Control of FNR Function of *Escherichia coli* by O$_2$ and Reducing Conditions

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Abstract

The synthesis of the enzymes constituting the electron transport chain of *Escherichia coli* is controlled by electron acceptors in order to achieve high ATP yields and high metabolic rates as well. High ATP yields (or efficiency) are obtained by the use of electron acceptors for respiration which allow high ATP yields, preferentially O$_2$, and nitrate in the absence of O$_2$. The rate of metabolism is adjusted by use of respiratory isoenzymes which differ in the rate and the efficiency of energy conservation, such as the non-coupling NADH dehydrogenase II (ndh gene) and the coupling NADH dehydrogenase I (nuo genes). By combination of the contrary principles (rate versus efficiency), growth is optimized for growth yields and rates.

One of the major transcriptional regulators controlling the switch from aerobic to anaerobic respiration is FNR (fumarate nitrate reductase regulator). FNR is located in the cytoplasm and contains a [4Fe–4S] cluster in the active (anaerobic) state. By reaction with O$_2$ the cluster is converted to a [2Fe–2S] cluster and finally to apoFNR. O$_2$ diffuses into the cytoplasm even at very low O$_2$-tensions (1 μM) where it inactivates [4Fe–4S] FNR. The formation of [4Fe–4S] FNR from apoFNR can use glutathione as a reducing agent in vitro. This process could also be important for the reductive activation of FNR in vivo. A model for the control of the functional state of FNR by O$_2$ and glutathione is discussed. According to this model the functional state of FNR is determined by a (rapid) inactivation of FNR by O$_2$, and a slow (constant) reactivation with glutathione as the reducing agent.

Introduction

In facultatively anaerobic bacteria, O$_2$ is an important environmental signal for the control of gene expression.

For O$_2$-sensing many different types of sensors have been identified in bacteria and archaea, most of which react directly with O$_2$ by O$_2$-reactive groups, like heme, FeS clusters, cysteine pairs, and FAD (for recent reviews see Bauer *et al.*, 1999; Patschkowski *et al.*, 2000). Other sensors like ArcB or PrrB sense the presence of O$_2$ indirectly, either by the function of respiratory chains or from characteristic metabolites of aerobic or anaerobic energy metabolism (Oh and Kaplan, 2000; Iuchi and Lin, 1993, Georgellis *et al.*, 2001). The proton potential Δp, or the membrane potential Δψ over the cytoplasmic membrane, as well as the energy charge of the bacteria decrease only slightly during transition from aerobic to anaerobic respiration (from −170 to −150 mV) (Tran and Unden, 1998). These parameters therefore most likely do not provide a signal for the switch from aerobic to anaerobic respiration.

Many of the O$_2$-sensors are located in the cytoplasmic membrane and directly accessible to signal molecules from the environment. Other O$_2$-sensors, like FNR (fumarate and nitrate reductase regulator) from *E. coli* or NifL from *Klebsiella* are located in the cytoplasm of the bacteria (Dixon, 1998; Klopprogge and Schmitz, 1999; Unden *et al.*, 1995; Becker *et al.*, 1996). This location requires presence of O$_2$ within the cells for reaction with the sensor (Unden *et al.*, 1995).

In *E. coli*, FNR is one of the major regulators controlling the switch from aerobic to anaerobic metabolism at the transcriptional level (Spiro and Guest, 1990; Gunsalus, 1992; Unden *et al.*, 1995; Kiley and Beinert, 1999). Three functionally different forms of FNR have been described (Figure 1). Under anoxic conditions, FNR is a dimer and carries a [4Fe–4S]$^{2+}$ cluster per monomer ([4Fe–4S]FNR) which is bound by 4 cysteine residues. This form binds to target DNA-sites, and controls the expression of the corresponding genes. The [4Fe–4S]$^{2+}$ cluster is essential for O$_2$-sensing, and is converted to a [2Fe–2S]$^{2+}$ cluster ([2Fe–2S]FNR) in the presence of O$_2$ (Kiley and Beinert, 1999; Khoroshilova *et al.*, 1995, 1997; Green *et al.*, 1996). [2Fe–2S]FNR is no longer active in transcriptional activation. After prolonged incubation with O$_2$, the [2Fe–2S] cluster further disintegrates, and FNR devoid of Fe and sulfide is formed (apoFNR) (Lazazzera *et al.*, 1996).

The review will first deal with the switch from aerobic to anaerobic metabolism which takes place in a way such as to optimize for adverse principles, energetic efficiency (ATP yield) and high growth rates. The second part will discuss low molecular weight factors controlling FNR function (cytoplasmic O$_2$ and...
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possibly glutathione), and how O\textsubscript{2} from the medium is able to react with FNR in the cytoplasm.

**Regulatory Rationale for the Switch from Aerobic to Anaerobic Metabolism**

*Synthesis of terminal reductases: Significance of high ATP yield ('efficiency').* The switch from aerobic to anaerobic respiration in *E. coli* has been studied in detail for the terminal reductases (for reviews see Spiro and Guest, 1990; Gunsalus, 1992; Iuchi and Lin, 1993; Stewart, 1993; Unden et al., 1995; Unden and Bongaerts, 1997). The genes encoding the terminal reductases are transcriptionally regulated by electron acceptors and other factors like growth phase and C-source. By this regulation the enzymes are synthesized in a hierarchical way ensuring preferential use of O\textsubscript{2}, whereas under anaerobic conditions nitrate is preferred (Figure 2). Only if neither O\textsubscript{2} nor nitrate are available, fumarate reductase and the fermentative enzymes are synthesized. The switch from aerobic to nitrate and fumarate respiration or fermentation corresponds to a progressive decrease in ATP yields (Gunsalus, 1992; Unden and Bongaerts, 1997). This regulation ensures preferential use of electron acceptors with high H\textsuperscript{+}/e\textsuperscript{-} ratios and ATP yield (maximal ATP yield; efficiency control). The respiratory dehydrogenases (here shown for NADH dehydrogenase I or Nuo and NADH dehydrogenase II or Ndh) differ in coupling efficiency (4 Na\textsuperscript{+}/2 e\textsuperscript{-} versus 0 H\textsuperscript{+}/2 e\textsuperscript{-}) and activity (indicated by the thickness of the arrow). When possible (e.g. in aerobic respiration), the Ndh enzyme with high activity, but low efficiency is used (maximal rate; rate control). The synthesis of the dehydrogenases is regulated at the transcriptional level by electron acceptors (O\textsubscript{2}, nitrate, fumarate), carbon source, and growth phase (Fis, IHF proteins). For details see text. Q, ubiquinone; MK, menaquinone.

**Synthesis of respiratory dehydrogenases: High metabolic rates.** The synthesis and use of the respiratory dehydrogenases, on the other hand, is not...
optimized for maximal ATP yields (Unden and Bongaerts, 1997; Tran et al., 1997; Wackwitz et al., 1999; Unden and Schirawski, 1997). *E. coli* contains many different respiratory dehydrogenases which all use the quinones as electron acceptors. Likewise, for the same electron acceptor often more than one respiratory dehydrogenase is found. For example, NADH is oxidized by the NADH dehydrogenase I and II isoenzymes (Calhoun et al., 1993; Tran et al., 1997; Bongaerts et al., 1995). NADH dehydrogenase I, or Nuo (nuo genes), couples the oxidation of NADH to $H^+$ translocation, whereas NADH dehydrogenase II, i.e. Ndh (ndh gene), does not (Figure 2). Thus the use of Nuo, but not of Ndh, generates a proton potential and drives phosphorylation of ADP. The enzyme activity (NADH:quinone oxidoreductase) of Ndh can be 3 times higher than that of Nuo and allows higher respiration rates.

The expression of the *nuo* and the *ndh* operons is regulated in response to electron acceptors (O$_2$, nitrate, fumarate), the C-source and the growth phase (Bongaerts et al., 1995; Wackwitz et al., 1999; Green and Guest, 1994; Green et al., 1996; Tran et al., 1997). The regulation is opposite for both operons. Thus the activity of the coupling enzyme (Nuo) is maximal in fumarate respiration, intermediate in aerobic respiration, and minimal in fermentation when it is not required. The non-coupling Ndh, on the other hand, is found mainly in aerobic respiration. In NADH–fumarate respiration the C-source and the growth phase (Bon- 

O$_2$ as the Signal for FNR Inactivation

For reaction with FNR, O$_2$ has to diffuse from the medium to FNR in the cytoplasm, and this process represents an important part of the signalling pathway. O$_2$ is able to react directly with FNR as shown in *vivo*, and also in the bacteria obviously no other mediators and intermediates are required for the reaction (Jordan et al., 1997). It has been shown, however, that ferricyanide is able in *vivo* and in *vivo* to promote inactivation of FNR function or [4Fe–4S]$^{2-}$ FNR destruction in an artificial reaction (Unden et al., 1990; Lazazzera et al., 1996), indicating that inactivation of FNR can also be achieved by oxidizing agents without direct need for O$_2$. As shown below, the properties of O$_2$ diffusion to the cytoplasm are consistent with the presence of O$_2$ in the cytoplasm under aerobic and microaerobic conditions, which is a prerequisite for direct interaction between FNR and O$_2$ (Becker et al., 1996; Unden et al., 1997; Tseng et al., 1996).

O$_2$ as the Regulatory Signal for FNR: The properties of O$_2$ as the regulatory signal for FNR were studied in growing bacteria in an oxystat by the use of FNR-dependent reporter gene fusions (Becker et al., 1996). The oxystat allows growth of the bacteria at defined and constant O$_2$ tensions (pO$_2$). The expression of the FNR-regulated genes decreases in a hyperbolic response with increasing O$_2$ tension in the medium. The O$_2$ tension corresponding to half-maximal induction or repression (pO$_2$) is in the range of 1 to 5 $\mu$M O$_2$ for most FNR regulated genes (equivalent to approx. 1 to 5 mbar, or 1 to 5 $\cdot$ 10$^{-5}$ Pa of O$_2$, or 0.5 to 2.5% air saturation). Only for *nirB* encoding the soluble nitrite reductase a distinctly higher pO$_2$ was found (Tran et al., 2000). Therefore at O$_2$ concentrations as low as 1 $\mu$M in the medium, sufficient O$_2$ should be present in the cytoplasm for FNR inactivation. The pO$_2$ of *sdh* expression which is regulated by the ArcBA system in response to O$_2$ is in a similar range (about 5 $\mu$M O$_2$) (Becker et al., 1996). The *cyd* and *cyo* operons encoding the quinol oxidases Cyo and Cyd which operate under aerobic or microaerobic conditions are subject to O$_2$ regulation by ArcA and FNR (Tseng et al., 1996; Fu et al., 1991). Expression of *cyd* is thus stimulated by ArcA under microaerobic conditions and repressed by FNR under
anaerobic conditions, resulting in a maximal expression under microaerobic conditions. The function of fermentative metabolism started only at very low pO₂ values (pO₂,5 ≤ 1 µM O₂) (Becker et al., 1997). Therefore aerobic, microaerobic and anaerobic respiration and fermentation take over with decreasing pO₂ sequentially. Aerobic respiration (mostly ArcBA regulated) is active at pO₂,5 = 5 µM O₂, whereas anaerobic respiration (mostly FNR regulated) and fermentation function at pO₂,5 = 5 µM O₂ and pO₂,5 = 1 µM O₂, respectively.

Presence of O₂ in the Cytoplasm for Catabolic Purposes: Degradation of aromatic compounds like benzoate requires O₂ for ring cleavage by oxygenases. Due to the cytoplasmic location of the oxygenases, growth at the expense of aromatic compounds depends on the presence of O₂ in the cytoplasm. This is in contrast to the aerobic metabolism of glucose or succinate which requires O₂ only for aerobic respiration by membraneous oxidases. The functioning of oxygenase dependent metabolic pathways, or growth on these substrates, therefore is indicative for the presence of O₂ in the cytoplasm (Arras et al., 1998).

Pseudomonas putida is able to grow at the expense of aromatic compounds under aerobic and microaerobic conditions. The oxygen tension decreasing the maximal growth rate by 50% (pO₂,5) is about 8 µM O₂ for aromatic compounds compared to about 1.5 µM O₂ for glucose or succinate (Arras et al., 1998). At the corresponding pO₂,5 value the oxygenases became limiting in catabolism, and the substrates of the oxygenases were excreted (Arras et al., 1998). Thus under aerobic and microaerobic conditions, the diffusion of O₂ into the cytoplasm occurs at high rates which is sufficient to support catabolic reactions with high O₂ requirement in the cytoplasm. The difference in the pO₂,5 values for FNR dependent regulation in E. coli (pO₂,5 = 1 to 5 µM O₂) and the growth on aromatic compounds (pO₂,5 = 8 µM O₂) should reflect the low rates of cytoplasmic O₂ consumption in the former and the high rates of O₂ consumption in the latter case.

Stability and Availability of O₂ within the Bacteria under Microaerobic Conditions?: In E. coli thiolis represent the major redox active compounds in the cytoplasm. For components like glutathione (GSH/ GSSG couple), glutaredoxin or thioredoxin (reduced and oxidized forms) redox potentials in the range of −200 mV to −300 mV are assumed (Tuggle and Fuchs, 1985; Hwang et al., 1992; Prinz et al., 1997). Reaction of O₂ (E⁰ = + 820 mV) with thiolis is slow compared to the high diffusion rates. Therefore O₂ is rather stable in this environment for kinetic reasons and is not in thermodynamic equilibrium with the thiol compounds. This explains why O₂ is present in the bacteria even under microaerobic conditions and despite the large difference in redox potential.

The supply of O₂ to the cytoplasm by diffusion can be calculated from the diffusion coefficients for O₂ in water, phospholipid, and protein solution (cytoplasm), and the cell dimensions (Unden et al., 1995; Becker et al., 1996). In E. coli and Pseudomonas the rate of O₂ diffusion into the bacteria can be compared to the respiration rate, i.e. the consumption of O₂ at the cytoplasmic membrane (Becker et al., 1996; Unden et al., 1995). Under microaerobic conditions with 1 µM dissolved O₂ in the medium (corresponding to about 0.5% air saturation) the diffusion of O₂ into the bacteria amounts to about 3 µmol O₂ × min⁻¹ × mg⁻¹ protein. The O₂ diffusion exceeds the O₂ consumption by respiration and other reactions (about 0.4 µmol O₂ × min⁻¹ × mg⁻¹ protein). Thus the experiments and the calculations concurrently demonstrate that under microaerobic conditions O₂ can be found and expected in the cytoplasm. This is in agreement with the measured pO₂,5 values for FNR regulation (1 to 5 µM O₂), oxygenase function (pO₂,5 ~ 8 µM O₂) and the switch to fermentation (pO₂,5 ≤ 1 µM O₂). Only at distinctively lower pO₂ values the cytoplasm might become anoxic due to consumption of O₂ by respiration and reaction with cytoplasmic thiols.

Glutathione and the Formation of Active FNR

Formation of Active FNR: FNR protein is present in E. coli in roughly the same concentrations under aerobic and anaerobic growth conditions (Spiro and Guest, 1987; Unden and Duchène, 1987). Therefore FNR function has to be controlled by changes in the functional state, and it has been shown that inactive FNR from aerobically grown E. coli can be converted under anaerobic conditions in vivo to the functional state without the need for protein synthesis (Engel et al., 1991). This suggests a reactivation of O₂-inactivated FNR in E. coli under anaerobic conditions.

According to the scheme of Figure 1 three biochemically and functionally different forms of FNR have been identified: [4Fe–4S] FNR and [2Fe–2S] FNR can be differentiated by Mössbauer spectroscopy and have been demonstrated in vitro, but also in vivo (Khoroshilova et al., 1997; Popescu et al., 1998; Kiley and Beinert, 1999) whereas formation of apoFNR as a product of O₂-inactivation has been demonstrated in vitro (Lazazzera et al., 1996; Tran et al., 2000). The interconversion of the different forms in Figure 1 has been demonstrated so far mainly in vitro, only conversion of [4Fe–4S] FNR to [2Fe–2S] FNR has been followed in vitro and in vivo (Khoroshilova et al., 1997; Popescu et al., 1998). Production of apoFNR from [2Fe–2S] FNR has been measured so far only in vitro due to the lack of appropriate methods in vivo (Lazazzera et al., 1996; Tran et al., 2000). The generation of [4Fe–4S] FNR has been studied mainly in vitro, but the involvement of the iscS desulphurase has been demonstrated in vivo by mutagenesis. The formation of [4Fe–4S] FNR from apoFNR obviously is part of de novo synthesis of active FNR. The reaction requires cysteine desulphurase (Khoroshilova et al., 1995, 1997; Green et al., 1996; Tran et al., 2000; Schwartz et al., 2000; Zheng et al., 1993) which catalyzes desulphuration of the cysteine providing HS⁻ (presumably via enzyme bound persulphide) for the FeS cluster formation. E. coli contains 3 genes homologous to the Azotobacter vinelandii nifS encoding cysteine desulphurase (NifS). The IscS isoenzyme (iscS gene) seems to be most important for
formulation of [4Fe–4S] FNR \textit{in vivo} (Schwartz \textit{et al.}, 2000). In addition, O\textsubscript{2} inactivated apoFNR can be used efficiently for reconstitution of active FNR in a very similar reaction with the same cofactors and enzymes (Tran \textit{et al.}, 2000). Therefore \textit{in vitro} FNR can be inactivated by O\textsubscript{2} and reactivated in a cyclic process as shown in Figure 1, and in this way O\textsubscript{2} inactivation of FNR is reversible. It is not known, however, whether the reactivation of O\textsubscript{2}-exposed apoFNR is of physiological relevance in \textit{E. coli}. Direct reactivation of [4Fe–4S] FNR from [2Fe–2S]-FNR has been demonstrated so far neither \textit{in vitro} nor \textit{in vivo}, and it is not known whether this reaction takes place.

Glutathione as the Reducing Agent for the Formation of [4Fe–4S] FNR. Mutants of \textit{E. coli} deficient of glutathione (gshA) show a shift in the switch point for FNR dependent regulation to distinctly lower pO\textsubscript{2} values, suggesting a role for glutathione in the control of FNR function in the bacteria (Tran \textit{et al.}, 2000). Complete loss of FNR function in the gshA mutant was not observed, suggesting that other cellular reductants can take over the function of glutathione in parts. This might apply in particular under anaerobic conditions when slow activation by the artificial reaction might be sufficient to retain FNR in the active state. \textit{In vitro}, glutathione can be used as the reducing agent for the formation of active FNR ([4Fe–4S] FNR) from apoFNR and from O\textsubscript{2}-inactivated apoFNR (Tran \textit{et al.}, 2000). Thus it is also possible to replace dihydrolipoamide which is used normally as a nonphysiological reducing agent. In the \textit{in vitro} reconstitution glutathione serves as the reducing agent for disulfide residues present in apoFNR and for the formation of HS\textsuperscript{−} from cysteine by the cysteine desulfurase. Due to the \textit{in vivo} effects of glutathione deficiency (gshA mutant) on FNR function and the role of glutathione as a major cellular reductant in \textit{E. coli}, it is possible that glutathione plays a similar role for FNR function in the bacteria as demonstrated \textit{in vitro}. Whether glutathione supports also the conversion of [2Fe–2S] FNR to [4Fe–4S] FNR is not known.

Control of the Functional State of FNR by O\textsubscript{2} and Glutathione

If the different forms of FNR can be interconverted as suggested in Figure 1, the function of FNR would depend on the ratio of active ([4Fe–4S] FNR) and inactive FNR ([2Fe–2S] FNR, apoFNR), and on the rates of [4Fe–4S] FNR formation and disassembly (Eq. 1).

\[
\text{FNR}_{\text{active}} + \frac{O_2}{GSH, \text{IscS, Fe}^{2+}, \text{Cys, others}} \rightarrow \text{FNR}_{\text{inactive}}
\]  

(1)

The only variable parameter affecting FNR function which has been identified so far, is the O\textsubscript{2} concentration in the cytoplasm (Becker \textit{et al.}, 1996). Therefore it is assumed that the inactivation of FNR by O\textsubscript{2} essentially is responsible for the control of the functional state of FNR, or the ratio of active and inactive FNR. According to Eq. 1 this can be explained assuming that the inactivation of FNR by O\textsubscript{2} is rapid compared to the formation of active FNR, and that the inactivation varies largely in response to the O\textsubscript{2} concentration. The reverse reaction, the formation of active FNR is assumed to be slow and to represent the sum of \textit{de novo} synthesis of FNR and of the reactivation of O\textsubscript{2}-inactivated FNR. The formation of active FNR is supposed to require essentially the same components identified \textit{in vitro}, i.e. glutathione as the reducing agent, cysteine, Fe ions, IscS and possibly some other factors (Eq. 1). As far as known, the factors are present in similar concentrations or activities under aerobic and anaerobic conditions. This applies to the total FNR contents (Unden and Duchêne, 1987), and presumably also to the glutathione content and GSSG/GSH ratio (Hwang \textit{et al.}, 1992; Prinz \textit{et al.}, 1997), supporting the view that the formation of active FNR occurs at roughly constant rates. However, there are no experimental data available so far which support these assumptions \textit{in vivo} directly.

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