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

de Graef, M.R.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4

Anaerobic/aerobic transitions in chemostat cultures of *Escherichia coli*: effects on product formation, redox state, energy state and DNA super coiling
ABSTRACT
In the previous chapters, differences with regard to product formation, internal redox state, gene expression and \textit{in vivo} enzyme activities were reported between \textit{steady state} cultures with various redox conditions. In order to understand how these differences are brought about, in this chapter we now focus on the sequence of events that accompany \textit{transitions} from aerobic to anaerobic, and conditions \textit{vice versa}.

Upon a switch from aerobic to anaerobic conditions the cells were able to activate the anaerobic pathways instantaneously. Within minutes, the typical fermentation products acetate, ethanol and formate were produced and from the rate of their formation, it could be calculated that the activity of the pyruvate dehydrogenase complex was inhibited immediately. The NADH/NAD ratio increased sharply and reached the level as observed in anaerobic cells after ca. 2 minutes. Supposedly, it is this high NADH/NAD ratio that blocks pyruvate dehydrogenase complex activity. In addition, it was found that the ATP/ADP ratio decreased rapidly with a concomitant rise of the linking number of the DNA (DNA supercoiling) which may be related to the onset of a modulated gene expression.

In the reverse experiment (transition from anaerobic to aerobic conditions), a more complex transient behaviour was seen. Initially, the cells were not able to carry out the aerobic catabolism as found during the steady state: a significant flux to pyruvate as an end product was observed. The NADH/NAD ratio decreased, but more slowly than during the reverse switch whereas the ATP/ADP ratio was found to increase during the first few minutes, followed by a decrease back to the steady state level. The linking number of the DNA showed an opposite trend.
INTRODUCTION
As highlighted in the general introduction, *Escherichia coli* is known for its ability to adapt to changes in the environment and in the preceding chapters we have focussed on a major environmental variable: the availability of oxygen and other electron acceptors and the effect of this on the catabolism of *E. coli*. There have been many, mostly molecular, studies on this subject and they have resulted in the identification of a number of global regulators (globulators) which play an important role in the adaptation to different redox environments. Two of these, Arc and Fnr, regulate the gene expression of specific aerobic and anaerobic genes (see the general introduction), but it is yet unclear what the primary signal is for these regulators. Arc is a member of the family of two component regulators. A model has been proposed in which the activation (phosphorylation) of the regulator protein ArcA depends on the accumulation of fermentation products (acetate, pyruvate) and/or the level of NADH in the cell (Iuchi, 1993). NADH is the major redox carrier in living cells, yet present in low concentrations. Hence during growth the produced NADH has to be re-oxidized: aerobically this is achieved by the respiratory chain, whereas under fermentative conditions the formed NADH is re-oxidized by the formation of products like lactate and ethanol. Earlier studies (Wimpenny and Firth, 1972; London and Knight, 1966; Takebe and Kitahara, 1963) revealed a large difference in NADH/NAD ratio between cells in the cell when grown aerobically or anaerobically. Assuming the NADH/NAD ratio to be a reflection of the cellular redox state, the redox state of the cell thus changes radically upon switching a culture from aerobic to aerobic conditions or vice versa. Later studies with *Enterococcus faecalis* showed the same effect (Snoep et al., 1992).

It is well known that product formation also changes dramatically during these environmental switches: aerobically the cell will respire, producing mainly CO₂, whereas anaerobically the cell will produce fermentation products (e.g. acetate, lactate, and ethanol), and therefore different enzymes need to be active under these conditions. The question then arises as to how these adaptive responses arise. Clearly, they may take place at different levels:

1. at the level of activity: by changing the environmental conditions, also the internal conditions change. Internal substrate/product and cofactor
concentrations may change and hence the \textit{in vivo} activity of the various enzymes. The extent of activity changes will depend on the kinetic properties of the enzyme and, for instance, allosteric control may play a role here.

2. at the level of gene expression and subsequent enzyme synthesis. This can take place by induction or repression by substrates or products, directly or via the above mentioned globulators (like FNR or Arc) which can play a more specific role in induction/repression.

3. Finally post translational changes and turnover may affect net enzyme synthesis.

So far, we have focussed on the presence or absence of specific enzymes (such as pyruvate formate lyase and the pyruvate dehydrogenase complex) and their relative contribution to the total catabolic flow under various steady state conditions. Here, we will try to gain some insights in the dynamics of adaptation. In this context, the phenomenon of supercoiling of DNA is of special interest as it is known that a) supercoiling changes rapidly upon disturbance of environmental conditions and b) it plays a role in gene expression (Gellert, 1981). In particular, it is known that supercoiling of the DNA changes upon a switch from aerobic to anaerobic conditions (Hsieh \textit{et al.}, 1991). Furthermore, a correlation has been found between the energetic status of the cell as reflected by the ATP/ADP ratio and the activity of the DNA gyrase (van Workum \textit{et al.}, 1996). Such a role of the energetic state can be understood, since two DNA topoisomerases control the level of (negative) supercoiling in bacterial cells of which one, DNA gyrase, is an ATP dependent enzyme that catalyses the underwinding of the DNA, whereas DNA topoisomerase I relaxes DNA (Drlica, 1992).

These two enzymes tend to maintain supercoiling within a fixed range. Since variations in the environmental conditions often leads to a difference in cellular energetics, these variations may result in changes in supercoiling (van Workum \textit{et al.}, 1996). It is tempting to consider changes in supercoiling as the very first and general response upon environmental disturbances with respect to the initiation of repression and induction mechanisms.

In this study we investigate the effect of a sudden change in the availability of oxygen using glucose-limited chemostat cultures of \textit{E. coli}. In these transient states, the effects on enzyme activities were studied, rather than
the effects on gene expression. These transient state experiments are used to study time hierarchies in the adaptation to a different redox environment.

**MATERIAL AND METHODS**

**Escherichia coli strain and growth conditions**

MC4100  
\[ F^- \text{araD139} \] (argF-lac) U169 rpsL150 relA1 deoC1 flb-5301 ptsI (Casabadan and Cohen, 1979) ('wild type')

MC 4100 (pBr322), this strain was used for ATP/ADP and DNA supercoiling measurements

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 (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 0.10±0.01 h⁻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 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 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 H₂SO₄ as eluent, using a 2142 refractive index detector (LKB) and an SP 4270 integrator (Spectra Physics). CO₂ production or O₂ consumption was measured by passing the effluent gas from the fermentor through a Servomex CO₂ analyser and a Servomex O₂ 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 PDHc was measured in a standard reaction mixture containing 50 mM sodium phosphate (pH 7.0), 5 mM pyruvate, 12.5 mM MgCl₂, 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 (ε=1030 M⁻¹cm⁻¹).

Lactate dehydrogenase activity was measured in a standard reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), NADH (350μM), MgCl₂ (10 mM), pyruvate (60 mM) and cell free extract. The reaction was started by the addition of pyruvate and the initial rate was monitored spectrophotometrically by following the oxidation of NADH at 340 nm (ε=6220 M⁻¹cm⁻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 neutralised, filtered extract, as described previously (Snoep et al., 1990).

**Sampling for ATP/ADP and DNA supercoiling measurements.**

Cell cultures were sampled (0.75 ml) into 0.75 ml 80°C phenol and Vortexed for about 10 s. Samples were centrifuged (12000 x g, 15 min), subsequently a chloroform extraction was performed on the water-phase of the sample/phenol mixture. A sample was taken from the water phase for ATP/ADP measurements, plasmid DNA was purified by a standard isopropanol precipitation. (Van Workum et al., 1996)
**ATP and ADP measurements**

ATP/ADP ratios were measured essentially as described by Van Workum *et al.* (1996). ATP was measured using a kit (LKB) based on the luciferin-luciferase method. The following changes were made: 10 mM KCl, glycerol-free pyruvate kinase, and 2.5 mM phosphoenolpyruvate were used in the ATP buffer. First ATP was measured and then ADP was converted to ATP using pyruvate kinase.

**DNA supercoiling**

The linking number of DNA was measured essentially as described by Van Workum *et al.* (1996)

DNA samples (plasmids) were put on an agarose gel (1.4%), in the presence of 10 μg ml⁻¹ chloroquine. After electrophoresis, DNA was transferred to Hybond membranes and hybridised with pBR322 labelled with [³²S]-dATP. The blots were autoradiographed by incubation on Kodak X-OMAT AR or Kodak MIN-RH film. The film was then digitised and the density in each lane was quantified at a number (ca. 200) of equally interspaced positions. From this a graph was made with points (d,x), where d is the density at point x. From this graph the peak positions where determined automatically using a 'peak-detecting algorithm', and a linking number was assigned to each peak. By fitting a third order polynomial and using the formula given by Van Workum *et al.* (1996), the average linking number was calculated.

**RESULTS**

**Steady states**

*E. coli* MC4100 was grown in chemostat cultures (D 0.1 h⁻¹, pH 6.5, 35°C) under glucose-limited conditions aerobically or anaerobically. Specific rates of product formation and substrate utilisation are given in table 4.1.

<table>
<thead>
<tr>
<th></th>
<th>$q_{O_2}$</th>
<th>$q_{CO_2}$</th>
<th>$q_{lac}$</th>
<th>$q_{lac}$</th>
<th>$q_{eto}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>-3.8</td>
<td>4.0</td>
<td>-1.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>7.8</td>
<td>-4.7</td>
<td>0.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table 4.1 Product formation and substrate utilisation rates of steady state cultures of *E. coli* MC 4100, pH 6.5, D=0.1 h⁻¹, T=35°C. Rates are in mmol h⁻¹ (g DRW)⁻¹. The values are the mean of 4 independent experiments. C-balances are within 90%-110%.
Chapter 4

Not surprisingly, under aerobic conditions all glucose is converted into biomass and CO₂ and no by-products are formed and the observed RQ value of 1 is according to the expected behaviour under these conditions. The NADH/NAD ratio is very low in these cultures, notably due to virtually undetectable levels of NADH. Anaerobically the culture showed the expected mixed acid fermentation pattern, acetate and ethanol are produced in equimolar amounts with the concomitant formate and carbon dioxide production. Succinate was a minor fermentation product (data not shown but see chapter 2). The NADH/NAD ratio is high under these conditions. All pyruvate produced during glycolysis is now further broken down via pyruvate formate lyase, which converts the pyruvate to acetyl-CoA, formate, CO₂ and H₂.

Aerobic/anaerobic transient state
At t=0 the air supply to a steady state aerobic chemostat culture was replaced by a supply of nitrogen gas. After approximately 20 seconds no oxygen could be detected anymore in the chemostat as measured by an oxygen electrode in the chemostat vessel. Upon switching an aerobic chemostat culture to anaerobic conditions, the catabolism changed dramatically. The fermentation stalled immediately, as is seen by a direct excretion of formate, acetate and ethanol (fig. 4.1). This is rather surprising as it indicates that all fermentative enzymes were present during aerobic growth as the time span after the switch was too short to allow for de novo synthesis. Apparently, the sudden absence of oxygen resulted in a direct activation of the fermentative enzyme machinery. Since the specific formate production rate equalled the ethanol plus the acetate flux, it can be concluded that, firstly, the pyruvate dehydrogenase complex was inactivated instantaneously and, secondly, no immediate activity of the formate hydrogen lyase occurred (which would have resulted in the formation of H₂ and CO₂ and the consumption of formate). The latter can be explained by the fact that the formate hydrogen lyase needs elevated levels of formate in order to be induced (Birkmann et al. 1987a,b). It is known that aerobically grown cells contain small amounts of the inactive (oxygen-insensitive) form of PFL (Knappe and Sawers, 1990; see also the General Introduction). This PFL must have been converted to the activated form upon depletion of oxygen. These results show that the amount of PFL in aerobic cells must have been considerable as it is able to sustain a specific
**Fig 4.1** Product formation during a transient state from aerobic to anaerobic conditions in a chemostat culture of *E. coli MC 4100*, *D*=0.1 h⁻¹, pH=6.5, *T*=35°C.

**Fig 4.2** NADH/NAD ratios during a transient state from aerobic to anaerobic conditions in a chemostat culture of *E. coli MC 4100*, *D*=0.1 h⁻¹, pH=6.5, *T*=35°C.

**Fig 4.3** PDHc enzyme activities (nmol reduced ferricyanide per minute per mg protein) during a transient state from aerobic to anaerobic conditions in a chemostat culture of *E. coli MC 4100*, *D*=0.1 h⁻¹, pH=6.5, *T*=35°C. PDHc washout was calculated starting with the amount of PDHc measured at *t*=0 min.
production rate of approximately 30% of the rate found in the final anaerobic, steady state condition.

The NADH/NAD ratio was also measured during this transient state (fig. 4.2). The ratio increased very rapidly to the steady state anaerobic level, which was reached within a few minutes which may explain the inactivation of the PDHc. In vitro measurements of the PDHc (fig. 4.3) suggest that the lowered PDHc content in anaerobic cells (see chapter 2) is due to a lowered synthesis but not to active breakdown of PDHc since the activity did not decrease faster than calculated from the wash out rate. However, the in vivo fluxes show that the PDHc is inhibited within minutes. The transition from aerobic to anaerobic conditions appears not to affect the cellular content of LDH (data not shown) suggesting that the synthesis of this enzyme is not subject to an oxygen-related regulation.

A threefold, temporary drop in the ATP/ADP ratio was observed upon the aerobic-anaerobic transition (fig 4.4). Restoration of the ratio starts within 5 minutes after the disturbance. The immediate decrease in ATP/ADP ratio was accompanied by a sudden increase in the linking number of the DNA (DNA is becoming now more relaxed). After this peak the linking number slowly decreased to the aerobic level (fig 4.4) although the sampling period has not been long enough to confirm a new steady state.

Fig 4.4 Change in ATP/ADP ratio (closed symbols) and linking number of the DNA (dLK, open symbols) during a transient state from aerobic to anaerobic conditions in a chemostat culture of E. coli MC 4100, D=0.1 h⁻¹, pH=6.5, T=35°C. The steady state value is the mean of 5 independent measurements.
**Transient state cultures**

**Anaerobic/aerobic transient state**

A steady state anaerobic chemostat culture of MC4100 was switched to aerobic conditions by replacing the nitrogen supply by an air supply. Within 20 seconds the culture was fully aerobic, as measured by an oxygen electrode in the chemostat. In fig. 4.5 and 4.6 the effects on catabolic fluxes respectively the NADH/NAD ratios are given. Clearly this switch has a profound effect on the cells. Apparently, the transition causes a large imbalance in the capacity of the glycolytic flux to pyruvate and the subsequent pyruvate dissimilating machinery with the consequence of a build-up and secretion of this intermediate, as they cannot longer convert all the pyruvate into acetyl CoA.

![Graph of product formation](image1)

**Fig 4.5** Product formation during a transient state from anaerobic to aerobic conditions in a chemostat culture of *E. coli* MC 4100, D=0.1 h⁻¹, pH=6.5, T=35°C.

![Graph of NADH/NAD ratios](image2)

**Fig 4.6** NADH/NAD ratios during a transient state from anaerobic to aerobic conditions in a chemostat culture of *E. coli* MC 4100, D=0.1 h⁻¹, pH=6.5, T=35°C.
As the PFL will be inactivated by oxygen, it is not surprising to see that the mixed acid fermentation pattern is interrupted (acetate and ethanol were washed out at a rate that equals the dilution rate) but, since LDH is present in anaerobically grown cells, it was rather unexpected to observe that significant amounts of pyruvate were excreted and no lactate was formed at all.

The changes in catabolic behaviour were accompanied by a decrease of the NADH/NAD ratio (fig 4.6) which is however much slower than the increase
during the aerobic/anaerobic switch. This may impede PDHc to come into action, although the amount of this enzyme in the cells is increasing (fig 4.7), but it cannot explain why no lactate formation is observed. With regard to the effects on the ATP/ADP ratio and DNA supercoiling a complex pattern is observed with an initial rise in ATP/ADP ratio and a subsequent fall to the steady state level within 10 minutes (fig.4.8). A corresponding pattern was observed for the linking number of DNA.

DISCUSSION

The above results show that cells can adapt rapidly when transferred from aerobic to anaerobic conditions as illustrated by the fact that the fermentation starts immediately without accumulation of either the substrate (glucose) or intermediates like pyruvate. Apparently, aerobic cells have the cellular makeup to cope with anaerobiosis. In fact, it can be easily envisaged that for such an adaptation only a certain level of PFL—in its oxygen insensitive form—has to be maintained in the cell and this doesn’t seem to be an energetic burden as aerobic conditions are energetically favourable. As soon as all oxygen in the environment (or intracellularly) is reduced and the NADH/NAD ratio has increased sufficiently, all criteria are fulfilled for PFL to function properly. Adaptation the other way around seems to be more complex as seen by the accumulation of pyruvate and the complicated pattern of ATP/ADP changes and the linking number of DNA. The observation that the organisms are not able to invoke a homolactic fermentation upon a switch to aerobicism seems anomalous as all conditions seem to have been fulfilled for such a catabolic response: LDH, high pyruvate concentrations and a sufficiently high NADH/NAD ratio. However, an instantaneous NADH reoxidation must have taken place upon the switch as, despite the fact that an oxidized compound (pyruvate) is excreted, the NADH/NAD ratio drops. This suggests that some respiratory potential is present in anaerobically growing cells. It may be that as a consequence of the differences in affinities of the respiratory machinery and LDH for NADH, a significant rate of lactate production can only proceed for a short period. Finally, upon the presence of an electron acceptor (in this case oxygen) NADH oxidation coupled to the generation of a proton motive force and subsequent ATP synthesis then would affect the ATP/ADP ratio positively. This seems to present a rather disturbing event that results in a severe imbalance of the various carbon
Chapter 4

fluxes which we cannot explain at this point. In this context, further quantitative studies with respect to the capacity of anaerobically grown cells to commence respiration instantaneously would be of interest. We have shown that transient conditions affect strongly the energy status (the ATP/ADP ratio) as well as the redox state (NADH/NAD) ratio of the cell and that the dynamics of the changes are quite different for the two transitions studied here.

It can be concluded that the responses in the activities of the fermentative enzyme systems, respectively the oxidative enzymes, cannot be generalized but are on the contrary very diverse. Thus, lactate dehydrogenase synthesis is not affected by a supply or depletion of oxygen whereas a minor but significant change is seen for the pyruvate dehydrogenase complex with respect to induction but strong effects are seen in activity. Finally, PFL activity changes instantaneously and it should be mentioned that synthesis of this fermentative enzyme is vastly different in steady state aerobic or anaerobic conditions (S. Alexeeva, pers. comm.).

The significance of the observed changes in DNA supercoiling is noteworthy. When either transition occurs, an energetic stress is invoked which causes a change in the DNA linking number. It is tempting to speculate that the observed adaptations are triggered by rapid changes in metabolic intermediates (ATP, NADH etc) that initiate general responses at the level of gene expression. These, finally, generate the signals that are responsible for adaptive mechanisms specific to the new environmental conditions. In this view, the timescale of the cellular response to a switch in oxygen availability in the environment can be divided into three stages:

1. The cells respond immediately (seconds) at the level of activity of the catabolic enzymes. For those enzymes that need oxygen as a substrate (cytochrome oxidases) or that are irreversibly inactivated by oxygen (pyruvate formate lyase), this is obvious and the response will be immediate. In addition, the environmental change may indirectly affect internal concentrations of metabolites that serve as effectors (e.g. allosteric effectors). The modified activity per se will result in differences in metabolite concentrations which may subsequently have an effect on gene expression (see 3.).
2. Supercoiling of DNA may change also very fast (0-1 minute), because the extent of coiling is directly related to a rapidly changing ATP/ADP ratio (seconds). This may constitute a general, non-specific response to stress conditions. It has been reported that many genes are switched on or off by such a mechanism. For example, it has been shown (Ni Bhriain et al., 1989) that during an osmotic shock, not only proteins involved in protecting the cells from such an osmotic shock are induced, but also many anaerobic genes. This response can be within minutes after a stress situation occurs.

3. More specific regulators (globulators) come into action (minutes) like FNR and Arc. These systems can fine tune the induction of genes on top of the global regulation brought about by DNA supercoiling. In this way only genes necessary for the current condition will remain induced. This response is slower than the other two, because it involves de novo synthesis after a certain stimulus has been sensed.

The sequence of events after an environmental transition thus would be as follows. Firstly, enzyme activities are altered due to changes in substrate, product and/or effector concentrations. Secondly, the subsequent change in the energetic state of the cell induces modification of DNA supercoiling causing a general response by inducing several stress related genes. Thirdly, the presence of condition-specific signals will invoke a fine tuning system that induces/represses the synthesis of enzymes as appropriate for the new condition, whereas the general stress response comes to an end when the metabolic network returns to a new steady state.

ACKNOWLEDGEMENTS
ATP/ADP ratios and DNA supercoiling were measured at the dept. of Microbial Physiology, Free University, Amsterdam, by Silvy van Dooren.

REFERENCES


Transient state cultures


