected and those with activities between 20 and 30 units mg^{-1} were pooled.

(*iv*) *Hydroxyapatite chromatography* A hydroxyapatite column (2.75 cm × 4.5 cm) was equilibrated with 4 volumes of 20 mM Tris-HCl, pH 7.6 containing 2 mM DTT. The pooled fractions from the previous step were applied to the column and washed with 4 volumes of 20 mM potassium phosphate, pH 7.0 containing 2 mM DTT. The lactate dehydrogenase was eluted with 200 ml of 60 mM phosphate, pH 7.0 containing 2 mM DTT. The active eluate was concentrated by ultrafiltration on a PM 10 membrane to 20 ml.

(*v*) *Ultrogel AcA 34 gel filtration* A column (2.75 cm × 100 cm) of Ultrogel AcA 34 was equilibrated with two column volumes of 10 mM Tris-HCl, pH 7.6 containing 2 mM DTT. The concentrated protein solution was applied to the column. The enzyme was eluted with the equilibrating solution. Fractions of 17 ml were collected and those containing 50 units mg^{-1} or greater were pooled.

(*vi*) *Matrex Gel Blue A affinity chromatography* The active fractions from step (v) were applied to the Matrex Gel Blue A affinity column. The enzyme was eluted as previously described (step (iii)). However, only those fractions containing greater than 200 units mg^{-1}, were pooled for the final purification step.

(*vii*) *DEAE 32 ion exchange chromatography* A column (1.7 cm × 3.5 cm) containing DEAE 32 was equilibrated with 100 ml of 20 mM Tris-HCl, pH 7.6 containing DTT. The pooled fractions containing the enzyme from step (vi) were applied to the column. The column was washed with 100 ml of the equilibrating solution and then 100 ml of the equilibrating solution containing 125 mM NaCl was applied to the column. The lactate dehydrogenase was eluted using 200 ml of the equilibrating solution containing 135 mM NaCl. The enzyme solution was concentrated by ultrafiltration on a PM 10 membrane to 9 ml.

RESULTS

Enzyme Purification

The L(+)-lactate hydrogenase from *T. ethanolicus* was purified using the steps shown in Table I. The final specific activity was 598 units per mg of protein for pyruvate reduction using standard assay conditions. The specific activity using L(+)-lactate and NAD^+ was 0.79 units per mg of protein using standard assay conditions. Concentrations of L(+)-lactate were varied between 16 mM to 1 M; temperature was varied between 25 to 70°C; several concentrations of NAD^+ and $Fru(1,6)P_2$ were attempted to obtain catalytic rates of L(+)-lactate oxidation without success. Polyacrylamide disc gel electrophoresis (Figure 1) displayed a single protein band after the final stage of purification. The protein band displayed activity staining in the presence of pyruvate, NADH and $Fru(1,6)P_2$ as shown by negative activity staining and in the presence of L(+)-lactate and NAD^+ as shown by positive activity staining. No activity staining was exhibited in the presence of D(−)-lactate. The same activity staining results were observed when crude extract was electrophoresed (not shown).

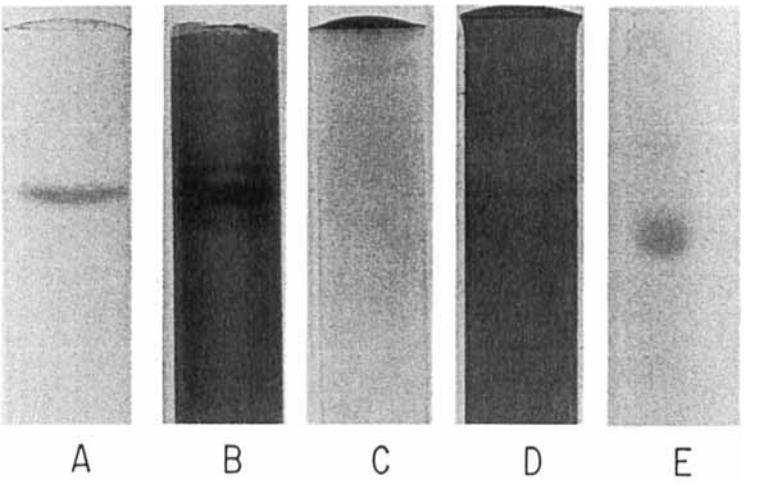

FIGURE 1 Polyacrylamide disc gel and SDS gel electrophoresis of lactate dehydrogenase from *T. ethanolicus*. (a) protein stain of L(+)-LDH, (b) positive activity stain of LDH in the presence of L(+)-lactate, (c) positive activity stain of L(+)-LDH in the presence of D(−)-lactate, (d) negative activity stain of L(+)-LDH in the presence of pyruvate, (e) protein stain of SDS gel electrophoresis. All disc gel were performed with 12 μg protein. The SDS gel was performed with 6 μg protein.

SDS polyacrylamide gel electrophoresis of the L(+)-lactate dehydrogenase from *T. ethanolicus* displayed a single protein band (Figure 1).

Molecular Weight Estimation

Molecular weight estimation of the native protein was determined using standardized gel filtration and found to be 134,000. Subunit molecular weight was estimated to be 33,500 by comparison with standard proteins electrophoresed on SDS polyacrylamide gels. These results indicated an apparent tetrameric structure of equivalent subunits for the L(+)-lactate dehydrogenase from *T. ethanolicus*. No metals were associated with the L(+)-lactate dehydrogenase as determined by plasma emission spectroscopy.

Substrate Specificity

Several compounds were examined to determine if they could substitute as substrates for pyruvate for the L(+)-LDH reaction under standard assay conditions. Oxobutyrate and oxoglutarate-dependent activity were both 82% of pyruvate dependent activity using standard assay conditions. Malate and oxalate were not reactive. The activity with NADPH was 34% of that obtained with NADH using standard assay conditions.

Heat Incubation and Heat Activation

Figure 2 shows pyruvate reductase activity of the L(+)-lactate dehydrogenase as a function of time of incubation at 50, 70, 80 and 90°C. At 50°C the enzyme appeared

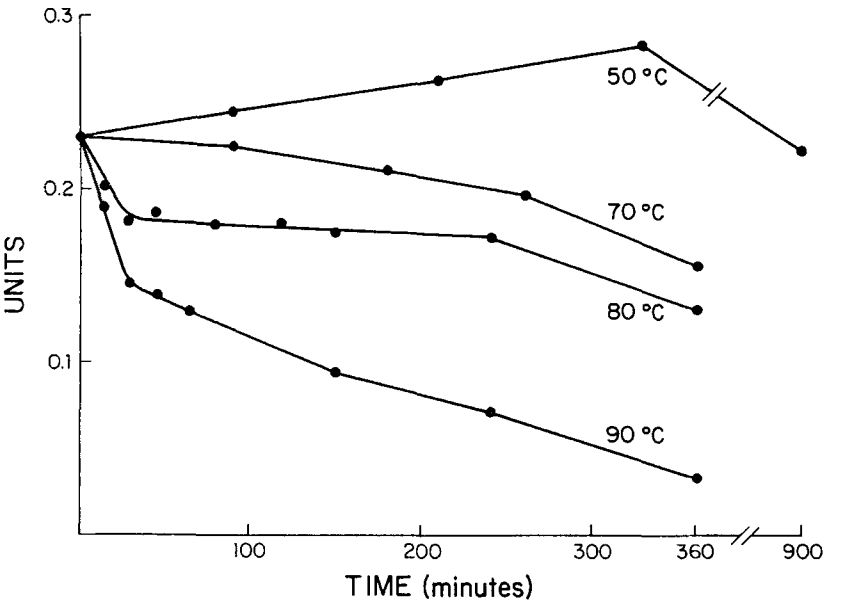

FIGURE 2 Heat incubations of lactate dehydrogenase. All reactions were run in the presence of 12 mM pyruvate, 0.2 mM NADH, 1 mM Fru(1,6)$_2$, 100 mM MES-0KOH pH 6.4 and 0.6 μg L(+)-LDH at 50°C.

to gain activity over 300 min of incubation. At 900 min the L(+)-LDH activity was about the same as at time zero. Loss of L(+)-LDH activity at 90°C was rapid in the early stages of incubation but activity was still present after 360 min. All incubations were stoppered to prevent loss of volume during the course of time. The Arrhenius plot (not shown) indicated that 80°C results in maximum specific activity for the L(+)-LDH. E_{act} was calculated to be 66,100 joules per mol for the reduction of pyruvate using standard assay conditions.

Activation by Fru(1,6)P_2 and Effect of pH

Fructose 1,6-bisphosphate was found to activate pyruvate reduction by the L(+)-lactate dehydrogenase from *T. ethanolicus*. Figure 3 demonstrates the combined effects of Fru(1,6)P_2 and pH on lactate dehydrogenase activity. At pH 7.5 lactate dehydrogenase activity was absent without Fru(1,6)P_2 but was induced in the presence of Fru(1,6)P_2. From pH 7 to 5, L(+)-lactate hydrogenase activity was present without Fru(1,6)P_2 but was greater in the presence of the activator. Fru(1,6)P_2 activation of pyruvate reduction by the L(+)-LDH was maximal at about pH 6.4.

The initial velocity curves of the L(+)-LDH at several Fru(1,6)P_2 concentrations and pH 6.0 are shown in Figure 4. Between 2.0–2.5 mM pyruvate, the initial velocity varied from 0.125 units the the absence of Fru(1,6)P_2 to 0.785 units in the presence of 1.0 mM Fru(1,6)P_2. The effect of Fru(1,6)P_2 on V_{max} and apparent K_m of the L(+)-LDH is shown in Table II. V_{max} values increased from 0.29 units in the absence of Fru(1,6)P_2 to 0.95 units in the presence of 1.0 mM Fru(1,6)P_2. Similarly, the apparent K_m values for pyruvate ranged from 2.2 mM in the absence of Fru(1,6)P_2 decreasing to 0.25 mM in the presence of 1.0 mM Fru(1,6)P_2. The double reciprocal

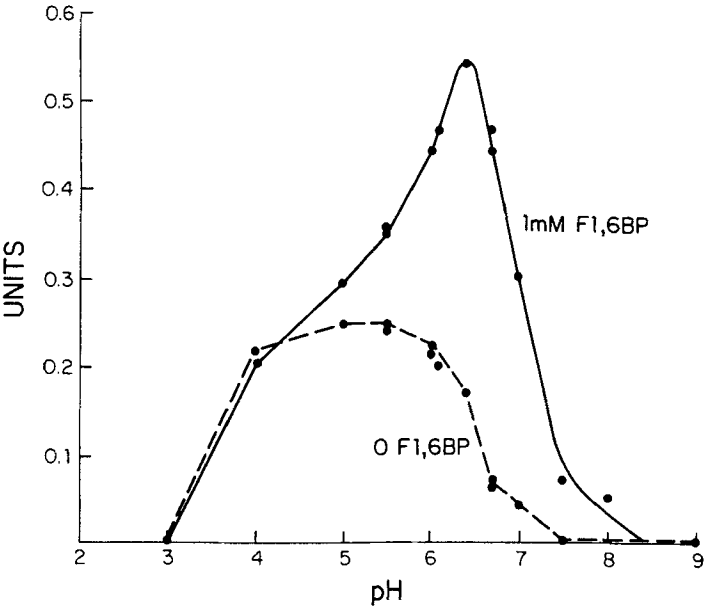

FIGURE 3 pH profile of L(+)-LDH. All reactions were run in 100 mM buffer, 2 mM pyruvate, 0.2 mM NADH, and 0.975 μg of L(+)-LDH at 50°C. Tris-HCl was used at pH 9.0, 8.5, 8.0, 7.5 and 6.0. Phosphate buffer was used at pH 7.0, 6.7, 6.4, 6.0, 5.5, 5.0, 4.0, and 3.0. MES-KOH buffer was used at pH 6.7, 6.1 and 5.5. Acetate buffer was used at pH 6.7 and 5.0. K-biphthalate was used at pH 4.0. KCl-HCl buffer was used at pH 2.0.

plot of the initial velocity data in Figure 4 indicated a sequential mechanism for the addition of substrate.[16] Similar initial velocity curves demonstrating Fru(1,6)P_2 activation were observed at several other pH values (not shown). Fru(1,6)P_2 did not replace pyruvate as a substrate.

Under standard assay conditions, a concentration greater than 1 mM Fru(1,6)P_2 did not result in an observable increase in the velocity of the L(+)-LDH reaction. No

TABLE II
Dependence of Kinetic Values of L(+)-Lactate Dehydrogenase from *T. ethanolicus* on concentration dependent activation by fructose 1,6-bisphosphate.

Fru(1,6)P_2(mM)	V_{max} (Units)[b]	K_m(mM)[a,b]
0.00	0.29	2.2
0.01	0.5	1.0
0.04	0.67	0.38
1.0	0.95	0.25

[a]Apparent K_m for pyruvate.
[b]Kinetic values were derived from the double reciprocal plots of the initial velocity data in Figure 4 (not shown). All reactions were performed in the presence of 0.2 mM NADH, 100 mM MES-KOH at pH 6.0 and 1.29 μg L(+)-LDH at 50°C. Reactions were performed as described in the text.

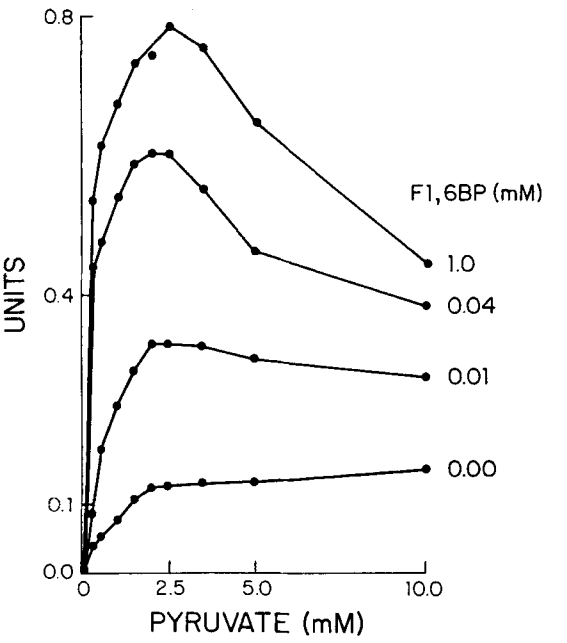

FIGURE 4 Fru(1,6)P_2 activation of L(+)-LDH initial velocities. All reactions were run in the presence of 100 mM MES-KOH pH 6.0, 0.2 mM NADH and 1.29 μg of L(+)-LDH at 50°C.

activation of lactate dehydrogenase was observed in the presence of phosphoenol-pyruvate, fructose, glucose, ribose, glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, fructose 1-phosphate, fructose 6-phosphate, phosphate buffer at concentrations of 1 mM or citrate and Mn^{2+}.

Effects of Pyruvate and NADH

Activation of the L(+)-lactate dehydrogenase by Fru(1,6)P_2 was found to be influenced by the concentration of pyruvate. At pH 6.0, the pyruvate initial velocity curves (Figure 4) displayed increasing reaction rates as pyruvate concentration was increased from 0 to 2.5 mM. However, as pyruvate concentration was increased beyond 2.5 mM, a reduction in reaction rates became evident in the presence of Fru(1,6)P_2. This effect was pronounced as Fru(1,6)P_2 concentration was increased from 0.01 to 1.0 mM.

Figure 5 demonstrates the effect of NADH concentration on L(+)-LDH activity. Fru(1,6)P_2 was maintained at 1 mM and the pH at 6.0 for all reactions. The initial velocities increased up to 0.2 mM NADH and then decreased beyond this concentration. Consistent with Figure 4, Figure 5 also shows that NADH initial velocity curves were influenced by the concentration of pyruvate (in the presence of Fru(1,6)P_2), being maximal around 2 mM pyruvate. The apparent K_m for NADH was estimated to be 0.05 mM from a double reciprocal plots of the data in Figure 5 (not shown). The apparent K_m for pyruvate was estimated to be 0.37 mM from a replot of the double reciprocal plot of the data in Figure 5 (not shown).

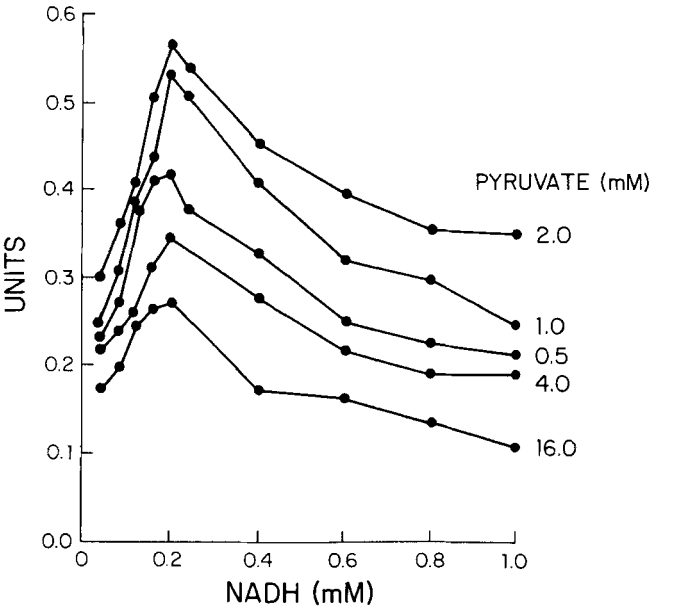

FIGURE 5 Pyruvate and NADH effect for L(+)-LDH initial velocities. All reactions were run in the presence of 100 mM MES-KOH pH 6.0, 1 mM Fru(1,6)P_2 and 1.2 μg of L(+)-LDH at 50°C.

An apparent V_{max} for pyruvate reduction at pH 6.0 was obtained by measuring the initial velocities at a constant ratio of pyruvate to NADH at different concentrations (data not shown). The curve of 1/v versus 1/pyruvate was extrapolated to a value of zero for 1/pyruvate to obtain $1/V_{max}$. The maximum specific activity in the presence of 1 mM Fru(1,6)P_2 was 1082 units per mg of L(+)-LDH.

Inhibition by NADPH

Several NADH analogues were tested for inhibitory effects including NADPH, $NADP^+$, NAD^+, ATP, ADP, GTP, GDP and L(+)-lactate. Most had little or

TABLE III
Dependence of kinetic values of the L(+)-lactate dehydrogenase from *T. ethanolicus* on concentration dependent inhibition by NADPH.

NADPH (mM)	V_{max} (Units)[b]	K_m (mM)[a,b]
0.0	1.1	0.08
0.1	0.8	0.07
0.5	0.53	0.06
0.7	0.32	0.036

[a]Apparent K_m for NADH.
[b]Kinetic values were derived from the double reciprocal plots of the initial velocity data of Figure 6 (not shown). All reactions were performed in the presence of 2 mM pyruvate, 1 mM Fru(1,6)P_2, 100 mM MES-KOH at pH 6.0 and 1.29 μg of L(+)-LDH at 50°C. Reactions were performed as described in the text.

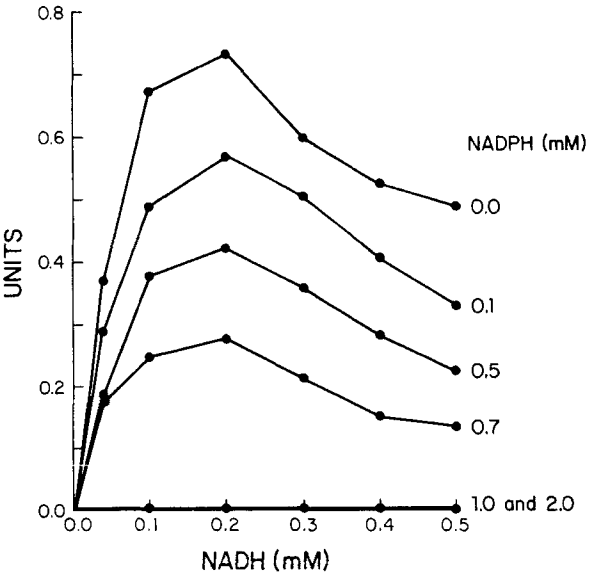

FIGURE 6 Inhibition of L(+)-LDH by NADPH. All reactions were run in the presence of 100 mM MES-KOH pH 6.0, 2 mM pyruvate, 1 mM Fru(1,6)P_2 and 1.29 μg L(+)-LDH at 50°C.

no inhibitory effect. Only NADPH at 1.0 mM or greater completely inhibited the lactate dehydrogenase activity under standard assay conditions. Figure 6 shows the inhibition pattern of the L(+)-lactate dehydrogenase reaction with increasing NADPH concentration. At 0.2 mM NADH, the initial velocity varied from 0.735 units in the absence of NADPH to 0.275 units in the presence of 0.7 mM NADPH. The effect of NADPH on V_{max} and apparent K_m is shown in Table III. The V_{max} values decreased from 1.1 units in the absence of NADPH to 0.32 units in the presence of 0.7 mM NADPH. The apparent K_m values for NADH ranged from 0.08 mM to 0.036 mM as NADPH was increased from 0.0 to 0.7 mM. The double reciprocal plot of this data (not shown) indicated an uncompetitive pattern.[16,17] The Dixon plot of the data in Figure 6 (not shown) indicated an apparent K_i value for NADPH of 0.375 mM. The similarity of Figure 6 to Figure 5 again demonstrates that maximal concentration of NADH is 0.2 mM.

DISCUSSION

The lactate dehydrogenase (E.C.1.1.1.27) from *T. ethanolicus* is NAD^+-linked, Fru(1,6)P_2-activated catalyzing the production of L(+)-lactate from pyruvate. There was no apparent activity of the lactate dehydrogenase from *T. ethanolicus* with D(−)-lactate as determined by activity staining of the enzyme after polyacrylamide gel electrophoresis.

Certain *Streptococcal* lactate dehydrogenases activated by Fru(1,6)P_2 were first reported by Wolin.[18] Since then Fru(1,6)P_2 has been shown to be an activator for numerous microbial L(+)-lactate dehydrogenases.[19-24,28-30] Fru(1,6)P_2 activation is only observed with L(+)-lactate dehydrogenases and not with D(−)-lactate

dehydrogenases.[19,25–27] Fru(1,6)P_2-activated, L(+)-LDHs have been isolated from thermophilic bacteria.[28,31] The L(+)-LDH from *T. ethanolicus* is this type of LDH since cooperative (heterotrophic) activation is observed with increasing concentration of Fru(1,6)P_2 (see Figure 4). Fru(1,6)P_2 is not reduced by NADH. The cooperative mechanism increases the velocity of the L(+)-LDH reaction and lowers the apparent K_m of the L(+)-LDH as shown in Figure 4 and Table II. Of interest is a L(+)-LDH from *Thermus aquaticus* YT-1 activated by citrate.[29] This effect was not observed for the L(+)-LDH from *T. ethanolicus* wt. Certain L(+)-LDHs are activated[13,19] by Mn^{2+} but this effect was not observed with the L(+)-lactate dehydrogenase from *T. ethanolicus*.

Fru(1,6)P_2 did not appear to activate the enzymatic oxidation of L(+)-lactate by NAD^+ as was observed for pyruvate reduction by NADH. At various concentrations of L(+)-lactate, NAD^+, Fru(1,6)P_2 and other likely activators, and at various pHs and ionic strengths, no oxidation of L(+)-lactate was observed except at 1 M L(+)-lactate, 1.25 mM NAD^+ at pH 9.5. Likewise, activity staining of the L(+)-LDH after polyacrylamide gel electrophoresis, developed only in the presence of similar concentrations of L(+)-lactate and NAD^+ at pH 9.5 after incubation for 6 hours. By contrast, the activity stain of the L(+)-lactate dehydrogenase for pyruvate reduction occurred under concentrations of reactants similar to those used to assay pyruvate reduction and required only minutes to develop. These observations were not surprising since characteristic of Fru(1,6)P_2-activated, L(+)-lactate dehydrogenases from procaryotes, the oxidation of L(+)-lactate by NAD^+ either does not occur or occurs only at low rates under physiological conditions *in vitro*.[19–24,28,30,31] By contrast, lactate dehydrogenases not activated by Fru(1,6)P_2, those specific for D(−)-lactate or those independent of NAD(H) as cofactor, usually catalyze both the oxidation of lactate and reduction of pyruvate under physiological conditions *in vitro*.[19,25,26,27,32]

It is likely that the tetramer was present as the predominant species of the L(+)-LDH from *T. ethanolicus*. Fru(1,6)P_2-activated, L(+)-lactate dehydrogenases from *Streptococcus uberis*[24] and *Bacillus stearothermophilus*[31,33] are stabilized in the tetrameric form (active) by Fru(1,6)P_2 and NADH. The dimeric form of these L(+)-LDHs are inactive or reduced in activity unless Fru(1,6)P_2 is present. However, Fru(1,6)P_2 induces the tetramer making such a distinction difficult. Gel electrophoresis activity staining indicated only one active species which co-electrophoresed with a single protein stain. Since the L(+)-LDH was routinely assayed in the presence of Fru(1,6)P_2 and NADH, the ratio of tetrameric to dimeric is presumed high.

Pyruvate reduction by L(+)-lactate dehydrogenase from *T. ethanolicus* was influenced by pH, pyruvate and NADH. The effect of pH on the L(+)-lactate dehydrogenase from *T. ethanolicus* (Figure 3) was such that at high pH (e.g., 9.0), pyruvate reductase activity did not occur (this effect was not due to denaturation since adjusting the pH back to 7.5 renewed L(+)-LDH activity). As the pH was lowered, i.e., from 8.5 to 6.4, reaction rates of L(+)-LDH were observed to increase (with or without Fru(1,6)P_2) consistent with a proton being a reactant component. The activation by Fru(1,6)P_2 was observed in a range of pH between 8.0–4.5 (Figure 3).

The observed maximal rate at pyruvate concentration around 2–2.5 mM and at NADH concentration around 0.2 mM is not fully understood. However, these optimal effects were only observed in the presence of Fru(1,6)P_2 and were most prominent at pHs at or below the optimal pH e.g., 6.0. From the double reciprocal plot of the data in Figure 5 (not shown), it was determined that the K_m values of NADH and pyruvate did not vary at 1 M Fru(1,6)P_2. For example, this effect was not observed at pH 7.5.

Other researchers have observed similar pyruvate or NADH optima[20,21,26,28] that tend to occur at lower pHs consistent with those observed for the L(+)-LDH from *T. ethanolicus*. One explanation for this observation is that the L(+)-LDH-NAD(H) complex does not dissociate as the substrate concentration increases resulting in decreasing rates of NADH oxidation.[34] This explanation also may account for the apparent unidirectional reaction (pyruvate reduction) observed for these types of LDHs. Since the K_m values for NADH (Table III) are low, indicating tight binding of NADH, such an explanation is consistent for the L(+)-LDH from *T. ethanolicus*.

Complete inhibition of pyruvate reduction was observed when NADPH was present of concentrations of 1 mM or greater. Given the NADPH-linked alcohol dehydrogenase in *T. ethanolicus* wt[10] and the high levels of ethanol produced by this bacterium, the high intracellular concentrations of NADPH that are likely during growth implicates NADPH as a physiological inhibitor of the L(+)-LDH. Inhibition of pyruvate reduction (NADH oxidation) may be a mechanism to direct pyruvate to ethanol to insure the oxidation of NADPH. Although competitive inhibition by NADPH is reasonable given that the L(+)-LDH reaction is NADH dependent, the pattern of inhibition was uncompetitive[17] indicating that NADPH binds to the enzyme substrate complex and not to the free L(+)-LDH. Since Fru(1,6)P_2 is a positive modifier for the L(+)-LDH, the inhibition pattern suggests that NADPH is a negative modifier.[17] Such negative cooperativity may result from NADPH binding to the modifier sites or to other sites when Fru(1,6)P_2 is bound. The shape of the curves in Figure 6 are quite similar to Figure 4 lending support to such an interpretation.

L(+)-LDHs, activated by Fru(1,6)P_2 have been shown to contain four modifier (anion) binding sites per tetramer (two per dimer).[31,33] The phosphate groups of two Fru(1,6)P_2 molecules bind to these sites on opposing dimers forming the active, tetrameric L(+)-LDH. Also, the L(+)-LDHs contain four catalytic sites in which pyruvate and NADH react forming L(+)-lactate and NAD^+. The catalytic sites are distinct and separate from the modifier sites. Since NADPH is an analogue of NADH, it is not surprising that NADPH displays some activity (34%) when replacing NADH by reacting with pyruvate at the catalytic site. Similarly, since NADPH contains one phosphate that could bind to the anion binding site, uncompetitive inhibition of the L(+)-LDH displaying negative cooperativity is reasonable. Competitive inhibition was not observed lending support to this interpretation. This is the first report of NADPH functioning in this capacity based on the literature reviewed.

The production of greater amounts of lactate by *T. ethanolicus* when grown on concentrations of glucose above 1% may be due to the activating effect of Fru(1,6)P_2 on the L(+)-lactate dehydrogenase since glucose is a metabolic precursor to Fru(1,6)P_2.

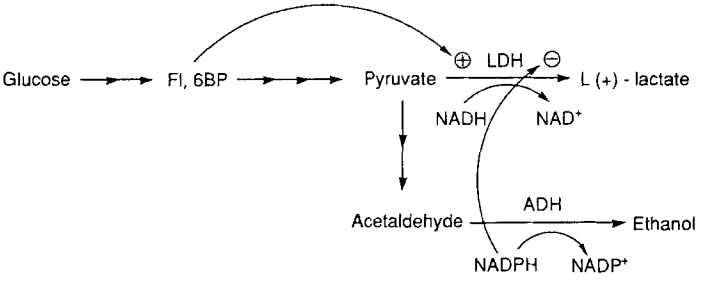

FIGURE 7 Pathways of end products formation as occurs in *T. ethanolicus*.

Similar explanations have been offered for the control of end product formation in *Thermoanaerobium brockii*[35] which is physiologically similar to *T. ethanolicus*. Low lactate production compared to ethanol under optimal growth conditions may be due to inhibition by NADPH and to the influence pH, pyruvate and NADH on the rate of L(+)-LDH reduction of pyruvate. However, intracellular concentrations of metabolites and the complete rate equation for the L(+)-LDH would be required to state unequivocally that the L(+)-lactate dehydrogenase from *T. ethanolicus* was responsible for the reduction of ethanol production. Figure 7 presents aspects of the metabolic pathway as believed to occur in *T. ethanolicus* wt.

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