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

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Citation for published version (APA):
Chapter 3

Growth of *Escherichia coli* at low oxygen concentrations, implications for catabolism and NADH/NAD ratio

Parts of this chapter together with chapter 2 have been submitted to *Journal of Bacteriology*. 
ABSTRACT

*Escherichia coli* wild type cells were cultured in glucose-limited chemostat cultures and the effects of varying the dissolved oxygen tension (DOT) on pyruvate catabolism were studied. At low dissolved oxygen tension (≤1%) fermentation and respiratory catabolism took place simultaneously, that is, the *in vivo* catabolic carbon flux from pyruvate was distributed over the pyruvate formate lyase and the pyruvate dehydrogenase complex. The cellular content of the pyruvate dehydrogenase complex in the cells decreased only slightly with decreasing DOT whereas the steady state NADH/NAD ratio increased sharply at low DOT, and presumably inhibited the activity of the pyruvate dehydrogenase complex.

The production of formate indicated that PFL was active at DOT values below 1%. Since active PFL is highly sensitive to oxygen, this activity can only be explained by assuming that the respiratory activity has sufficient capacity to deplete the cytosol of oxygen under these conditions.
Growth at low oxygen concentrations

INTRODUCTION

In the regulation of catabolism of *Escherichia coli* the ambient redox potential is an important parameter. In chapter 2 experiments have been described in which the redox potential of the medium is changed by using electron acceptors with different midpoint potentials. Another way to change the redox potential is to use different concentrations of the same electron acceptor. Here we report on the effects of different levels of O$_2$ supply on the pyruvate catabolism of *E. coli*. Lower oxygen tensions may result in numerous changes in the cellular makeup and activity. In the early 70's Wimpenny *et al.* (1971) already observed changes in the external redox potential of the culture when the partial oxygen pressure was changed. Also, it seems logical to assume that changes will occur with respect to the reduction state of the components of the respiratory chain since the rate of NADH reoxidation is submaximal whenever terminal oxidases are not provided with sufficient oxygen. In addition, the lowering of the oxygen pressure lowers the redox potential of the medium significantly and such a drop in itself could have an important effect on the physiology of the cell, either directly by altered enzyme activities due to kinetic effects or by effects on enzyme synthesis via redox related regulators such as Arc or FNR (see the general introduction). For example, with FNR it was found indeed that this regulator responds to changes in the environmental redox potential (Unden *et al.*, 1990) rather than to oxygen *per se.* Similarly, it is known that low oxygen conditions invokes the synthesis of the high affinity (towards oxygen) cytochrome $bd$ oxidase preferential to the cytochrome $bo$ oxidase which is active under fully aerobic conditions (Tseng *et al.*, 1996; Puustinen *et al.*, 1991; Fu *et al.* 1991). The level of induction of either of the cytochrome oxidases varies over a broad range of DOT values around 0.5% (DOT = dissolved oxygen tension), which corresponds to approx. 500 nM under physiological conditions (De Jonge, 1996). Again the Arc and/or FNR systems may play a role here (Fu *et al.*, 1991; Cotter and Gunsalus, 1992; Tseng *et al.*, 1996). In chapter 2 it was shown that by using various external electron acceptors, the NADH/NAD ratio in the cell could be manipulated. Moreover, when the external redox state was changed, the internal redox state followed the same trend. It seems reasonable to assume that varying the steady state DOT
value would give similar results, cells growing at lower DOT values supposedly will show an increased the NADH/NAD ratio. In this study we have focussed on the fluxes through the pyruvate dehydrogenase complex (PDHc) and the pyruvate formate lyase (PFL) at low oxygen conditions. It has been always assumed that the PFL works anaerobically and the PDHc aerobically. However, as shown in chapter 2, the PDHc can be active anaerobically, provided that the redox potential of the NADH/NAD couple is high enough, but it remains to be seen whether aerobic conditions can be obtained in which the cellular redox potential is sufficiently low to allow for PFL activity and to (partially) inhibit PDHc activity and/or synthesis.

**MATERIAL AND METHODS**

Escherichia coli strain and growth conditions

MC4100 F' araD139 (argF-lac) U169 rpsL150 relA1 deoC1 flb-5301

*ptsF1* (Casabadan and Cohen, 1979) ('wild type')

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

Organisms were cultured in a 3 liter fermentor, Bioflow III (New Brunswick). Growth media were simple salts media as specified by Evans *et al.* (1970) but instead of citrate, nitrilotriacetic acid (2mM) was used as chelator. Selenite (30 μg/l) and thiamine (15 mg/l) were added to the medium. The dilution rate was set at 0.3 ±0.01 hr⁻¹. The pH value of the culture was maintained at 6.5±0.1 using sterile 4M NaOH. The temperature was set to 35°C. The DOT (dissolved oxygen tension) of the culture was measured using a polarographic electrode (Ingold). The DOT was maintained constant by controlling the stirrer speed. 100% DOT was set by sparging the chemostat with air, 0% DOT by sparging with nitrogen gas. 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 and Tempest, 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 a SP 4270 integrator (Spectra Physics). CO$_2$ production and O$_2$ consumption were measured by passing the effluent gas from the fermentor through a Servomex CO$_2$ analyser and a Servomex O$_2$ analyser.

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 PDHe was measured in a standard reaction mixture containing 50mM sodium phosphate (pH 7.0), 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 (1030 M$^{-1}$.cm$^{-1}$)

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).

H$_2$ measurements
Gas samples were taken from chemostat cultures and analysed for H$_2$ on a Chrompack gas chromatograph, using a Molsieve 5Å PLOT column (Moezelaar and Stal, 1994)
RESULTS

*E. coli* MC 4100 was cultured under different steady state dissolved oxygen tensions (DOT) ranging from fully anaerobic to fully aerobic conditions. From the observed steady state fluxes (table 3.1) it can be concluded that at oxygen tensions values around 1% and lower, fermentation and respiration occur simultaneously as can be deduced from the production of formate and consumption of oxygen. In these micro-aerobic cultures, significant amounts of acetate and ethanol are being produced. This is rather surprising because the PFL is irreversibly damaged by small traces of oxygen (Knappe and Sawers, 1990). It may well be that under these conditions the respiratory chain has sufficient capacity to consume oxygen at such a rate that the cytoplasm remains devoid of oxygen, resulting in an environment where the enzyme can be active.

<table>
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<th>q_{succ}</th>
<th>q_{lac}</th>
<th>q_{form}</th>
<th>q_{ace}</th>
<th>q_{etoh}</th>
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Table 3.1 Steady state fluxes in glucose limited chemostat cultures of *E. coli* MC4100, pH 6.5, D=0.3 h^-1, q in mmol h^-1 g^{-1} (g DRW)^{-1}. DOT is given in percentage of air saturation. The values are the means of at least 4 independent measurements SD is ± 10%.

Aerobically acetyl-CoA is converted via the TCA cycle. In the micro-aerobic cultures, not all of the acetyl-CoA is converted via the TCA cycle as large amounts of acetate and ethanol are being produced. NADH is re-oxidized in these cultures in two ways: (i) by the formation of ethanol and (ii) by the respiratory chain although it cannot be excluded that cytosolic NADH oxidases play also a role in the oxidation of NADH. The acetate/ethanol ratio in the anaerobic culture is 1, as expected, and only small amounts of succinate are produced. In the cultures at low DOT (1% and 0.5%) the ratio is higher, as NADH is re-oxidized by oxygen.

The produced formate can be cleaved in H_2 and CO_2, either by a formate dehydrogenase or a formate hydrogen lyase (Kessler and Knappe, 1996). In
an effort to make a proper estimation of the flux through the pyruvate formate lyase, hydrogen gas production was measured. In cultures of 1\% and 0.5\% DOT hydrogen gas is produced as was measured by gas chromatography (data not shown). However, it should be mentioned that the hydrogen production is too small and the measurement too inaccurate to allow for a reliable calculation of the total PFL flux.

At 1\% DOT a high flux to formate is observed, indicating a specific PFL activity of minimally 11.3 mmol h\(^{-1}\) (g DRW)\(^{-1}\). Thus, it can be concluded that there is a distribution of the pyruvate flux at DOT's below 2\% between PFL and PDHc.

![Fig 3.1 NADH/NAD ratio versus DOT in glucose limited chemostat cultures of E. coli MC4100 (D=0.3, pH=6.5). The values are the means of at least 4 independent measurements.](image)

In fig 3.1 the steady state NADH/NAD ratios of the various cultures are given. At a DOT of appr. 2\% the NADH/NAD ratio increases sharply, which seems to coincide with a shift from respiration to fermentation. Apparently, now the PDHc is inhibited and a large part of the pyruvate flux is branched via the PFL. From fig 3.1, it can be seen that there is a good correlation between the DOT and the NADH/NAD ratio in these cells. At low DOT (below 2\%) a vastly larger part of NADH oxidation takes place via fermentation. The trend is the same as seen in chapter 2, where external electron acceptors were used to manipulate the redox potential. A lower external redox potential is reflected by a higher NADH/NAD ratio and results in a more fermentative behaviour.
Besides the *in vivo* flux through PDHc, the synthesis of the PDHc was monitored for the various cultures. The total amount of PDHc was found to decrease only slightly with decreasing DOT (fig 3.2) as measured by *in vitro* activity determination in cell free extracts. As the regulation of the PDHc genes is under control of a general regulator of aerobic/anaerobic catabolism (Arc; Quail *et al.*, 1994;) the minor (though significant) changes may be surprising. However, it should be mentioned that a similar difference in PDHc synthesis between aerobic and anaerobic conditions has been reported by Kaiser and Sawers (1994). What is interesting is that it can be seen here that the regulation of expression is not an on/off switch but a gradual decrease/increase of gene expression. In contrast to the gradual change in gene expression of the PDHc in the cells at the different oxygen tensions, the NADH/NAD ratio shows a ‘switch’ point (at ca. 2% DOT). Here we can see there is no direct correlation between the flux through PDHc and the redox state of the cell, as measured by the NADH/NAD ratio.

**Discussion**

In the micro-aerobic cultures studied here an interesting feature of catabolism is seen: simultaneous respiratory and fermentative catabolism. The transition from respiratory catabolism to fermentative catabolism is not an abrupt switch but a gradual process. At DOT values between >70% and appr. 1-2% the
cells show a virtually complete aerobic catabolism. Fermentation starts at 2% DOT, and the NADH/NAD ratio increases in parallel. A high NADH/NAD ratio will allow the alcohol dehydrogenase to be active as its \textit{in vivo} activity is regulated positively both at the kinetic and the expression level by the NADH/NAD ratio (Leonardo \textit{et al.}, 1996). Surprisingly the PFL is active, although the enzyme is oxygen sensitive. We observed that the flux from pyruvate to acetyl-CoA is distributed over the PDHc and the PFL under microaerobic conditions. An exact calculation of the separate fluxes is impossible, however, as the end products of these enzymes are the same (CO$_2$, acetate, ethanol) and H$_2$ measurements were not accurate enough to allow calculation of the exact PFL flux. Tseng \textit{et al.} (1996) have reported that under microaerobic conditions (<10% air saturation) the anaerobic respiratory enzymes are induced (e.g. \textit{narGHJI}, \textit{frdABCD} and \textit{dmsABC}). These enzymes are induced by the FNR system (see General Introduction). As the PFL is also regulated by this system, it is very likely to be induced under microaerobic conditions. Recent studies have revealed that indeed at these low oxygen pressures PFL is expressed, even to higher levels than those observed under fully anaerobic conditions (pers. comm. S. Alexeeva, E.C. Slater Inst., Amsterdam).

The formate that is being produced by the PFL can be used in formate respiration. In formate respiration formate is the electron donor and oxygen can serve as the electron acceptor. Formate respiration generates a protonmotive force, which can be used to generate ATP (Jones, 1980). In this respiratory chain the primary electron acceptor is either formate dehydrogenase N (Berg \textit{et al.}, 1991; Enoch and Lester, 1975; Jones, 1980), which is maximally active under anaerobic conditions with nitrate, or formate dehydrogenase O, which is synthesized at relatively low levels irrespective of the presence of oxygen (Pommier \textit{et al.}, 1992). Hence, the actual flux to formate may even be higher than the measured flux, because of this formate respiration.

It has been reported earlier that under these micro-aerobic conditions, as applied in our study, cytochrome $d$ oxidase is induced (De Jonge, 1996; Tseng \textit{et al.}, 1996). The physiological rationale for this phenomenon is that cytochrome $bd$ oxidase has a high affinity for oxygen (Green \textit{et al.}, 1988; $K_m = 0.2\mu$M), as compared to cytochrome $bo$ oxidase (Chepuri \textit{et al.}, 1990; $K_m = 2 \mu$M) which may provide the cell with additional scavenging capacity in
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order 1) to keep up a high respiration rate at low oxygen tensions (a common response to nutrient-limited growth conditions, see Teixeira de Mattos and Neijssel, 1997) and 2) to maintain an intracellular oxygen concentration that is low enough to allow the PFL to be active. A followup of the studies presented here would therefore be to see how deletion of either of the cytochrome oxidases would affect the in vivo activity of both enzyme systems. If the above-mentioned function were indeed the case, the DOT threshold for PFL activity would be lowered for cells lacking the high affinity oxidase.

It should be mentioned here that it was observed that very often at lower oxygen tensions (below 2% DOT), the cultures showed an oscillating behaviour in the reading of the oxygen electrode with a periodicity of a few seconds. It may be that small changes in the oxygen tension, due to the imperfectness of the chemostat apparatus have a strong effect on the signalling mechanisms (presumably Arc and FNR) resulting in a continuous switching on and off. This would hamper the cells to achieve a steady cellular makeup.

REFERENCES


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