Regulation of pyruvate catabolism in Escherichia coli: the role of redox environment

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Chapter 2

The steady state internal redox state (NADH/NAD) reflects the external redox state and correlates to catabolic adaptation in *Escherichia coli*.

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Chapter 2

**ABSTRACT**

*Escherichia coli* (MC4100) has been grown in anaerobic glucose-limited chemostat cultures, either in the presence of an electron acceptor (fumarate, nitrate or oxygen) or fully fermentative. The steady state NADH/NAD ratio was found to be related to the presence and nature of the electron acceptor. Anaerobically the ratio was highest and gradually decreased with increasing midpoint potential of the electron acceptor.

As pyruvate catabolism is a major switchpoint between fermentative and respiratory metabolism, the fluxes through the different pyruvate consuming enzymes were calculated.

In anaerobic cultures with fumarate or nitrate as an electron acceptor, a flux through the pyruvate dehydrogenase complex was calculated, a finding which is in contrast to the general assumption that the complex cannot be active under these conditions. *In vitro* activity measurements of PDHc showed the cellular content of the enzyme to vary with the internal redox state.

Whereas western blots showed the E3 subunit (dihydrolipoamide dehydrogenase) not to vary dramatically under the conditions tested, the amount of the E2 subunit (dihydrolipoamide acetyltransferase) amount followed the trend that was found for the *in vitro* PDHc activity. From this it is concluded that regulation of PDHc expression is exerted on the E1/E2 operon (*aceEF*). We propose that the internal redox state is reflected by the external redox state. The latter may subsequently govern both expression and activity of the two pyruvate catabolizing enzymes: pyruvate formate lyase (PFL) and PDHc.
INTRODUCTION

In Enterobacteriaceae—as in many prokaryotes and all eukaryotes—the nucleotides NAD and NADH play a central role in catabolism. They function as the most important redox carriers involved in metabolism. These nucleotides not only serve as electron acceptors in the breakdown of catabolic substrates, but in addition provide the cell with the reducing power needed in energy conserving redox reactions such as occur in anaerobic and aerobic respiration. A balance in the rates of oxidation and reduction of these nucleotides is a prerequisite for continuation of both catabolism and anabolism since the turnover of the nucleotides is very high, compared to their concentrations. Whereas for a given carbon and energy source, catabolic NADH formation occurs under all conditions by a rather limited set of redox reactions (e.g. glycolysis), a wide variety of mechanisms has evolved to fulfill the requirement of NADH reoxidation. Thus, in many bacterial species, e.g. *E. coli*, a variety of compounds can serve as acceptor of the electrons from NADH. These acceptors may either be present in the environment (external acceptors) or they may be generated intracellularly. Electron transfer may occur either in a cytoplasmatic, non-vectorial process or in a membrane-bound vectorial process. The former reactions (fermentation) result in the reoxidation of NADH and the formation of reduced compounds only, whereas with the latter NADH oxidation may be coupled to the conservation of free energy (respiration).

The regulatory mechanisms that underlay the expression of genes coding the enzymes specific to respiration and fermentation have been studied extensively (Iuchi and Weiner, 1996; Lynch and Lin, 1996). In *E. coli* these genes are under control of at least 3 global regulators which exert their effects in dependence of the redox environment of the cell. These are, firstly, FNR which is involved in the regulation of expression of some fermentation related enzymes (Spiro and Guest, 1990) and secondly the two-component regulatory systems Nar (Stewart, 1993) and Arc (Iuchi and Lin, 1993).

FNR can function as both an activator and repressor of many anaerobically controlled genes. Its regulatory mechanism is thought to reside in binding to the promoter regions of the relevant genes with affinities that depend on the redox state of the cystein rich N-terminus (Green and Guest, 1993).

Nar, which serves primarily as a nitrate sensing system (Stewart, 1993)
belongs to the two component redox regulation systems. It comprises a membrane spanning sensor (NarX) that may act as a kinase under the proper environmental conditions, causing phosphorylation of the regulator (NarL). This regulator activates transcription of nitrate reductase genes and represses the fumarate reductase gene (see for a review see Stewart, 1993). The Arc system also belongs to the two component regulation systems. In its active form it mainly represses enzymes of aerobic catabolism (e.g. the TCA cycle and the respiratory chain) (Iuchi and Lin, 1993). Basically, the mechanism of this system involves a transphosphorylation from the sensor ArcB to the regulator ArcA although no precise mechanism has been put forward unequivocally (Iuchi, 1993; Tsuzuki, 1995). Although in vitro studies have shown that both lactate and NADH stimulate the activation (phosphorylation) of Arc (Iuchi, 1993; Iuchi et al., 1994), it is not known what the biochemical signal is that results in activation of the system.

Pyruvate is a key intermediate in the catabolism of *E. coli* and is for most if not all free energy sources a common product, irrespective of environmental conditions. Its subsequent conversion by either pyruvate formate lyase (PFL) or the pyruvate dehydrogenase complex (PDHc) can be considered as a major switch point between fermentative routes (mixed acid fermentation) and oxidative routes (the citric acid cycle and subsequent respiration).

Both PFL and PDHc have been subject to extensive molecular studies (Guest et al., 1989; Knappe and Sawers, 1990; Mattevi et al., 1992). In *E. coli* expression of PFL is regulated by the Fnr and Arc systems whereas PDHc synthesis is regulated by the Arc system (Iuchi and Lin, 1988; Quail et al., 1994).

It has been assumed for many years that the PDHc could not be active under anaerobic conditions. The absence of PDHc-activity under anaerobic conditions in *E. coli* has been explained by the high sensitivity towards NADH inhibition (Hansen and Henning, 1966). However, in *E. faecalis* it was found that the distribution over PDHc and PFL of the catabolic flux correlated with the in vivo steady state redox potential of the NADH/NAD couple (Snoep et al., 1990). Thus, in this organism even under anaerobic conditions high in vivo activities of the PDHc were found provided that growth conditions were such that the steady state NADH/NAD ratio was sufficiently low (Snoep et al., 1991). This, for example could be achieved
Redox sensing

when pyruvate was used as the sole energy source.

In contrast to *E. faecalis*, *E. coli* is capable of both anaerobic (nitrate and fumarate) and aerobic respiration. It is to be expected that these respiration types affect the redox state of the cell to various extents and hence possibly the level and/or activity of the PDHc. Indeed, Kaiser and Sawers (1994) provided circumstantial evidence that the pyruvate dehydrogenase complex of *E. coli* can be active anaerobically when the cells are provided with nitrate as an external electron acceptor.

Here we report on the effect of different electron acceptors on the steady state metabolic fluxes in *E. coli* grown in glucose-limited chemostat cultures. For all conditions, the steady state flux distribution through PFL and PDHc rates were determined for both wild type cells and mutant strains lacking PFL or PDHc. In addition, the steady state cellular redox state (as reflected by the NADH/NAD ratio) and *in vitro* activity of PDHc were determined. The steady state NADH/NAD ratio appeared to be dependent on the nature of external electron acceptors. It is demonstrated that synthesis and activity of the PDHc does occur under anaerobic respiratory conditions and that both correlate with the cellular steady state redox state.

**MATERIAL AND METHODS**

*Escherichia coli* strains and growth conditions

MC4100  F* araD139 (argF-lac) U169 rpsL150 relA1 deoC1 flb-5301 ptsF* (Casabadan and Cohen, 1979) ('wild type')

RM201 see MC4100 Δpfl-25 Ω(pfl::cat pACYC184) (Sawers and Böck, 1988)

RM319 see MC4100 Δ(aroP-aceEF) (Kaiser and Sawers, 1994)

The strains were maintained on beads in LB medium with 50% (w/v) glycerol at -20°C.

Organisms were cultured in a 700 ml fermentor, Modular Fermentor Series III (L.H. Engineering Co. Lt, England). Growth media were simple salts media as specified by Evans *et al.* (1970) but instead of citrate, nitrilotriacetic acid (2 mM) was used as chelator. Selenite (30 μg/l) and thiamine (15 mg/l) were added to the medium. In cultures of RM201 without an external electron acceptor, 5 mM acetate was added to the medium. The dilution rate was set at 0.10 h⁻¹. The pH value of the culture was maintained at 6.5±0.1 using sterile 4M NaOH, in cultures of RM201
without an external electron acceptor 1M Na$_2$CO$_3$ was added to the NaOH, to supply the culture with CO$_2$. The temperature was set to 35°C. Cultures were stirred at 1000 rpm. To prevent excessive foaming silicone antifoaming agent (BDH; 1% w/v) was added at a rate of approximately 0.5 ml h$^{-1}$. Anaerobiosis was maintained by the method described previously (Teixeira de Mattos et al., 1983).

**Analyses**

Steady state bacterial dry weight was measured by the procedure of Herbert et al. (1971). Glucose, pyruvate, lactate, formate, acetate, succinate and ethanol were determined by HPLC (LKB) with an Aminex HPX 87H organic acid analysis column (Biorad) at a temperature of 65°C with 5 mM H$_2$SO$_4$ as eluent, using a 2142 refractive index detector (LKB) and an SP 4270 integrator (Spectra Physics). CO$_2$ production or O$_2$ consumption was measured by passing the effluent gas from the fermentor through a Servomex CO$_2$ analyzer and a Servomex O$_2$ analyzer.

**Enzyme activities**

**In vitro**

To obtain cell free extracts, cells were taken from a steady state culture, centrifuged (3020xg, 10 min), washed twice with 50 mM sodium phosphate buffer (pH 7.0), and sonified using a Branson Sonifier 250 (4 minutes, duty cycle 50%, output control 35%). The cell debris was removed by centrifugation (12100xg, 15 min).

The overall activity of the PDHc was measured in a standard reaction mixture containing 50mM sodium phosphate (pH 7.0), 5 mM pyruvate, 12.5 mM MgCl$_2$, 0.18 mM thiamine pyrophosphate (TPP), 0.175 mM coenzyme A, 2 mM NAD, 1 mM potassium ferricyanide. The reaction was started by the addition of cell free extract and the initial rate was monitored spectrophotometrically by following the reduction of ferricyanide at 430 nm ($e=1030$ M$^{-1}$cm$^{-1}$).

The activity of the E2 component (dihydrolipoamide acetyltransferase) of the PDHc was measured in a standard reaction mixture containing 10 mM acetylphosphate, 4 mM lipoamide(SH)$_2$NH$_2$, 0.13 mM CoA and 2 units PTA (phospho-trans-acetylase) in 50 mM Tris (pH 7.0). The reaction was started by the addition of cell free extract and the initial rate was monitored
following the production of acetyl lipoate at 240nm ($\epsilon=5000 \, M^{-1} \, cm^{-1}$).

**Western blotting**

Equal amounts of cell free extracts of chemostat cultures of *E. coli* were loaded and run on a SDS-PAGE gel (12.5%). The gel was then incubated in a transfer buffer (20mM TrisCl, pH 8.7; 150 mM glycine; 20% methanol) for 30 minutes. The proteins were blotted on a nitrocellulose filter by electrophoretic transfer. The membrane was then floated in TBS (10mM TrisCl, pH 7.6; 150 mM NaCl). Excess protein binding sites were blocked by TBST (TBS + 0.05% Tween 20) and incubated in TBST with 1% BSA (Blot Qualified). Next, the primary antibody was applied to the filter for 30 minutes. The membrane was washed in TBST for 5 minutes to remove unbound antibody. After this the secondary antibody was applied (anti-IgG alkaline phosphatase conjugate) for 30 minutes. The membrane was washed again in TBST for 5 minutes to remove unbound antibody and subsequently dried on filter paper. The color reaction was started by transferring the membrane to the color development solution (NBT substrate in AP buffer (100 mM TrisHCl, pH9.5; 100 mM NaCl, 5 mM MgCl$_2$ mixed with BCIP substrate). Within 1-15 minutes the color developed and the reaction was stopped by rinsing the filter in deionized water.

**NADH and NAD$^+$**

Levels of nucleotides were measured by first extracting the nucleotides from a culture sample and then assaying for the nucleotides in the neutralized, filtered extract, as described previously (Snoep et al., 1990)

**RESULTS**

**Catabolic fluxes**

*Anaerobic conditions without external electron acceptors*

All *E. coli* strains were grown in glucose-limited chemostat cultures at a dilution rate of 0.1 h$^{-1}$, pH 6.5. Anaerobic growth of strain RM 201 (PFL mutant) was only observed when the culture medium was supplied with CO$_2$ and acetate. Presumably this is due to the need for C$_2$-compounds for biosynthetic purposes (Varenne et al., 1975). Therefore with this strain carbonate was used as a titrant and acetate (5 mM) was added to the medium. For all
Fig. 2.1 Production rates of lactate, acetate and ethanol in anaerobic glucose limited chemostat cultures of *E. coli* MC4100, RM201 and RM319 respectively. The cells were grown at a dilution rate of 0.1 h⁻¹ at pH 6.5, T=35°C.

Fig. 2.2 Production rates of lactate, acetate ethanol and electron flow rates (accept) in anaerobic glucose limited chemostat cultures of *E. coli* MC4100, RM201 and RM319 respectively with 25 mM fumarate added to the medium. The cells were grown at a dilution rate of 0.1 h⁻¹ at pH 6.5, T=35°C.

Fig. 2.3 Production rates of lactate, acetate ethanol and electron flow rates (accept) in anaerobic glucose limited chemostat cultures of *E. coli* MC4100, RM201 and RM319 respectively with 5 mM nitrate added to the medium. The cells were grown at a dilution rate of 0.1 h⁻¹ at pH 6.5, T=35°C.
cultures, carbon balances of 100 ±10 % could be calculated on the basis of glucose consumption rates and product formation rates (fig 2.1). The main fermentation product of strain RM 201 was found to be lactate, confirming the absence of PFL and indicating that under these conditions PDHc was not active. It should be noted here that the specific rate of succinate production was invariably found to be the same for all strains (0.8 mmol/g. dry weight/h). Lactate formation from glucose is a redox neutral process and by taking into account that the overall metabolism must fulfill the demand of redox neutrality, it must be concluded that the summation of all NAD(H) dependent reactions leading to succinate and biomass add up to a closed redox balance as well. This is confirmed by the observation that strain RM319 produced ethanol and acetate in equimolar amounts. This must have occurred obviously via PFL (the strain lacks PDHc) which, again, is an overall redox neutral process. Similarly, it can be concluded that in wild type *E. coli* no *in vivo* activity of PDHc occurs (fig 2.4a).

**Anaerobic conditions with fumarate or nitrate as electron acceptor**

In the presence of fumarate (25 mM medium concentration) a significant shift in the relative production rates was observed (fig 2.2) as compared to fermentative conditions. Succinate production rates (from glucose) similar to those observed under fermentative conditions were measured (data not shown). With all strains it was found that the fumarate added to the medium was fully reduced to succinate thus providing the cells with an additional NADH sink. When this NADH oxidizing flux is included in the calculation of the redox balance, it follows that for the wild type cells breakdown of pyruvate solely by PFL would not provide sufficient reducing equivalents to allow for the observed fumarate reduction rate since in that case the excess of acetate production relative to ethanol should equal the fumarate reduction rate. Indeed, this was observed for RM 319, which can produce acetate solely via PFL, the difference between the specific production rates of acetate and ethanol equaled the fumarate reduction rate. As in the wild type the difference between acetate and ethanol production rates is less than the fumarate reduction rates, an additional NADH generating pathway must have been operational to feed fumarate reduction. We conclude that here PDHc activity provides the reducing equivalents to fumarate respiration.
Further support for this conclusion is provided by the observation that now strain RM 201 produces acetate rather than being dependent for its growth on the availability in the medium as acetate production in the absence of PFL can only be ascribed to PDHc activity. On the basis of the abovementioned argument, the \textit{in vivo} fluxes via the PDHc have been calculated and the results are presented in fig. 2.4b.

A similar approach was followed by analyzing the relative fluxes to end products when the three strains where grown anaerobically in glucose-limited chemostat cultures with 5 mM nitrate added to the medium. Specific product formation and nitrate utilization rates are given in fig 2.3. The results show that the presence of nitrate as an electron acceptor resulted in a shift in flux distribution over PFL and PDHc qualitatively similar to the shift found with fumarate. Again, the observed acetate production by strain RM 201 can only be ascribed to the activity of PDHc whereas in wild type cells \textit{in vivo} PDHc activity can be inferred from the difference between acetate and ethanol production being lower than the rate of NADH oxidation by nitrate reduction. In contrast, with the control strain RM 319 this difference was found to be in accordance with the NADH oxidation rate via nitrate respiration assuming that here no flux through PDHc occurred.
**Growth in the presence of oxygen**

Under fully aerobic conditions, the PFL is de-activated (Knappe and Sawers, 1990) and the conversion of pyruvate is solely catalyzed by the PDHc. In table 2.1 the specific consumption and production rates in aerobic chemostat cultures are given. As expected with glucose-limited cultures (Holms, 1996), with the wild type strain all glucose is catabolized completely to CO_2 and the same was found for *E. coli* RM 201. Strain RM 319 behaved quite differently. Lacking the ability to feed the TCA cycle with acetyl-CoA, part of the glycolytically formed pyruvate was reduced to lactate, but apparently the activity of the lactate dehydrogenase was not sufficient to cope with the glycolytic flux to pyruvate: high concentrations of pyruvate were found in the effluent (up to 50 mM). The question remains as to how CO_2 is formed in these cultures but it may well be that this is produced via the pentose phosphate pathway. This would yield also the necessary C_2 compounds needed for biosynthesis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>q_{glc}</th>
<th>q_{pyr}</th>
<th>q_{lac}</th>
<th>q_{CO2}</th>
<th>q_{O_2}</th>
</tr>
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<tbody>
<tr>
<td>MC4100</td>
<td>-1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>RM201</td>
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<td>0.0</td>
<td>0.0</td>
<td>4.8</td>
<td>4.6</td>
</tr>
<tr>
<td>RM319</td>
<td>-8.1</td>
<td>8.0</td>
<td>3.8</td>
<td>5.7</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 2.1. Specific rates of substrate utilization and product formation in glucose limited chemostat cultures of various *E. coli* strains grown aerobically at D=0.1 h^{-1}, pH 6.5. The specific rates are expressed in mmol (g dry wt)^{-1} h^{-1}. glc, glucose; pyr, pyruvate; lac, lactate;

**Internal redox state**

For all growth conditions tested, the steady state internal redox state, as reflected by the NADH/NAD ratio, was determined. The results are shown in fig. 2.5. For all strains, the highest ratios were seen during fermentative growth, irrespective of the route of pyruvate conversion. Fumarate respiration resulted in a slightly lower NADH/NAD ratio and conditions of nitrate respiration resulted in a further lowering of the ratio. It was observed that a further increase of the nitrate concentration in the growth medium (7.5 mM) resulted in an even lower NADH/NAD ratio (0.27), but it should
be stressed that no steady state could be obtained. When the cells were grown under fully aerobic conditions, the NADH/NAD ratio dropped to values roughly tenfold lower than found for fermenting cells. For strain RM 319, however, a higher ratio was observed.

![Graph showing NADH/NAD ratios in E. coli MC 4100, RM 201, and RM 319 grown in glucose limited chemostat cultures (D=0.1, pH=6.5) with no acceptor, 25 mM fumarate, 5 mM nitrate or fully aerobic. The values are the means of at least 4 independent measurements. ND, not determined.]

*PDHc subunit expression and in vitro activity.*

In addition to the physiological characterizations given above, the overall in vitro activity of PDHc was determined for a number of growth conditions. It can be seen (table 2.2) that the activity —i.e. the cellular content of PDHc— increased when growth occurred in the presence of fumarate and even more so with nitrate and was highest under fully aerobic conditions. Furthermore, a similar trend was found with regard to the expression of dihydrolipoamide

<table>
<thead>
<tr>
<th></th>
<th>anaerobic</th>
<th>fumarate</th>
<th>nitrate</th>
<th>aerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM201</td>
<td>26±2.1</td>
<td>42±3.1</td>
<td>83±4.2</td>
<td>131±8.5</td>
</tr>
<tr>
<td>MC4100</td>
<td>22±1.4</td>
<td>30±5.3</td>
<td>67±4.6</td>
<td>128±6.7</td>
</tr>
</tbody>
</table>

*Table 2.2: In vitro measurements of the PDHc (in nmol ferricyanide reduced/min/mg protein) of Escherichia coli RM201 and MC4100 cell free extract. Cells were obtained from anaerobic glucose limited chemostat cultures with nitrate (5mM) fumarate (25mM), no electron acceptor, or aerobically (pH 6.5, D 0.1 h⁻¹).*
transacetylase (the E2 subunit of PDHc) as determined for a limited number of growth conditions by specifically assaying the activity of this subunit (table 2.3). The control strain RM 319 showed no activity (data not shown) whereas for both other strains it was found that aerobiosis resulted in a four- to fivefold increase in E2-expression as compared to fermentative conditions.

Finally, the expression of the E3 (dihydrolipoamide dehydrogenase) subunit of the PDH complex was specifically assayed by Western blotting of cell free extracts of cells grown under the conditions as outlined above (figure 2.6). Surprisingly, no significant difference for the E3 component was observed for any condition nor when comparing the strains. It should be noted that E. coli RM 319 is mutated in the E1/E2 component (Kaiser and Sawers, 1994) and that the E3 component is under control of a specific promoter. So, even in anaerobic cells the E3 subunit is expressed, although there is no in vivo PDHc activity in these cultures.

**Table 2.3. In vitro measurements of the E2 component of the PDHc (in nmol acetyl lipoate formed/min / mg protein) of Escherichia coli MC4100 or RM201 cell free extracts. Cells were obtained from anaerobic chemostat cultures with nitrate (5mM), fumarate (12mM), no electron acceptor or aerobic chemostat culture. (pH 6.5, D 0.1 h⁻¹).**

<table>
<thead>
<tr>
<th></th>
<th>anaerobic</th>
<th>fumarate</th>
<th>nitrate</th>
<th>aerobic</th>
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</thead>
<tbody>
<tr>
<td>MC 4100</td>
<td>24±5.4</td>
<td>ND</td>
<td>ND</td>
<td>98±5.8</td>
</tr>
<tr>
<td>RM 201</td>
<td>25±2.1</td>
<td>51±4.1</td>
<td>85±3.2</td>
<td>116±6.2</td>
</tr>
</tbody>
</table>

**fig 2.6:** Western blot analysis of cell free extracts of different chemostat cultures of E. coli strains with antibody against the E3 subunit of the PDHc. lane 1: RM 319, aerobic; lane 2: MC 4100, anaerobic with fumarate; lane 3: MC 4100 anaerobic with nitrate; lane 4: idem; lane 5: MC 4100 anaerobic; lane 6: RM 201 aerobic; lane 7: RM 201 anaerobic with nitrate; lane 8: idem; lane 9: anaerobic with fumarate; lane 10: RM 201 anaerobic; lane 11 idem lane 12: MC 4100 aerobic.
DISCUSSION

In facultatively aerobic organisms, pyruvate conversion constitutes a major branching point between the fermentative and the respiratory mode of catabolism. Central to the relative flux distributions at this point are the relative \textit{in vivo} activities of pyruvate dehydrogenase on the one hand and pyruvate formate lyase on the other. In this paper the \textit{in vivo} flux distribution through these enzymes has been compared in dependence of the availability of electron acceptors. It is concluded firstly that the internal steady state redox state of the cell - as reflected by the NADH/NAD ratio - is strongly influenced by the availability and nature of external electron acceptors. A similar finding had already been made with \textit{Enterococcus faecalis} (Snoep \textit{et al.}, 1990). Secondly, a positive correlation is established between the redox state and the PDHc flux: under more oxidizing conditions the flux is higher.

It has been assumed that in Enterobacteriaceae, PDHc is active aerobically only (Guest \textit{et al.} 1989) but from the flux calculations presented here, the conclusion can be drawn that in \textit{E. coli} this enzyme complex can contribute to catabolism in the absence of oxygen as has been suggested before by Kaiser and Sawers (1994). Hence, the general conclusion is valid that PDHc activity is not dependent on the presence of oxygen \textit{per se} but rather on the external redox condition. Our studies suggest further that the cellular response to the redox state of the environment is mediated by the internal redox state.

Our conclusion that the \textit{in vivo} activity of the PDHc is controlled by the cell's redox state is in agreement with the finding that the rate of an NAD dependent reaction is related to the actual redox potential of the acceptor. More importantly, however, is the fact that our results indicate that control on PDHc synthesis is exerted by a redox dependent regulation of E2 synthesis. Whether the regulation is exerted by NADH \textit{per se} cannot be concluded here but it should be remarked that such a type of regulation resembles strongly the proposition made for the role of the NADH/NAD ratio with regard to the synthesis of alcohol dehydrogenase (Leonardo \textit{et al.}, 1993,1996). Considering the steps involved in pyruvate oxidation by PDHc, it may seem surprising that it was observed that the synthesis of the E3 subunit, which catalyzes a redox dependent reaction, apparently is not affected by the redox state of the cell. However, a possible explanation may
be that E3 is also involved in the synthesis of branched-chain amino acid synthesis (Randle et al., 1987; Reed and Yeaman, 1987) and hence the anabolic activity would be hampered if synthesis of this subunit would be redox dependent.

The question remains by what mechanism PDHc synthesis is repressed and/or activated. In this context, it is interesting to note that Iuchi (1993) found that in vitro phosphorylation of the sensor (ArcB) of the ArcA/B two component regulatory system is enhanced by NADH. In addition, it is known (Quail et al., 1994) that PDHc expression is repressed by a phosphorylation cascade via the Arc system whereas derepression occurs when the Arc system is in its unphosphorylated form. This is compatible with the results presented here as well as with Iuchi’s finding (Iuchi, 1993) that nitrate addition to anaerobic cultures resulted in the induction of succinate dehydrogenase and flavin-linked D-lactate dehydrogenase (both enzymes typically linked to respiration, the former playing a role in the tricarboxylic acid cycle and the latter being flavin dependent). It may very well be that under conditions in which reoxidation of NADH is hampered by a lack of electron acceptor (fermentative catabolism) or occurs at a decreased rate in the presence of acceptors with a lower midpoint potential (anaerobic respiration) build up of reduced nucleotides serves as a signal for the repression of PDHc via the Arc system. It should not be excluded that other catabolic intermediates have a signalling role and that the presumed effect of NAD(H) is an indirect one. Considering kinetic effects of a changed NAD(H) level on the various enzymes involved, one may expect the changes in the availability of acceptors to be accompanied by changes in intracellular concentrations of intermediates such as pyruvate, lactate and acetyl-CoA. Interestingly, these metabolites have been proposed by Iuchi (Iuchi, 1993) as having a signalling function for the ArcA/B system. Whether this is indeed the case is currently under investigation. However, in view of the fact that pyridine nucleotides play a central role in both respiration (as electron donors to the respiratory chain) and fermentation (as they govern product formation by the demand of redox neutrality) it seems logical to attribute to them a regulatory role in the synthesis of those enzymes for which they serve as a substrate.

In principle, the carbon fluxes from pyruvate can be distributed over three enzymes: PFL, PDHc and LDH. The absence of in vivo flux via the LDH in
MC4100 and RM319 under anaerobic conditions is puzzling. In vitro activity of the enzyme is high (a rate of ca. 400 nmol/min/mg protein was measured in cell free extracts under saturating conditions) and pyruvate and NADH are available (2 mM (1) and 1.4 µmol/g dry weight). Yet, when grown glucose-limited, no flux through the LDH is observed, which suggests some downregulation of the activity of LDH that is controlled by the limiting availability of the carbon and energy source (under glucose excess conditions, such as occur in batch cultures, invariably lactate is formed). Similar observations have been made with the lactic acid bacteria and with these organisms it has been shown that the LDH needs fructose-1,6 bisphosphate as an activator. Presumably, under glucose-limited conditions this glycolytic intermediate is present in too low concentrations to allow LDH activity. We are not aware of a similar mechanism in E. coli. We, and others (Kaiser and Sawers, 1994) observed that under all conditions tested PDHc is synthesized and the level of expression varies only about four- to fivefold between fully aerobic and fermentative conditions. We consider the availability of this complex to be yet another example of maximization of metabolic flexibility so often encountered with micro-organisms (Teixeira de Mattos and Neijssel, 1997). Although regulation of metabolic activity at the level of gene expression may optimize the cell’s enzymatic make up for a particular steady state condition, the constitutive presence of essential enzymes guarantees a rapid response to sudden changes in the environment. Thus, in the case of pyruvate metabolism, the cell can cope with sudden transitions to aerobic conditions as the immediate and irreversible deactivation of PFL can be compensated for by the PDHc provided that the kinetic constraint by the high NADH/NAD ratio is relieved. In this context, it is not surprising that fermenting cells retain respiratory capacity (see chapter 4). The time scales at which these responses occur under aerobic-anaerobic (and vice versa) transitions and the accompanying intracellular changes are presented in chapter 4.

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Redox sensing


