Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of Enterococcus faecalis, Lactococcus lactis, Azotobacter vinelandii and Escherichia coli: Implications for their activity in vivo

Jacky L. Snoep a, Mark R. de Graef a, Adrie H. Westphal b, Arie de Kok b, M. Joost Teixeira de Mattos *a and Oense M. Neijssel a

a Department of Microbiology, E.C. Slater Institute, Biocentrum, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, the Netherlands, and b Department of Biochemistry, Agricultural University, Wageningen, the Netherlands

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Abstract: The effect of NADH on the activity of the purified pyruvate dehydrogenase complexes (PDHc) of Enterococcus (Ec.) faecalis, Lactococcus lactis, Azotobacter vinelandii and Escherichia coli was determined in vitro. It was found that the PDHc of E. coli and L. lactis was active only at relatively low NADH/NAD ratios, whereas the PDHc of Ec. faecalis was inhibited only at high NADH/NAD ratios. The PDHc of Azotobacter vinelandii showed an intermediate sensitivity. The organisms were grown in chemostat culture under conditions that led to different intracellular NADH/NAD ratios and the PDHc activities in vivo could be calculated from the specific rates of product formation. Under anaerobic growth conditions, only Ec. faecalis expressed PDHc activity in vivo. The activities in vivo of the complexes of the different organisms were in good agreement with their properties determined in vitro. The physiological consequences of these results are discussed.

Key words: Enterococcus faecalis; Lactococcus lactis; Azotobacter vinelandii; Escherichia coli; NADH/NAD ratio; Enzyme activity in vivo

Introduction

In most microbial species, pyruvate is catabolized under aerobic conditions primarily via the pyruvate dehydrogenase complex (PDHc). PDHc occupies a key position between glycolysis and the citric acid cycle and it is generally thought to be active only under aerobic growth conditions. The complex is subject to regulation both at the level of gene expression [1,2] and of enzyme activity [3–5]. Surprisingly, however, in Enterococcus (Ec.) faecalis the PDHc was found to be active...
under certain anaerobic growth conditions [6]. It was suggested that the low sensitivity of this particular PDHc towards NADH was responsible for this unique activity [7]. Therefore we studied the sensitivity towards NADH of the purified complexes of *Ec. faecalis*, *Lactococcus lactis*, *Azotobacter vinelandii* and *Escherichia coli* and compared this to the activities of the PDHc in vivo in the different organisms grown in chemostat culture under various growth conditions that were supposed to cause large differences in the cells’ NADH/NAD ratios.

**Materials and Methods**

**Organisms**

*Enterococcus faecalis* NCTC 775, *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* c17 (a strain from the Netherlands Institute for Dairy Research collection) and *Escherichia coli* B ATCC 11303 were maintained on beads in a reinforced clostridial medium (Oxoid) with 50% (w/v) glycerol at −20°C. *Azotobacter vinelandii* ATCC 478 was maintained on simple salts agar plates.

**Growth conditions**

*Ec. faecalis* and *L. lactis* were cultured as described previously [6]. *E. coli* and *A. vinelandii* were cultured on the simple salts media as specified by Evans et al. [8] with nitrilo-triacetic acid as chelating agent. Carbon source-limiting conditions were obtained by adding approximately 50 mM glucose to the media under anaerobic conditions and 20 mM (35 mM for *A. vinelandii*) under aerobic conditions. When *A. vinelandii* was grown under nitrogen-fixing conditions, the molybdate concentration was increased to 2.5 mg 1−1 and 65–140 mM glucose was added to the medium. Cultures were stirred at 1000 rpm (unless stated otherwise) and the pH of the culture was maintained automatically ±0.1 pH unit by titration with 4 M NaOH. The temperature was set at 37°C for *E. coli* and *Ec. faecalis* and at 30°C for *L. lactis* and *A. vinelandii*. Silicone anti-foaming agent (BDH, Poole, UK) was added at regular time intervals so as to prevent excessive foaming. The pyruvate solution was set at pH 3.5 by the addition of NaOH and subsequently sterilised by filtration (Seitz filter plates, type EKS). Glucose was sterilised at 110°C.

**NADH / NAD ratios**

Concentrations of nucleotides were measured by extraction from a culture sample and subsequent assaying for the nucleotides in the neutralised, centrifuged extract, as described previously [6]. In cells grown under glucose excess conditions, the extraction of NADH was performed at room temperature at pH 12.5.

**NADH inhibition of the PDHc in vitro**

The purification of the PDHc from *Ec. faecalis* and from *L. lactis* has been described previously [7,9]. Overall activity of the PDHc was measured spectrophotometrically at 340 nm by the formation of NADH at 25°C according to Schwartz and Reed [5]. Sensitivity of the complexes from different organisms to NADH was determined by using various concentrations of NAD and NADH in the PDHc activity assay.

**Results and Discussion**

Previous experiments have shown that, in *Ec. faecalis* grown under anaerobic conditions with pyruvate as the energy source, the pyruvate dehydrogenase complex is active. This finding was unexpected because it is commonly thought (mainly based on observations made with *E. coli*) that this enzyme is inactive under anaerobic conditions due to inhibition by NADH. We therefore measured the sensitivity to NADH of the PDHc of *Ec. faecalis* and of other species of which the purified PDHc was available.

The isolated enzyme complexes showed marked differences in their sensitivity to NADH (Fig. 1). The PDHc of *E. coli* and *L. lactis* were most sensitive, whereas the PDHc of *Ec. faecalis* was inhibited only at high NADH/NAD ratios. The PDHc of *A. vinelandii* showed an intermediate sensitivity. Thus, in comparison with the other enzymes, the PDHc of *Ec. faecalis* is indeed the least sensitive to NADH.
Fig. 1. Relationship between ln ([NAD]/[NADH]) and activity of the pyruvate dehydrogenase complex of *Ec. faecalis* (○) (data from [7]), *L. lactis* (●) (data from [9]), *A. vinelandii* (▲) and *E. coli* (△). Activity is expressed as percentage of the activity that was found with the same NAD concentration, but with no NADH added.

To investigate whether these data were in agreement with the activity of PDHc in vivo, *Ec. faecalis*, *L. lactis*, *E. coli* and *A. vinelandii* were grown in chemostat cultures under conditions that were expected to cause different intracellular NADH/NAD ratios and the specific rates of product formation were determined (Table 1). Under anaerobic glucose-limited conditions, *Ec. faecalis* and *L. lactis* showed the well-known mixed-acid type of fermentation: acetate, ethanol, lactate, and formate were the main products of glucose catabolism. Aerobically, these species reacted differently: *Ec. faecalis* produced almost only acetate plus CO₂, whereas *L. lactis* produced more acetoin (Table 1). Under anaerobic conditions, *E. coli* showed also a mixed-acid type of fermentation, but with lactate production rates lower than those of the streptococci and the additional production of succinate (Table 1). Under aerobic conditions glucose was completely oxidised to CO₂ (Table 1). *A. vinelandii* was cultured glucose-limited both with ammonium added to the medium and without a fixed nitrogen source. The sole products that were formed were invariably CO₂ and biomass (Table 1). During nitrogen-fixing conditions a marked increase in steady state dry weight was observed upon increasing the stirring rate from 750 to 1000 rpm.

Table 1
Specific rates of substrate utilization and product formation and NADH/NAD ratios in aerobic and anaerobic glucose-limited chemostat cultures of *Ec. faecalis* (A, dilution rate (*D*) 0.3 h⁻¹), *L. lactis* (B, *D* = 0.2 h⁻¹), *E. coli* (C, *D* = 0.3 h⁻¹), and specific rates of substrate utilization and product formation and NADH/NAD ratios in chemostat cultures of *A. vinelandii* (D, *D* = 0.1 h⁻¹)

<table>
<thead>
<tr>
<th>O₂</th>
<th>q_Glc</th>
<th>q_Ac</th>
<th>q_EtOH</th>
<th>q_Lac</th>
<th>q_CO₂</th>
<th>q_O₂</th>
<th>q_For</th>
<th>q_Suc</th>
<th>q_Acet</th>
<th>NADH/NAD</th>
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<tr>
<td>A</td>
<td>−</td>
<td>−7.4</td>
<td>4.0</td>
<td>6.7</td>
<td>4.1</td>
<td>1.4</td>
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<td>8.1</td>
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<td>+</td>
<td>−6.6</td>
<td>7.7</td>
<td>0.0</td>
<td>3.0</td>
<td>11</td>
<td>−11</td>
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<td>0.3</td>
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<tr>
<td>B</td>
<td>−</td>
<td>−6.3</td>
<td>1.9</td>
<td>1.9</td>
<td>7.5</td>
<td>0.4</td>
<td>n.a.</td>
<td>3.9</td>
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<td>+</td>
<td>−5.2</td>
<td>1.9</td>
<td>0.0</td>
<td>1.4</td>
<td>9.6</td>
<td>−5.8</td>
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<tr>
<td>C</td>
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<td>−13</td>
<td>8.4</td>
<td>7.6</td>
<td>0.1</td>
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<td>D</td>
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For *L. lactis* pH = 6.0, all other pH = 7.0. Rates are expressed as mmol (g dry weight h⁻¹), consumption and production are indicated with negative and positive signs, respectively. Glc, glucose; Ac, acetate; EtOH, ethanol; Lac, lactate; For, formate; Acet, acetoin; Suc, succinate; n.a., not applicable. Data are means of three independent experiments, standard deviations in *q* values are < 5% of the mean, standard deviations in NADH/NAD ratios < 10%.

a Glucose-limited with ammonium as N-source.
b Nitrogen-fixing conditions at a stirring rate of 750 rpm.
c Nitrogen-fixing conditions at a stirring rate of 1000 rpm.
This suggests that at 750 rpm the oxygen supply rate was limiting the rate of nitrogen fixation. It should be realized that the demand for a high rate of ATP synthesis (i.e. a high respiration rate), a high rate of generation of reducing equivalents (for nitrogen reduction) and a low oxygen tension (to protect the nitrogenase) renders definition of the nature of the limiting nutrient under nitrogen fixing conditions highly complicated [10].

From Table 1 it can be seen that in *E. faecalis* the PDHc was active in vivo under anaerobic conditions, but not in *L. lactis*. In the latter organism, all acetyl-CoA was formed via pyruvate formate lyase (PFL). Under aerobic conditions, PFL was inactive and pyruvate oxidation occurred solely via PDHc. In *E. coli*, the activity in vivo of PDHc cannot be calculated as with the lactic acid bacteria due to conversion of formate to H₂ and CO₂ via formate-hydrogen lyase. However, a distinction can be made indirectly between the PFL and the PDHc activity as an extra mol NADH is formed via the latter which must be reoxidized (e.g. by the formation of ethanol). Redox balances of 92 and 98% were calculated on the assumption that PDHc had not been active. Hence, the flux via the PDHc must have been very low. Under aerobic conditions the situation is the same as in all the other organisms: pyruvate oxidation occurred solely via the PDHc.

Intracellular NADH/NAD ratios were measured to determine whether this factor is also an important regulatory parameter in vivo. These ratios did not differ much in the different organisms when they were cultured under comparable conditions (Table 1). Under anaerobic conditions the measured NADH/NAD ratio was too high for the PDHc of *E. coli* and *L. lactis* to be active (c.f. Fig. 1). However, under aerobic conditions a NADH/NAD ratio was measured in these organisms that was inhibitory in vitro, but in vivo the enzyme complex was still active. This can be explained by a direct reaction of oxygen with the complex, because it is known that inhibition by NADH is due to an overreduction of a component (E3) of the complex [11]. A direct reaction with oxygen [12] would prevent this overreduction.

In *A. vinelandii*, very high NADH/NAD ratios were measured when the organism was grown under nitrogen-fixing conditions. Nevertheless, a high catabolic rate via the PDHc was sustained. One may speculate that under nitrogen-fixing conditions the vicinity of nitrogenase is kept essentially anaerobically, whereas other parts would be more aerobic. Inevitably, this would affect the NADH/NAD ratio locally. Hence, no conclusions about the ratio in the vicinity of PDHc could be drawn from the data presented.

Clearly, the various organisms studied contain pyruvate dehydrogenase complexes that are vastly different with regards to their sensitivity towards NADH. As PDHc plays a major role in the carbon metabolism, it is to be expected that the observed differences have some physiological relevance. With the obligately aerobic *A. vinelandii*, growing under N₂ fixing conditions, a high reducing capacity (i.e. a high NADH/NAD ratio) is needed: firstly to reduce molecular nitrogen; secondly to maintain a high respiration rate to meet the high energetic demands of nitrogen fixation; and thirdly to establish a virtually anaerobic environment for the extremely oxygen-sensitive nitrogenase [10]. This can be achieved by maintaining a high glycolytic flux, provided that PDHc is relatively insensitive.

Of the four organisms studied, *E. coli* contained the most sensitive PDHc. This organism is very well adapted to both respiratory and fermentative conditions and clearly the PDHc is limited to the former conditions and is functionally replaced by the PFL under the latter. In *L. lactis*, with a PDHc that is almost as sensitive as the *E. coli* PDHc, a similar mechanism is found in which PDHc activity is taken over by PFL and LDH under anaerobic conditions. In contrast to all the other organisms tested, in *Ec. faecalis* the PDHc is active under anaerobic conditions as well as under aerobic conditions, which is in agreement with its lower sensitivity to NADH. This special characteristic endows *Ec. faecalis* with a great flexibility even compared to an organism like *E. coli*, as is reflected in its responses to shifts between aerobic and anaerobic growth conditions. We have shown that, due to its expression of PDHc under anaerobic conditions, *Ec. faecalis*
can adapt rapidly to a shift to aerobic conditions [13]. As PFL cannot be active aerobically, *E. coli* cannot continue its pyruvate catabolism upon such a shift but first has to synthesize PDHc. This results in transient pyruvate excretion (data not shown) and hence in a highly inefficient use of the energy source.

**References**