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A novel mode of lactate metabolism in strictly anaerobic bacteria

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Summary

Lactate is a common substrate for major groups of strictly anaerobic bacteria but the biochemistry and bioenergetics of lactate oxidation is obscure. The high redox potential of the pyruvate/lactate pair of $E^{0'}$ = -190 mV excludes direct NAD^+ reduction ($E^{0'}$ = -320 mV). To identify the hitherto unknown electron acceptor, we have purified the lactate dehydrogenase (LDH) from the strictly anaerobic, acetogenic bacterium *Acetobacterium woodii*. The LDH forms a stable complex with an electron-transferring flavoprotein (Etf) that exhibited NAD^+ reduction only when reduced ferredoxin (Fd^{2-}) was present. Biochemical analyses revealed that the LDH/Etf complex of *A. woodii* uses flavin-based electron confurcation to drive endergonic lactate oxidation with NAD^+ as oxidant at the expense of simultaneous exergonic electron flow from reduced ferredoxin ($E^{0'} \approx$-500 mV) to NAD^+ according to: lactate + Fd^{2-} + 2 NAD^+ → pyruvate + Fd + 2 NADH. The reduced ferredoxin is regenerated from NADH by a sequence of events that involves conversion of chemical (ATP) to electrochemical ($\Delta_{\tilde{\mu}}{Na}^+$) and finally redox energy (Fd^{2-} from NADH) *via* reversed electron transport catalyzed by the Rnf complex. Inspection of genomes revealed that this metabolic scenario for lactate oxidation may also apply to many other anaerobes.

Introduction

Substrate-level phosphorylation (SLP) coupled to glycolysis with lactate as (an) end product *via* pyruvate reduction is found in many fermenting anaerobic bacteria as a principle mode of energy conservation. The prototype of this metabolic scheme is found in homofermentative lactic acid bacteria that ferment one mol of glucose to two mol of lactate that are excreted into the environment. Other fermenting bacteria produce lactate alongside with other fermentation

end products. The key enzyme in lactate production from pyruvate is the lactate dehydrogenase that quantitatively consumes the electrons (in form of NADH) generated during glycolysis to reduce pyruvate to lactate. These NADH-oxidizing LDHs are classified as nLDHs (Garvie, 1980).

Lactate produced by primary fermenters does not accumulate in the environment since it is a good growth substrate for many bacteria (Balch*, et al.*, 1977, Diez-Gonzalez*, et al.*, 1995, Yang*, et al.*, 1987, Brockman and Wood, 1975). Lactate that diffuses into oxic zones of the enviroment is oxidized to carbon dioxide with concomitant reduction of oxygen to water. The first step is its oxidation to pyruvate, catalyzed by lactate dehydrogenases. The redox potential ($E^{0'}$) of lactate/pyruvate is only -190 mV (Thauer*, et al.*, 1977) and the concomitant equilibrium constant (K_{eq}) for the reaction

$$\text{lactate} + NAD^+ \leftrightarrow \text{pyruvate} + NADH \qquad \Delta G^{0'} = +25 \text{ kJ/mol} \qquad \text{(eq. 1)}$$

is 4 x 10^{-5}. Thus, under standard conditions only 0.01 permill of lactate are oxidized to pyruvate. K_{eq} may be changed to more favourable values by removal of pyruvate, allowing for a slow, but complete oxidation of lactate. The low K_{eq} is the result of the low redox potential of the NAD^+/NADH pair ($E^{0'}$ = -320 mV) which introduces a serious energetic barrier for lactate oxidation coupled to NAD^+ reduction. The electrons derived from lactate oxidation have to travel energetically "uphill" to NAD^+ in an energy-dependent process. Thus, bacteria that grow on lactate as sole energy and carbon source have a serious energetic problem due to the high redox potential of the pyruvate/lactate pair. Aerobic bacteria have solved this problem simply by not using NAD^+ as electron acceptor but a membrane integral electron acceptor that channels the lactate-derived electrons into the aerobic electron transport chain (Kaback and Milner, 1970, Ma*, et al.*, 2007). The primary electron acceptor in, for example, *Escherichia coli* is ubiquinone with an $E^{0'}$ of +113 mV (Anraku and Gennis, 1987). These

LDHs are membrane-bound or at least membrane-associated and classified as NAD^+-independent LDHs, iLDHs. Lactate can also be used under anoxic conditions as growth substrate by bacteria that have no membrane-bound LDHs. These bacteria include the ecologically important groups of sulfate-reducing bacteria or acetogenic bacteria. How these bacteria oxidize lactate to pyruvate is completely obscure. We have addressed this question using the acetogenic model organism *Acetobacterium woodii*. In this communication we will not only demonstrate a novel mode of lactate oxidation widespread in anaerobic microorganisms, but also a novel mode of energy coupling during lactate metabolism.

Results

Growth of A. woodii *on lactate*

To determine the kinetic parameters for growth of *A. woodii* on lactate, media containing lactate as carbon and energy source were inoculated to a final OD_{600} of 0.05 with a preculture adapted to growth on DL-lactate for several generations. Growth of the cells started immediately. Cultures that did not receive lactate reached a final OD of 0.2, probably due to residual DL-lactate contained in the preculture. The growth rate and the final optical densities increased with increasing lactate concentrations. The growth rate reached a maximum at 10 and 20 mM DL-lactate, where it was 0.12 h^{-1} and the doubling time was 5.0 h. The final optical densities increased with higher lactate concentrations and reached a maximum with OD = 1.4 at 100 mM DL-lactate. Growth on 80 mM D- or L- lactate was similar.

Purification and characterization of an LDH/Etf complex

Establishing a purification protocol for the LDH required the prior establishment of an enzyme assay to determine LDH activity. The assay usually used for measuring nLDHs was not applicable as there was no lactate-dependent NAD^+ reduction in cell-free extracts of *A. woodii*. The artificial electron acceptor dichlorophenolindophenol (DCPIP, 100 µM) that has been used before to measure NAD^+-independent lactate dehydrogenases (Garvie, 1980) was reduced with lactate as reductant but the background activity was rather high. Therefore, other electron acceptors were also tested, namely methylviologen and ferricyanide ($K_3Fe(CN)_6$). Ferricyanide was the most suitable electron acceptor because of reproducibility and negligible background activity. Thus, LDH activity during the purification process was routinely measured using lactate as electron donor and ferricyanide as electron acceptor.

To purify the lactate dehydrogenase, *A. woodii* was grown to the late exponential growth phase on DL-lactate, harvested and disrupted with a French pressure cell. Membranes were removed by ultracentrifugation and the lactate dehydrogenase was purified from the cytoplasm by ion exchange chromatography on Q-Sepharose and Phenyl-Sepharose followed by Butyl-Sepharose. FAD (5 µM) was present during all purification steps, as the LDH precipitated in the absence of FAD. FMN could not substitute FAD. Using this procedure, the enzyme was purified 156-fold to apparent homogenity with an average specific activity of 30 U/mg (Tab. 1). When the preparation was analyzed on a denaturing gel, three distinct proteins with apparent molecular masses of 51, 46 and 29 kDa were detected (Fig. 1A). These were identified by peptide mass fingerprinting to be encoded by the genes *lctB (Awo 0871)*, *lctC (Awo 0872)* and *lctD (Awo 0873)*, that potentially encode the small and large subunits of an electron transferring flavoprotein (*lctB* [EtfB] and *lctC* [EtfA], respectively) and a lactate dehydrogenase (*lctD*). All three proteins apparently form a stable complex whose molecular mass was estimated by native gel electrophoresis to 151 and 211 kDa and analytical gel filtration to 138 kDa (Fig. 1B). Since electron transfer flavoproteins have invariably been reported as EtfAB dimers (Bertsch*, et al.*, 2013, Li*, et al.*, 2008, Sato*, et al.*, 2003), the 211

kDa fragment encountered during native gel electrophoresis presumably shows an artefact corresponding to a 1 LDH : 2 EtfAB stoichiometry. Both other values obtained are consistent with a heterotrimer of EtfA, EtfB and LDH in a 1:1:1 stoichiometry.

Basic biochemical properties of the LDH/Etf complex in A. woodii

Lactate:ferricyanide oxidoreductase activity was rather insensitive to a pH ranging from 5.5 to 9.0. The highest activity was encountered at pH 7. Activity was similar in Bis-Tris (50 mM), PIPES (50 mM) and KP_i (50 mM) buffer but decreased by 25% in MOPS (50 mM) buffer. LDH activity was optimal between 20°C and 30°C. Activity above 30°C decreased by 20% and dropped down to only residual activity at 50°C. LDH activity at low temperatures of 10°C was still at 65%. Divalent cations stimulated activity, highest stimulation observed with $CaCl_2$ was 2-fold. Activity increased linearly with increasing $CaCl_2$ concentration and reached saturation at 40 mM. Therefore, the assay used to measure LDH activity was 50 mM Bis-Tris buffer, pH 7, containing 50 mM $CaCl_2$ at room temperature.

NAD^+ is the electron acceptor of the LDH/Etf complex but its reduction requires lactate and reduced ferredoxin

As expected from the fact that cell free extract did not catalyze lactate-dependent NAD^+ reduction, the purified LDH/Etf complex did not reduce NAD^+ with lactate as electron donor either. However, the presence of a flavin-containing Etf protein building a complex with the LDH led to the hypothesis that the enzyme uses flavin-dependent electron bifurcation to drive endergonic NAD^+ reduction with lactate and reduced ferredoxin as reductant (Bertsch *et al.,* 2013, Li *et al.*, 2008, Buckel and Thauer, 2012). A similar process was described for a hydrogen evolving hydrogenase. There the endergonic hydrogen production ($E^{0'}$ $[2H^+/H_2]$ = -

414 mV) from NADH ($E^{0'}$ = -320 mV) is driven by a simultaneous electron transfer from reduced ferredoxin to protons producing molecular hydrogen (Schut and Adams, 2009). To analyze whether or not ferredoxin is involved in lactate oxidation catalyzed by the LDH/Etf complex of *A. woodii*, ferredoxin was purified from *C. pasteurianum* (Schönheit *et al.*, 1978) and reduced with CO ($E^{0'}$ = -520 mV) using CO dehydrogenase (CODH) purified from *A. woodii* (Hess *et al.*, 2013). This ensured a constant ferredoxin reduction and regeneration in a CO atmosphere. After preincubation of CODH with ferredoxin under 100% CO, the latter was reduced, which was measured spectroscopically at 430 nm (Fig. 2). Addition of NAD^+ (4 mM) and purified LDH/Etf led to an immediate increase in absorbance at 340 nm, showing NADH formation (Fig. 2). The specific NAD^+ reduction activity was 2.9 ± 0.02 (SD) U/mg in the presence of 70 µM ferredoxin. There was no NAD^+ reduction in the absence of ferredoxin, or in the absence of a reducing system or in the absence of LDH/Etf. The affinities for the substrates were determined to 31 µM for ferredoxin and 430 µM for NAD^+, and the growth substrates had K_m values of 3.6 mM for D-lactate and 112 mM for L-lactate. These experiments revealed that reduced ferredoxin is required for lactate-dependent NAD^+ reduction. Unfortunately, oxidation of reduced ferredoxin could not be measured since a continuous ferredoxin reducing system was used.

Pyruvate reduction is coupled to NADH oxidation only in the presence of ferredoxin

To determine whether ferredoxin is involved in electron transfer, we searched for a reaction that would reduce ferredoxin. Therefore, we analyzed whether the complex catalyzes the reverse reaction. Pyruvate reduction to lactate with NADH as electron donor is highly exergonic. However, multiple measurements showed that there was no NADH oxidation with pyruvate as electron acceptor. Only when oxidized ferredoxin was added to the system, the reaction was initiated and NADH oxidation was detected by a decrease of absorbance at 340

nm (Fig. 3A). The activity was 1.2 ± 0.3 (SD) U/mg. The LDH thus favours the physiologically more relevant lactate oxidation over pyruvate reduction more than two-fold. This experiment not only exemplified the reversibility of the reaction catalyzed by the LDH/Etf complex. Moreover, it revealed a strict requirement for ferredoxin also in the backward reaction.

Simultaneous reduction of ferredoxin and pyruvate with NADH as electron donor

After having established that the reverse reaction also required ferredoxin, we could address the question whether ferredoxin is reduced in the course of the reaction. Upon addition of NADH to the assay mixture containing pyruvate and oxidized ferredoxin, NADH was oxidized (Fig. 3A). At the same time, ferredoxin was reduced, as visible by a decrease in absorbance at 430 nm (Fig. 3A). NADH-dependent ferredoxin reduction was strictly dependent on the presence of lactate. To determine the coupling ratio, the oxidation of NADH and reduction of ferredoxin were monitored simultaneously. The amount of mol NADH oxidized per mol of ferredoxin reduced was calculated using the absorbance changes at 430 and 340 nm. From a number of experiments a stoichiometry of NAD^+ : ferredoxin of 2 : 1 was obtained (Fig. 3B).

Discussion

The lactate dehydrogenase of *A. woodii* forms a stable complex with an electron-transferring flavoprotein and apparently uses the recently established mechanism of flavin-based electron bifurcation for energetic coupling (Buckel and Thauer, 2012). Apparently, exergonic electron flow from reduced ferredoxin to NAD^+ drives endergonic electron flow from lactate to NAD^+ according to: lactate + Fd^{2-} + 2 NAD^+ → pyruvate + Fd + 2 NADH. A similar subset of an

electron bifurcation reaction, electron confurcation, is found in the hydrogen evolving iron-hydrogenase from *Thermotoga maritima* where oxidation of reduced ferredoxin with concomitant reduction of protons to hydrogen drives production of molecular hydrogen from NADH (Schut and Adams, 2009). Since the discovery of electron bifurcation in 2008, a number of electron bifurcating reactions have been identified (Bertsch and Müller, 2013; Li and Thauer, 2008; Schut and Adams, 2009; Wang*, et al.*, 2010; Kaster*, et al.*, 2011; Schuchmann and Müller, 2012; Wang*, et al.*, 2013a; Wang*, et al.*, 2013b). Electron bifurcating reactions can be classified in three classes, the heterodisulfide reductase (Hdr)-, the transhydrogenase (Nfn)- and the electron transfer flavoprotein (Etf)-type. In all cases, a flavin is essential for electron bifurcation. The LDH/Etf complex has three predicted FADs and one 4Fe-4S cluster (Fig. 4) and FAD is required for stability and activity. The mechanistic course of flavin-dependent electron flow during electron bifurcation is still subject of speculation. Generally, two different theories for the mechanism of electron bifurcation have been postulated. On the one hand, it was proposed by Kaster *et al.* that the three different states of flavoproteins (FP) give rise to different redox potentials for FP/FPH2 (n=2), FP/FPH (n=1) and FPH/FPH2 (n=1). FPH2 oxidation through two different electron acceptors with different redox potentials gives rise to a bifurcating FPH2 oxidation (Kaster *et al.,* 2011). Therefore, the flavoprotein exhibits a stable semiquinone state and thus electrons from lactate and ferredoxin should be transferred consecutively. In the other scenario outlined by Nitschke and Russel (2012), the flavoprotein should be fully reduced before sequentially one electron reduces the high potential compound which leaves behind a highly reactive radical able to reduce the low potential acceptor (Nitschke and Russel, 2012).

The discovery of an electron-bifurcating LDH/Etf complex allows to postulate a pathway for acetogenesis from lactate in *A. woodii* and its coupling to ATP synthesis. As depicted in Fig. 5, lactate is oxidized to pyruvate at the expense of simultaneous oxidation of reduced ferredoxin. Pyruvate oxidation yields acetyl-CoA, CO_2 and Fd^{2-}. Half of the reduced

ferredoxin is oxidized by the acetyl-CoA synthase/CO dehydrogenase and the hydrogen-dependent CO_2 reductase. Both enzymes are known to use ferredoxin as electron carriers (Hess *et al.*, 2013). Thus, the question emerges how the reduced ferredoxin required for lactate oxidation is regenerated. Most likely from NADH by reverse electron transfer, mediated by the Rnf complex and driven by $\Delta_{\tilde{\mu}Na^+}$. That the Rnf complex drives ferredoxin reduction with NADH as reductant at the expense of $\Delta_{\tilde{\mu}Na^+}$ was demonstrated very recently (Hess *et al.*, 2013). The $\Delta_{\tilde{\mu}Na^+}$ is established by the Na^+ F_1F_O ATP synthase at the expense of ATP hydrolysis (Fritz and Müller, 2007; Brandt*, et al.*, 2012). During lactate oxidation to acetate in *A. woodii*, ATP is only synthesized by SLP, and part of the ATP has to be reinvested to generate $\Delta_{\tilde{\mu}Na^+}$ that then generates the reducing power for the first step in substrate oxdiation. Overall, this pathway allows lactate oxidation to acetate according to:

4 lactate + 1.5 ADP ↔ 6 acetate + 1.5 ATP

SLP drives chemiosmosis which leads to ferredoxin reduction at the Rnf complex. This then finally drives lactate oxidation. It allows the synthesis of only 0.25 ATP/mol acetate, the lowest ATP yields described for *A. woodii* (Fig. 5). This extremely low yield is reflected by the high amounts of substrate required to reach appreciable optical densities. The metabolic scheme developed for lactate oxidation in *A. woodii* may also apply to other anaerobes oxidizing lactate or other high potential substrates such as ethanol ($E^{0'}$ acetaldehyde/ethanol = -197 mV (Thauer *et al.,* 1977)).

The gene encoding the lactate dehydrogenase (*lctD*) in *A. woodii* is accompanied by two genes encoding the electron transfer flavoprotein which forms a stable complex with the LDH (Fig. 4). These three genes are adjacent to each other on the chromosome. Downstream of the *lctD* is a gene encoding a potential lactate transporter (*lctE*) and a racemase (*lctF*). The latter is consistent with the observation that D- as well as L-lactate promoted growth. The

gene downstream of *lctF* is in opposite orientation to *lctF* and thus not part of the operon. Upstream of, but in the opposite orientation to *lctB,* is a gene whose product is similar to a GntR-like transcriptional regulator (TR). Since it is conserved in a number of *lct* operons in anaerobes, we speculate that it regulates transcription of the *lct* operon. A substrate-dependent transcriptional regulator is consistent with the fact that cells grown on fructose did not have LDH activity.

A search of databases revealed that more than 20 different organisms have LDH genes next to Etf genes. These organisms include strains in the order of *Halanaerobiales* with *Acetohalobium arabaticum* DSM 5501 as a representative, *Fusobacteriales, Thermotogales, Thermoanaerobacteriales* and many different strains from the order *Clostridiales* (Fig. 6). All of them have a lactate permease gene and some of them also harbour a lactate racemase gene. All organisms have in common that they are anaerobes, however they are metabolically versatile. *Desulfotomaculum reducens* MI-1, for example, is a sulfate-reducer, whereas *Heliobacterium modesticaldum* Ice1 is a phototrophic organism. A similar set of genes is also encountered in the metal reducer *Alkaliphilus metalliredigens* QYMF or the nitrate reducer *Clostridium perfringens* ATCC 13124. Thus, we propose that the electron-bifurcating lactate dehydrogenase of the model acetogen *A. woodii* is probably also present in a wide range of anaerobes independent of the type of anaerobic metabolism. It is noteworthy, that all of these anaerobes either have Rnf and/or Ech to drive reduction of ferredoxin with NADH or H_2 as donor.

Thus, the electron bifurcating lactate dehydrogenase of *A. woodii* solves the energetic enigma imposed by the low energy substrate lactate in anaerobes without cytochromes, quinones or other membrane-soluble electron carriers and gives a rationale for the presence of the Rnf complex in these anaerobes.

Experimental procedures

Growth of cells and purification of the lactate dehydrogenase

A. woodii (DSM 1030) was grown at 30°C under anaerobic conditions in 20-l-liter flasks (Glasgerätebau Ochs, Bovenden-Lenglern, Germany) using 80 mM lactate to an OD_{600} of ~1.5. The medium and all buffers were prepared using the anaerobic techniques described previously (Bryant, 1972; Hungate, 1969). All buffers used for preparation of cell extracts and purification contained 2 mM DTE, 4 µM resazurin and 5 µM FAD. All purification steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan, USA) filled with 95-98% N_2 and 2-5% H_2 as described (Heise *et al.*, 1992). Cells of *A. woodii* were harvested and washed twice in 50 mM Tris-HCl (pH 7.0) with 420 mM sucrose. The cells were resuspended in 50 mM Tris-HCl (pH 8.0) with 420 mM sucrose and 600 mg lysozyme and incubated for 1 h at 37°C. After centrifugation the protoplasts were resuspended in buffer A (50 mM Tris/HCl, 20 mM $MgSO_4$, 20% glycerol, pH 7.5) with 0.5 mM PMSF and 0.1 mg/ml DNAseI and passed two times through a French pressure cell (110 MPa). Cell debris was removed by centrifugation at 24000 g for 40 minutes. Membranes were removed by centrifugation at 130000 g for 90 minutes. The supernatant containing the cytoplasmic fraction with approximately 2000 mg protein was applied to a Q-Sepharose high performance column (2.6 cm x 5 cm) equilibrated with buffer A. Protein was eluted with a linear gradient of 300 ml from 0 M to 1 M NaCl in buffer A. Ferricyanide-dependent lactate dehydrogenase activity eluted at around 190 mM NaCl. Ammonium sulfate (0.8 M) was added to the pooled fractions and these were loaded onto a Phenyl-Sepharose high performance column (1.6 cm x 10 cm) equilibrated with buffer B (50 mM Tris/HCl, 20 mM $MgSO_4$, 0.8 M $(NH_4)_2SO_4$, 20% glycerol, pH 7.5). Protein was eluted with a linear gradient of 100 ml from 0.8 to 0 M $(NH_4)_2SO_4$ in buffer A. LDH activity eluted in a peak around 0.24 M $(NH_4)_2SO_4$. Pooled

fractions were desalted using ultrafiltration in 10-kDa VIVASPIN tubes (Sartorius Stedim Biotech GmbH, Germany) and ammonium sulfate (0.8 M) was added. The sample was applied to a Butyl-Sepharose high performance column (0.7 × 2.5 cm) equilibrated with buffer B and eluted with a linear gradient of 20 ml from 0.8 to 0 M $(NH_4)_2SO_4$ in buffer A. Purified LDH/Etf eluted in a peak around 0.52 M $(NH_4)_2SO_4$. Approximately half of the activity was encountered along with contaminations around 40 mM $(NH_4)_2SO_4$. Fractions corresponding to the first peak were pooled, desalted and stored in buffer A at 4°C.

Measurement of LDH/Etf activity

All enzymatic assays where performed at 30°C in 1.8 ml anaerobic cuvettes (Glasgerätebau Ochs, Bovenden-Lenglern, Germany) sealed by rubber stoppers in a N_2 atmosphere with 1 ml of 50 mM Bis-Tris (pH 7.0) containing 50 mM $CaCl_2$ and 2 mM DTE. Lactate dehydrogenase activity was measured with 1 mM ferricyanide as electron acceptor at 420 nm (ε = 1 mM^{-1} cm^{-1}). The reaction was started by adding 50 mM lactate. Physiological LDH activity with NADH and ferredoxin was measured at 340 nm and 430 nm respectively (ε_{NADH} = 6.2 mM^{-1} cm^{-1}, ε_{Fd} = 13.1 mM^{-1} cm^{-1}). Ferredoxin was purified from *Clostridium pasteurianum* as described (Schönheit *et al.*, 1978). Reduced ferredoxin was constantly regenerated through the addition of purified CODH from *A. woodii* using a gasphase of 100% CO (Hess *et al.,* 2013). The forward reaction contained 30 µM ferredoxin, 4 mM NAD^+, 17 µg CODH and was initiated by the addition of 50 mM D-lactate. The reverse reaction contained 20 µM ferredoxin and 200 µM NADH and was initiated by the addition of 50 mM pyruvate.

Analytical methods

The concentration of proteins was measured according to Bradford (Bradford, 1976). Proteins were separated in 12% polyacrylamide gels and stained with Coomassie brilliant blue G250. The molecular mass of the purified LDH/Etf complex was determined using a calibrated superose 6 column equilibrated with buffer A (50 mM Tris/HCl, 20 mM $MgSO_4$, 20% glycerol, pH 7.5). Superose 6 was calibrated using defined size standards (ovalbumin: 43 kDa; albumin: 158 kDa; catalase: 232 kDa; ferritin: 440 kDa). The purified LDH/Etf complex was separated on a native polyacrylamide gel (5-13%) at 4°C.

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Figure legends

Fig. 1. SDS-PAGE monitoring of the purification process of the LDH/Etf complex and native size determination by native PAGE. A. After each purification step, fractions containing highest LDH activity were pooled and 10 µg of each pool was applied onto a 12% polyacrylamide gel (Laemmli, 1970). B. 10 µg of the purified LDH/Etf complex was separated on a native polyacrylamide gel (5-13%) at 4°C. The native mass of the complex was determined from the migration distance of the native complex in comparison to known molecular standards as reference points. Coomassie Brilliant Blue G250 was used for protein staining.

Fig. 2. The LDH/Etf reduces NAD^+ in a lactate- and ferredoxin-dependent manner. The assay contained 30 µM ferredoxin, 17 µg CODH, 4 mM NAD^+ and 4.6 µg LDH/Etf in a 100% CO gas atmosphere. The reaction was started upon addition of 50 mM D-lactate. The continuous reduction of Fd was measured at 430 nm […] and the reduction of NAD^+ was measured at 340 nm [─], simultaneously.

Fig. 3. The LDH/Etf oxidizes NADH while pyruvate and ferredoxin are reduced. A. The assay contained 20 µM ferredoxin, 200 µM NADH and 4.6 µg LDH/Etf in a 100% N_2 gas atmosphere. The reaction was started upon addition of 50 mM pyruvate. The reduction of Fd was measured at 430 nm […] and the oxidation of NADH was measured at 340 nm [─], simultaneously. B. Oxidation of NADH and reduction of ferredoxin was monitored simultaneously at 340 and 430 nm, respectively. 20 µM ferredoxin, 50 mM pyruvate and 100 µM NADH were incubated until absorbance reached a constant level. The amount of reduced electron carrier was calculated from the absorbance difference and the molar extinction coefficient.

Fig 4. Proposed model of the electron bifurcating LDH/Etf complex. Composition of cofactors is based on sequence analysis.

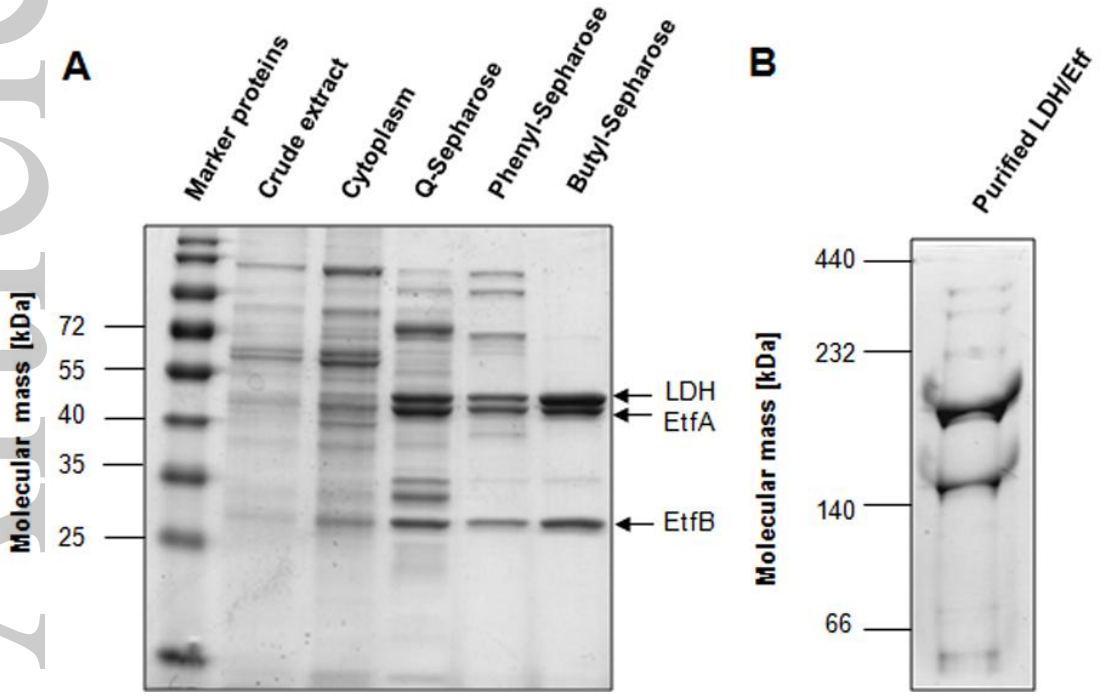
Fig. 5. Enzymology and bioenergetics of lactate metabolism in *A. woodii*. The hydrogen-dependent CO_2 reductase is assumed to use Fd^{2-} as reductant (Schuchmann and Müller, 2013). CoFeS-P, corrinoid-iron-sulfur protein. The stoichiometry of the ATPase is 3.3 Na^+ per Na^+ ATP hydrolyzed. Values for the amount of ATP synthesized or hydrolyzed by the acetate kinase and the ATPase are rounded.

Fig. 6. Structure of the putative lactate-utilization cluster of *A. woodii* and other organisms. Sequence identities are indicated in percent below the protein product. TR: transcription regulator. *C. botulinum, Clostridium botulinum; C. ljungdahlii, Clostridium ljungdahlii; I. polytropus, Ilyobacter polytropus; E. limosum, Eubacterium limosum.*

Purification of the LDH/Etf complex

	Volume activity [U/ml]	Volume [ml]	Protein [mg]	Specific activity* [U/mg]	Yield [%]	Purification fold
Cytoplasm	8	50	2076	0.20	100	1
Q-Sepharose	23	40	273	3.9	230	19.5
Phenyl-Sepharose	13.5	13	7.9	22.3	44	112
Butyl-Sepharose	19.5	1.4	0.9	30	7	150

***** LDH activity was determined monitoring the lactate-dependent reduction of ferricyanide at 430 nm. The assay mixture contained 50 mM Bis-Tris, 50 mM $CaCl_2$ at pH 7. Measurements were performed at room temperature in anaerobic cuvettes. The data from one representative purification are shown.



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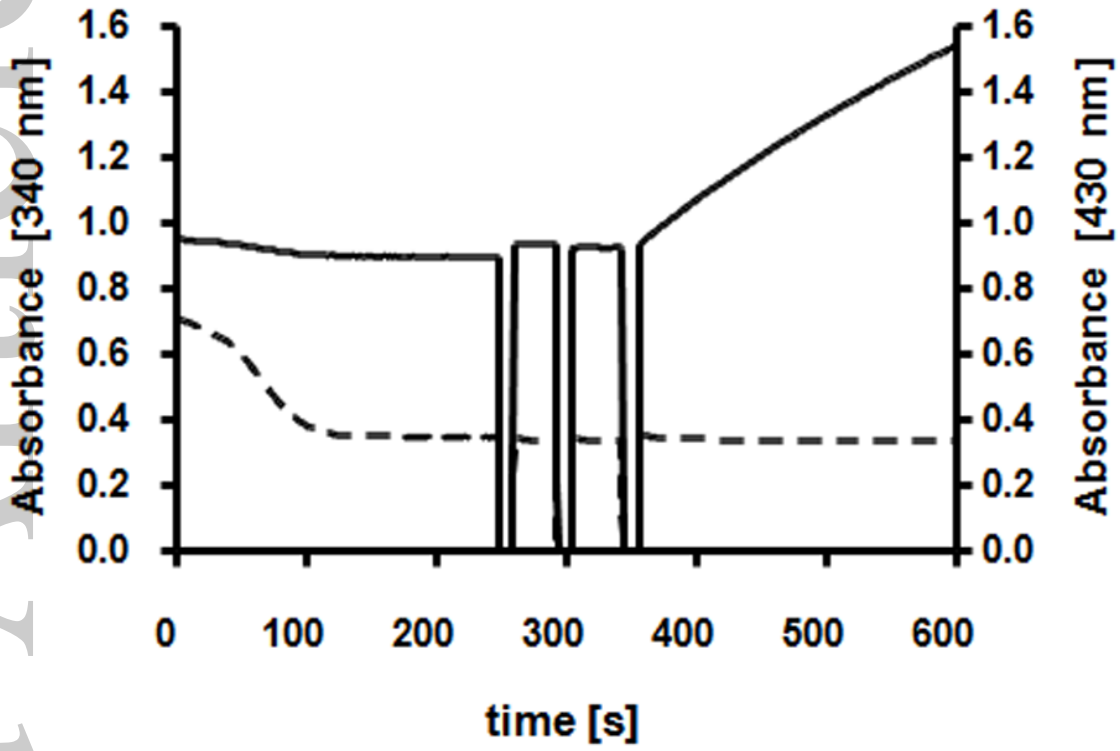

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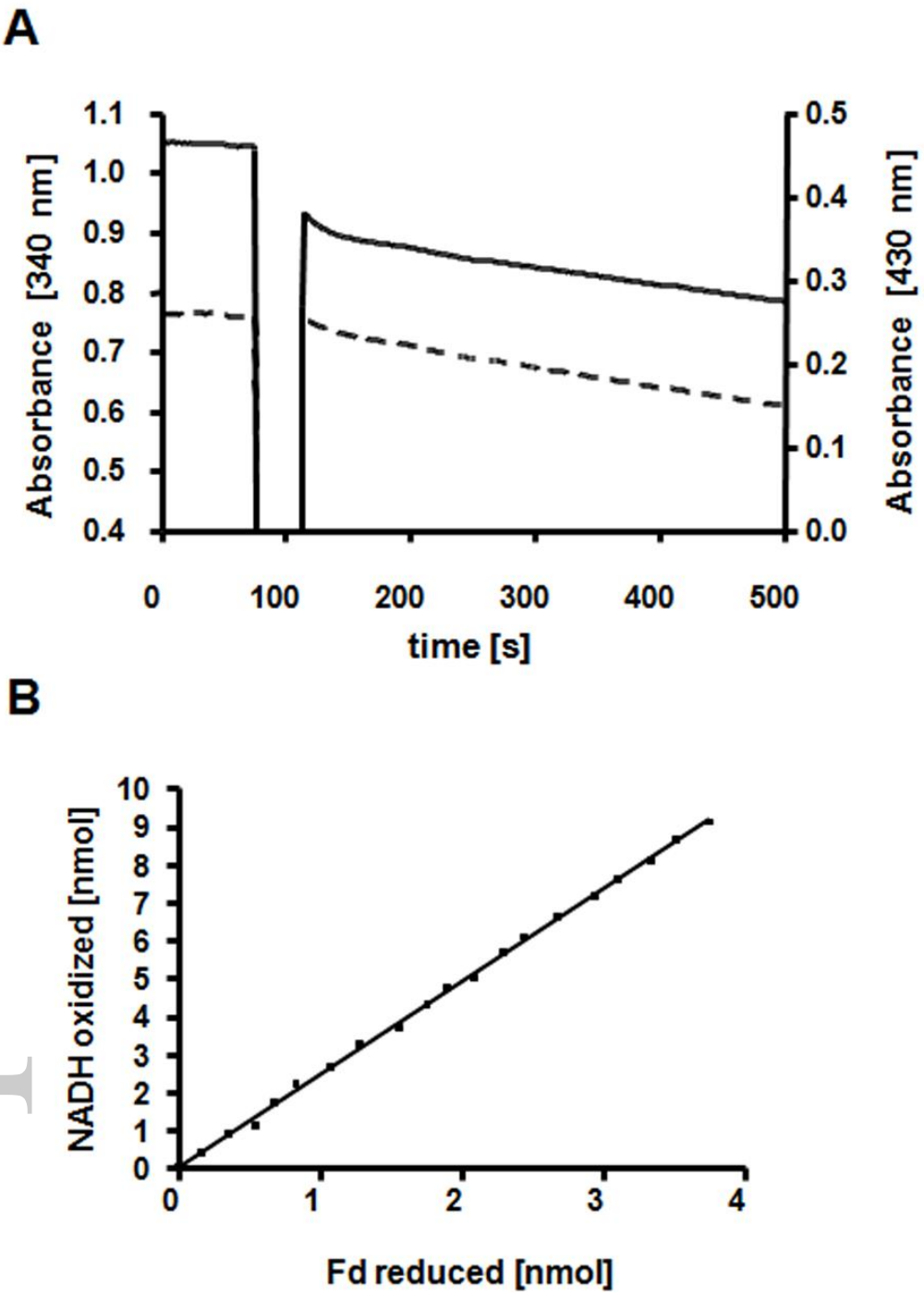

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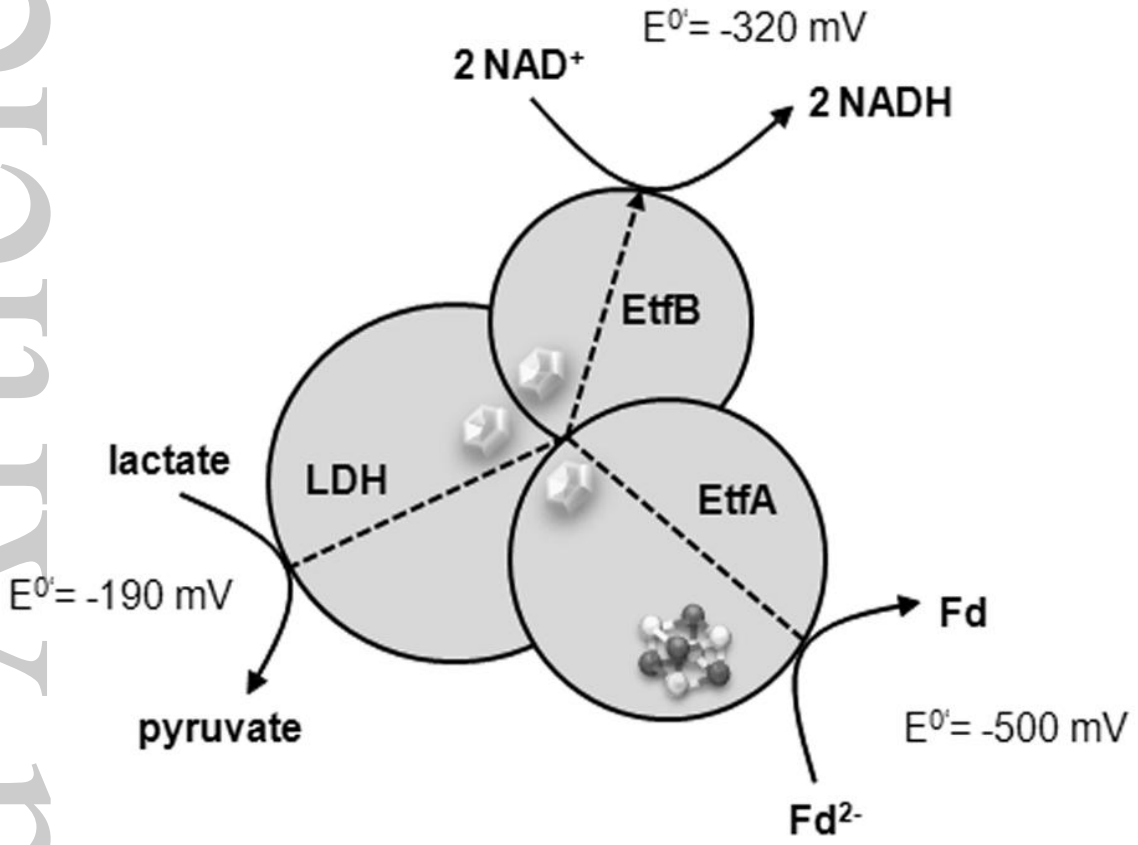

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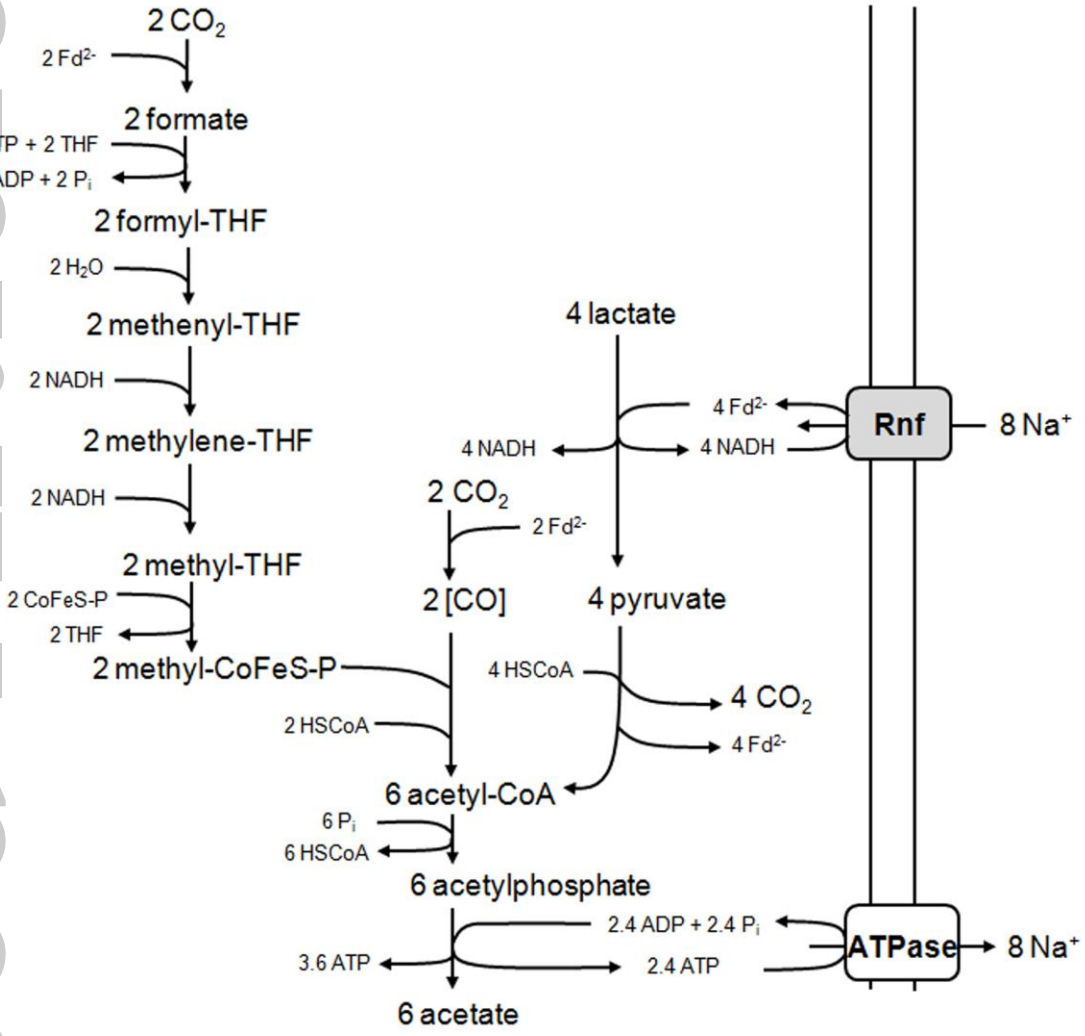

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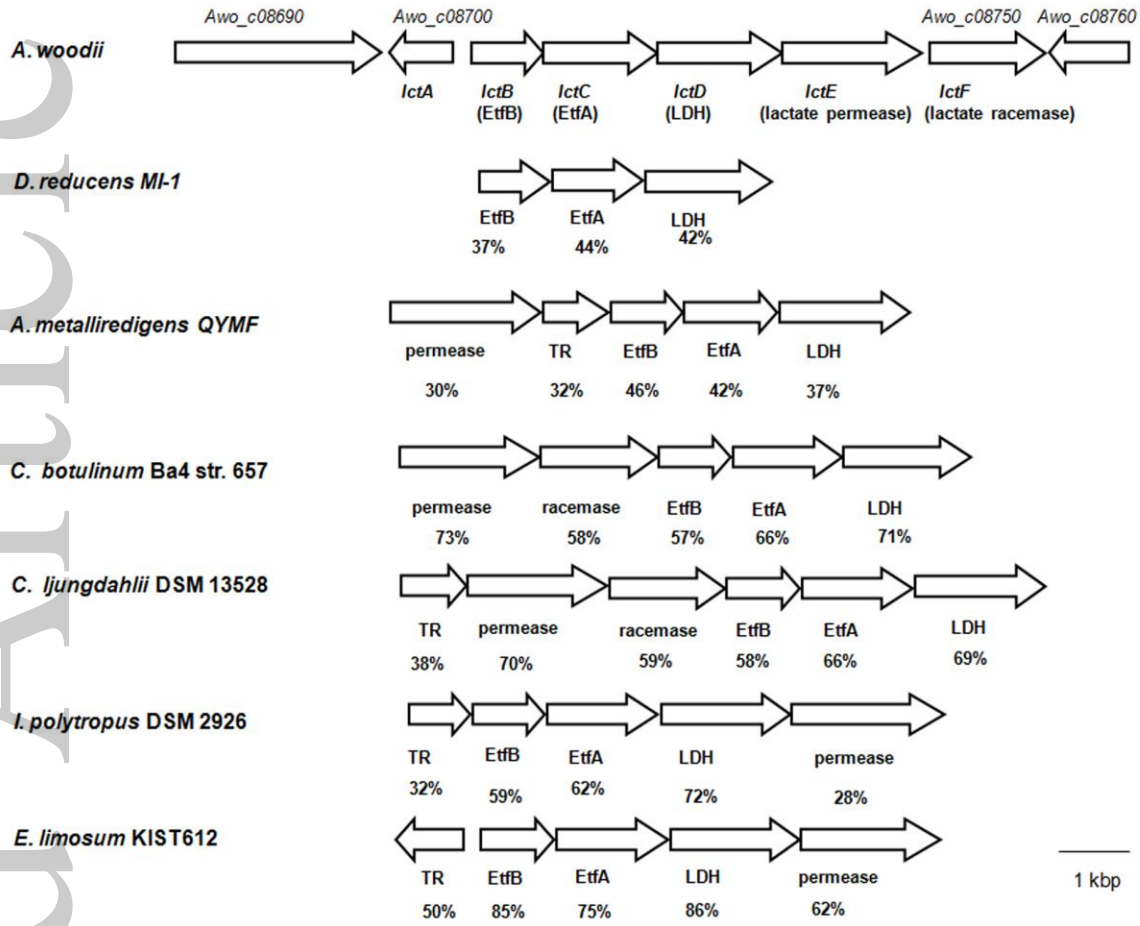

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