In vivo cycling of the *Escherichia coli* transcription factor FNR between active and inactive states

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FNR proteins are transcription regulators that sense changes in oxygen availability via assembly–disassembly of [4Fe–4S] clusters. The *Escherichia coli* FNR protein is present in bacteria grown under aerobic and anaerobic conditions. Under aerobic conditions, FNR is isolated as an inactive monomeric apoprotein, whereas under anaerobic conditions, FNR is present as an active dimeric holoprotein containing one [4Fe–4S] cluster per subunit. It has been suggested that the active and inactive forms of FNR are interconverted *in vivo*, or that iron–sulphur clusters are mostly incorporated into newly synthesized FNR. Here, experiments using a thermo-inducible *fnr* expression plasmid showed that a model FNR-dependent promoter is activated under anaerobic conditions by FNR that was synthesized under aerobic conditions. Immunoblots suggested that FNR was more prone to degradation under aerobic compared with anaerobic conditions, and that the ClpXP protease contributes to this degradation. Nevertheless, FNR was sufficiently long lived (half-life under aerobic conditions, ~45 min) to allow cycling between active and inactive forms. Measuring the abundance of the FNR-activated *dms* transcript when chloramphenicol-treated cultures were switched between aerobic and anaerobic conditions showed that it increased when cultures were switched to anaerobic conditions, and decreased when aerobic conditions were restored. In contrast, measurement of the abundance of the FNR-repressed *ndh* transcript under the same conditions showed that it decreased upon switching to anaerobic conditions, and then increased when aerobic conditions were restored. The abundance of the FNR- and oxygen-independent *tatE* transcript was unaffected by changes in oxygen availability. Thus, the simplest explanation for the observations reported here is that the FNR protein can be switched between inactive and active forms *in vivo* in the absence of *de novo* protein synthesis.

Received 6 June 2005

Revised 1 September 2005

Accepted 12 September 2005

INTRODUCTION

The FNR protein is an oxygen-responsive transcription regulator that controls the expression of more than 100 transcriptional units in *Escherichia coli*, and coordinates the transition from aerobic to anaerobic growth (Becker *et al.*, 1996; Covert *et al.*, 2004; Gonzalez *et al.*, 2003; Guest *et al.*, 1996; Kang *et al.*, 2005; Salmon *et al.*, 2003; Sawers, 1999; Unden & Bongaerts, 1997). The intracellular concentration of FNR is similar under both aerobic and anaerobic conditions (Sutton *et al.*, 2004a; Unden & Duchene, 1987), but FNR is activated under anaerobic conditions by the acquisition of [4Fe–4S] clusters that promote the formation of homodimers, and enhance DNA binding at sites resembling the consensus sequence TTGAT-N₄-ATCAA (Bauer *et al.*, 1999; Eiglmeier *et al.*, 1989; Green *et al.*, 1996, 2001; Green & Paget, 2004; Khoroshilova *et al.*, 1995, 1997; Kiley & Beinert, 1999, 2003; Lazazzera *et al.*, 1993, 1996; Moore & Kiley, 2001; Patschkowski *et al.*, 2000; Popescu *et al.*, 1998). Four essential cysteine residues (Cys20, 23, 29 and 122) ligate the FNR [4Fe–4S] cluster, which is

incorporated in a process involving the *isc* operon (Schwartz *et al.*, 2000). Accordingly, deletion of the *iscS* gene (a cysteine desulphurase that provides the sulphur for iron–sulphur cluster assembly) impairs FNR activity *in vivo* (Schwartz *et al.*, 2000). Furthermore, comparison of the effects on FNR, which requires an iron–sulphur cluster for activity, and those on FNR*, an FNR variant that is active in the absence of an iron–sulphur cluster, suggests that the *isc* operon plays a major role in the assembly of the FNR iron–sulphur cluster (Schwartz *et al.*, 2000).

The [4Fe–4S] form of FNR acts as both a positive and a negative regulator of gene expression, activating transcription by recruiting RNA polymerase, or repressing transcription by inhibiting the formation of productive promoter–RNA polymerase interactions (Barnard *et al.*, 2004; Bell & Busby, 1994; Blake *et al.*, 2003; Browning *et al.*, 2003; Green & Marshall, 1999; Green *et al.*, 1998; Lamberg & Kiley, 2000; Lamberg *et al.*, 2002; Li *et al.*, 1998; Lonetto *et al.*, 1998; Marshall *et al.*, 2001; Meng *et al.*, 1997; Williams *et al.*, 1997; Wing *et al.*, 2000). Exposure of *E. coli* to air is sensed by the

disassembly of the FNR iron–sulphur clusters. The [4Fe–4S] clusters are first converted to [2Fe–2S] clusters (Crack *et al.*, 2004; Jordan *et al.*, 1997; Khoroshilova *et al.*, 1997; Sutton *et al.*, 2004a, b). This inactivates FNR by promoting the formation of FNR monomers, and inhibition of DNA binding (Lazazzera *et al.*, 1996). When oxygen persists in the environment, the [2Fe–2S] clusters disassemble, yielding apo-FNR (Green *et al.*, 1991; Achebach *et al.*, 2005) in a process that can be driven by superoxide, a by-product of aerobic metabolism (Sutton *et al.*, 2004b). Consequently, FNR-activated genes are switched off, and FNR-repressed genes are switched on under aerobic conditions.

Studies *in vitro* have shown that under anaerobic conditions iron–sulphur clusters can be incorporated into the isolated apo-FNR protein, and the reconstituted protein can then bind to DNA with high affinity, and regulate transcription from target promoters (Green *et al.*, 1996; Jordan *et al.*, 1997; Khoroshilova *et al.*, 1995). Furthermore, *in vivo* studies of the expression of an FNR-activated gene, and the reactivity of the cysteine thiol groups (Cys20, 23, 29 and 122) of FNR that ligate the iron–sulphur clusters, suggest that inactive apo-FNR and active iron–sulphur-containing FNR can be interconverted when cultures are shifted between aerobic and anaerobic conditions (Engel *et al.*, 1991; Six *et al.*, 1996). Thus, under aerobic conditions, the cysteinyl residues that are used to ligate both the [4Fe–4S] and [2Fe–2S] clusters of FNR are reactive, and can be carboxymethylated, but when cultures are shifted to anaerobic conditions, the cysteinyl residues are protected (Engel *et al.*, 1991). Moreover, it has recently been suggested that aerobically grown *E. coli* cells contain significant amounts of apo-FNR (Achebach *et al.*, 2005). The simplest explanation for these observations is that a significant amount of FNR is present in the apo form under aerobic conditions, and, upon transfer to anaerobic conditions, apo-FNR acquires iron–sulphur clusters. However, it has been suggested that rather than being available to receive an iron–sulphur cluster, apo-FNR protein is mostly degraded, and that iron–sulphur clusters are mostly incorporated into newly synthesized FNR (Patschkowski *et al.*, 2000). Here, evidence is presented to support the view that FNR is cycled between active and inactive states in the absence of *de novo* FNR synthesis.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains used were: *E. coli* MC1000 (*lac*), and the isogenic *fnr* mutant JRG1728 (Spiro & Guest, 1987a); *E. coli* MG1655, and the isogenic *clpX* mutant. The plasmids used were: pJLA502 (Ap^R), an expression vector with a temperature-inducible promoter (Schauder *et al.*, 1987); pGS199 (Ap^R), a pJLA502-based *fnr* expression plasmid (Spiro & Guest, 1987b); and pFF-41.5 (Tet^R), a low-copy-number plasmid with a class II FNR-activated promoter fused to *lacZ* (Wing *et al.*, 1995). Standard methods were used to transform bacteria, and isolate DNA (Sambrook & Russell, 2001). Bacterial cultures were grown in Lennox broth (Lennox, 1955) containing tryptone (10 g l⁻¹), yeast extract (5 g l⁻¹), NaCl (5 g l⁻¹), and supplemented with antibiotics as indicated: tetracycline (35 mg l⁻¹), ampicillin (100 mg l⁻¹), kanamycin (50 mg l⁻¹). Cultures were grown from

colonies on agar plates at 37 °C, either aerobically in conical flasks (10–20 ml in a 250 ml flask, shaking at 250 r.p.m.), or anaerobically in sealed bottles (7–25 ml, without shaking). The MIC values for chloramphenicol (to inhibit protein synthesis) and rifampicin (to inhibit transcription) were determined by measuring growth of aerobic cultures in the presence of increasing concentrations of the antibiotics. The MICs were 10 µg ml⁻¹ for chloramphenicol, and 6 µg ml⁻¹ for rifampicin.

***lacZ* reporter experiments.** *E. coli* JRG1728 (*fnr*), containing the reporter plasmid FF-41.5, was transformed with either pJLA502 (vector) or pGS199 (encoding FNR). Three independent cultures (20 ml) of both strains were grown under aerobic conditions at 25 °C for 5.5 h, before increasing the temperature to 42 °C for 30 min to induce FNR synthesis. The cultures were then returned to 25 °C for 1 h to switch off further *fnr* transcription. At this stage, one group of cultures was transferred to an anaerobic workstation (Don Whitley MK3), and poured into universal vials, which were sealed and placed in anaerobic jars (Oxoid). These anaerobic cultures were then incubated at 25 °C for 3.5 h. A second group of cultures was maintained under aerobic conditions. At each stage, samples of the cultures (2–3 ml) were taken for the measurement of β -galactosidase activity in triplicate (Miller, 1972), and to estimate the amounts of FNR in the cytoplasm by using Western blotting. For immunoblotting, crude cell extracts were prepared by alkaline lysis, and, after matching the protein contents of the samples, polypeptides were separated by SDS-PAGE (10% polyacrylamide). The polypeptides were then transferred to nitrocellulose membranes for detection of FNR using a polyclonal antibody (1:10 000 working dilution) and the ECL Plus system (Amersham). The relative amount of FNR in each sample was determined using ImageMaster software (Amersham).

Addition of ¹⁴C-labelled amino acids (0.185 MBq; Amersham) to post-induction cultures of JRG1728 (*fnr*) containing pGS199 was used to determine whether the shift to 25 °C (non-inducing condition) from 42 °C (inducing condition) halts FNR synthesis. FNR and the control protein aconitase were collected from cell-free extracts (0.8 ml) using anti-FNR and anti-aconitase sera (1 h incubation at 20 °C), followed by trapping of the antibody–protein complexes with Protein A Sepharose (0.1 ml). Aliquots were separated on SDS-polyacrylamide gels for autoradiography (as described below) and Western blotting (as described above; the aconitase antibody was used at a 1:10 000 working dilution).

Determination of protein and transcript stabilities. Cultures of *E. coli* MC1000, MG1655 and MG1655 *clpX* were grown to mid-exponential phase under aerobic and anaerobic conditions, at which point chloramphenicol was added to a final concentration of 30 µg ml⁻¹, which is threefold greater than the MIC. Aliquots (2 ml) were removed from the cultures at various time points after the addition of the antibiotic. After normalizing the protein contents of crude cell lysates, the polypeptides were separated by SDS-PAGE (10% polyacrylamide), and transferred to a nitrocellulose membrane for detection of FNR, as described above.

To determine the stabilities of the *ndh* and *dmsA* transcripts, cultures of *E. coli* MC1000 were grown to mid-exponential phase (OD₆₀₀ ~ 0.4–0.6), at which point transcription was inhibited by addition of rifampicin to a final concentration of 250 µg ml⁻¹. At various time points after exposure to rifampicin, aliquots (1 ml) of the cultures were removed, and mixed with RNAlprotect (Qiagen) to inhibit RNA degradation. Total RNA was then isolated using Qiagen RNeasy Mini-prep kits, according to the manufacturer's instructions. Any contaminating DNA was degraded by incubation with 10 units RNase-free DNase I (Roche) for 30 min at 37 °C. The DNase I was then inactivated by incubation at 70 °C for 5 min. Aliquots (2 µl) of the DNA-free RNA samples were mixed with 2 µl 200 mM MOPS (pH 7.0), 4 µl 37%

formaldehyde solution, 10 µl formamide, and 1 µl ethidium bromide (200 µg ml⁻¹). The mixtures were incubated at 55 °C for 1 h, and then chilled on ice for 10 min, before visualization on 1.5 % (w/v) denaturing (formaldehyde) agarose gels.

To determine the relative abundance of the *dmsA* and *ndh* transcripts, the total RNA samples were used as the templates in RT-PCR reactions. These reactions contained equal amounts of template (~2 µg RNA) and 25 units reverse transcriptase (ABgene), 5 µl Reddymix (ABgene), 2.5 µM forward primer, and 2.5 µM reverse primer, in a total volume of 10 µl. The primers used were: DPD007 (5'-GAGTAGTCGCCGT-AATGGT-3') and DPD007a (5'-ATGCGGTATTGGCTGC-3'), which yield a 600 bp product for *dmsA*; and DPD008 (5'-GGCCTGAC-CAACGAAG-3') and DPD008a (5'-CCAGCTGGTGTGCAGC-3'), which yield a 400 bp product for *ndh*. The RT-PCR program consisted of the following steps: 1 cycle of 30 min at 47 °C, and 2 min at 95 °C, followed by 30 cycles of 95 °C for 20 s, 58 °C for 1 min, and 72 °C for 5 min. A final step of 5 min at 72 °C was added to the end of the program. The resulting cDNAs were visualized after separation on 1.8 % (w/v) agarose gels buffered with TBE (90 mM Tris, 90 mM borate, 50 mM EDTA), and staining with ethidium bromide (200 µg ml⁻¹). Control reactions in which Reverse Transcriptase was omitted were used to ensure that the RNA samples were DNA free. The relative abundance of the cDNAs was determined using ImageMaster software (Amersham).

Determination of transcript abundance in the presence and absence of oxygen. To ensure that chloramphenicol (30 µg ml⁻¹) was sufficient to inhibit protein synthesis, cultures of *E. coli* MC1000 were incubated at 37 °C to mid-exponential phase, and chloramphenicol was added to half of the samples. Incubation was then continued for 30 min, before ¹⁴C-labelled amino acids (0.185 MBq; Amersham) were added. All cultures were then incubated at 37 °C for a further 1 h, before collecting the bacteria by centrifugation. Crude cell lysates were separated by SDS-PAGE using a 10 % (w/v) gel. After fixing in 2-propanol:water:acetic acid (25:65:10) for 30 min, and soaking in Amplify fluorographic reagent (Amersham) for a further 30 min, the gel was dried and exposed to Hyperfilm MP (Amersham) at -70 °C.

Cultures of *E. coli* MC1000 were grown at 37 °C under aerobic conditions to mid-exponential growth phase. Chloramphenicol was then added to a final concentration of 30 µg ml⁻¹, and the incubation was continued for a further 30 min. At this point, samples (1 ml) were taken for total RNA isolation, and the cultures were then made anaerobic in a Don Whitley MK3 anaerobic workstation to transfer the cultures to sealed universal vials, which were incubated at 37 °C for 1 h in anaerobic jars (Oxoid). Further samples (1 ml) were taken for RNA isolation. The remaining culture was then transferred back into sterile 250 ml conical flasks for a final 1 h incubation at 37 °C, before the last set of samples (1 ml) for RNA isolation were obtained. Each aliquot of culture (1 ml) was rapidly mixed with 2 ml RNeasy Protect (Qiagen) to inhibit RNA degradation, and total RNA was isolated using a Qiagen RNeasy Mini-prep kit. After treatment with DNase I (see above), the quality of the resulting RNA samples was determined by electrophoresis, as described above. The mRNA transcripts for *ndh* and *dmsA* were amplified using RT-PCR, as described above. A control transcript, *tatE*, which is unresponsive to oxygen availability (Jack *et al.*, 2001), was used to standardize the reactions, and was amplified using primers DPD005 (5'-CACTCAAAATGAGAGAGCTTTTC-3') and DPD005a (5'-GGTGAGATTAGTATTACCAAACTG-3') to yield a 200 bp fragment of *tatE*. The resulting cDNAs were visualized after separation by electrophoresis on 1.8 % (w/v) agarose TBE gels, as described above.

The effect of the ClpXP protease on FNR-mediated regulation of *dmsA* and *ndh* transcription was investigated by isolating total RNA from

anaerobic cultures of MG1655 and MG1655 *clpX*, and estimating the abundance of the transcripts by RT-PCR, as described above. Once again, the *tatE* transcript served as a control.

RESULTS

The starting point for this work was the suggestion that after exposure of the *E. coli* FNR protein to oxygen, and consequent degradation of the [4Fe-4S] clusters, the resulting monomeric apoprotein is mostly degraded rather than recycled (Patschkowski *et al.*, 2000). Earlier studies, in which the expression of an FNR-activated gene (*frd*, encoding fumarate reductase), and the reactivity of the cysteine thiol groups of FNR, were determined upon shifting cultures between aerobic and anaerobic conditions in the absence of *de novo* FNR synthesis, suggested that apo-FNR is recycled (Engel *et al.*, 1991). Therefore, experiments were formulated to test the hypothesis that apo-FNR is recycled *in vivo*.

FNR synthesized under aerobic conditions is activated upon switching cultures to anaerobic conditions

The strategy adopted was to control FNR synthesis by using the thermo-inducible FNR expression plasmid pGS199 in the *fnr lac* double-mutant JRG1728. A model FNR-dependent promoter FF-41.5 fused to *lacZ* in the low-copy-number plasmid pFF-41.5 (Wing *et al.*, 1995) was chosen as a reporter of FNR activity, because many natural FNR-regulated promoters are also dependent on other transcription factors for activation. Thus, JRG5438 carries the *lacZ* reporter pFF-41.5 and the FNR expression plasmid pGS199. An equivalent strain JRG5437 carrying the vector pJLA502 in place of pGS199 was used as a control. After pre-culturing under aerobic conditions at 25 °C, FNR synthesis was then induced by incubation at 42 °C, before returning the cultures to 25 °C to halt further FNR synthesis. Finally, the cultures were either transferred to anaerobic conditions at 25 °C, or held under aerobic conditions at 25 °C. At each stage, aliquots were removed to determine whether FNR was present in the bacteria, and the consequent effect on β-galactosidase activity. This showed that before FNR synthesis was induced, expression from the FNR-dependent FF-41.5 promoter was low in both the test and control cultures (Fig. 1a, bars labelled 1). After induction of FNR synthesis, expression of the reporter gene was enhanced even under aerobic conditions (Fig. 1a, bars labelled 2 and 3). This apparent aerobic FNR activity is probably caused by the increase in the intracellular concentration of FNR (approx. three- to fivefold greater than that observed for chromosomally encoded FNR, as estimated by immunoblotting) after induction. Addition of ¹⁴C-labelled amino acids to post-induction cultures, and analysis of the radiolabelled cell-free extracts, revealed that although polypeptides with molecular masses corresponding to FNR and the control protein aconitase B were detected in Western blots of immunoprecipitates obtained using anti-FNR and anti-aconitase B sera, only the higher-molecular-mass species (aconitase B) was detected in the

Fig. 1. Activation of an FNR-dependent model promoter upon transfer to anaerobic conditions in the absence of *de novo* FNR synthesis. The expression of *fnr* in JRG5438 was controlled by the thermo-inducible (42 °C) promoter of pGS199, and FNR activity was reported by activation of the FNR-dependent FF-41.5 promoter fused to the reporter gene *lacZ*. The control strain JRG5437 contained the vector (pJLA502) in place of pGS199. (a) Cultures of JRG5437 (FNR⁻; black bars) and JRG5438 (FNR⁺; open and grey bars) were grown under aerobic conditions at 25 °C for 5.5 h. Aliquots were taken for β -galactosidase assays (1). FNR synthesis was then induced by incubation at 42 °C for 30 min under aerobic conditions. Further samples to monitor FNR activity were taken (2). FNR synthesis was then halted by incubation at 25 °C for 1 h (3). Finally, the cultures were either maintained under aerobic conditions (4, grey bar) or transferred to anaerobic conditions (4, open and black bars) for 3.5 h at 25 °C, before further samples were taken for β -galactosidase assays. β -Galactosidase activity was measured according to Miller (1972), using triplicate assays of triplicate cultures. The mean values and standard deviations are shown. (b) Representative anti-FNR immunoblots of bacterial lysates obtained from the samples indicated above. Before immunoblotting, the lysates were separated on denaturing polyacrylamide gels, and stained with Coomassie blue to allow equalization of the amounts of protein applied to each lane. Samples in lanes 1–4 are as described above for 1–4 in (a). (c) The relative amounts of FNR present at the various stages of the experiment, as determined using the ImageMaster software package with the immunoblots shown in (b). The amount of FNR present in lane 2 of each blot in (b) was set at 100 %.

corresponding autoradiographs. The absence of FNR on the autoradiographs suggests, but does not prove, that FNR protein is not synthesized when cultures are shifted back to the non-permissive condition (25 °C) after induction of FNR synthesis at 42 °C (not shown). Despite the apparent absence of *de novo* FNR synthesis, upon transfer to anaerobic conditions β -galactosidase activity was enhanced by approximately fourfold compared with cultures maintained under aerobic conditions (Fig. 1a, bars labelled 4). This response is consistent with the hypothesis that a portion of the FNR protein synthesized during the aerobic 42 °C phase, which was incapable of activating transcription (apo-FNR or [2Fe–2S] FNR), is converted into a transcriptionally competent form when the culture is transferred to anaerobic conditions.

Determination of the relative amounts of FNR present at each stage of the experiment showed that FNR synthesis was initiated upon transfer to the higher temperature [Fig. 1b (lane 2) and c (bar 2)]. After the cultures were returned to 25 °C (to inhibit further FNR synthesis) for 1 h, the cellular content of FNR protein was lowered by ~35 % [Fig. 1b (lane 3) and c (bar 3)]. Upon transfer to anaerobic conditions for 3.5 h, the cellular content of FNR was maintained, whereas under aerobic conditions, the relative amount of FNR decreased to ~10 % of the original amount [Fig. 1b (lane 4) and c (bar 4)]. This suggests that FNR protein turnover might be greater under aerobic compared with anaerobic conditions.

Stability of the *E. coli* FNR protein *in vivo*

It has been shown that the *E. coli* FNR protein can be activated *in vitro* by reconstituting the [4Fe–4S] clusters into apo-FNR under anaerobic conditions (Green *et al.*, 1996; Jordan *et al.*, 1997; Khoroshilova *et al.*, 1995). This suggests that if apo-FNR is not quickly degraded under aerobic conditions *in vivo* then it could be recycled. Therefore, to further investigate the stability of the FNR protein *in vivo* under aerobic conditions, immunoblotting with anti-FNR serum was used to probe a series of samples taken from cultures treated with chloramphenicol to inhibit protein synthesis. Inhibition of protein synthesis was confirmed by the inability to detect radiolabelled polypeptides on autoradiographs of cell-free extracts obtained from cultures to which ¹⁴C-labelled amino acids had been added 30 min after chloramphenicol treatment. Labelled polypeptides were readily detected in equivalent cultures that had not been treated with chloramphenicol (not shown). The half-life of the FNR protein under aerobic conditions was estimated to be ~45 min (Fig. 2a). It has been reported that FNR is a substrate for the ClpXP protease (E. L. Mettert and P. J. Kiley, personal communication reported in Flynn *et al.*, 2003), and thus FNR degradation in a *clpX* mutant was investigated. This revealed that, under aerobic conditions, FNR half-life in the *clpX* mutant was increased to ~80 min, suggesting that the ClpXP system contributes to, but is not the only agent mediating, the degradation of FNR under aerobic conditions (Fig. 2a). Measurements of the rate of

Fig. 2. Effect of a *clpX* mutation on FNR degradation and activity. (a) Quantification of immunoblots of FNR in crude lysates of cultures of MG1655 (■) and the MG1655 *clpX* mutant (▲) maintained under aerobic conditions in the presence of chloramphenicol. Cultures were grown to mid-exponential phase, and then chloramphenicol (30 $\mu\text{g ml}^{-1}$) was added, and aliquots were removed for immunoblotting with anti-FNR serum at the indicated times. The signals obtained were quantified using the ImageMaster software package. (b) RT-PCR products obtained from using total RNA from anaerobic cultures of MG1655 (lane 1), and MG1655 *clpX* (lane 2) as the template, and *dmsA*, *ndh* and *tatE* transcript specific primers.

[Fe-S] cluster incorporation into apo-FNR *in vitro* indicate a reconstitution rate of 0.02–0.09 nmol FNR min^{-1} , equivalent to 10^{12} – 10^{13} molecules of FNR reconstituted per minute (Achebach *et al.*, 2004). As the intracellular concentration of FNR is ~ 3000 – 4000 molecules per *E. coli* cell (Sutton *et al.*, 2004a; Uden & Duchene, 1987), it would appear that FNR is sufficiently long lived *in vivo* under aerobic conditions to offer the possibility that it can be cycled between active and inactive forms.

Similar experiments carried out under anaerobic conditions suggested that the FNR protein is more stable than under aerobic conditions. In contrast to the aerobic experiments, for both the wild-type and the *clpX* mutant, 20–30 % of FNR protein was still detectable after 24 h (not shown).

Measurement of the relative amounts of an FNR-activated transcript (*dmsA*; Lamberg & Kiley, 2000) and an

FNR-repressed transcript (*ndh*; Green & Guest, 1994; Meng *et al.*, 1997; Spiro *et al.*, 1989) in anaerobic cultures of MG1655 and MG1655 *clpX* revealed that the *dmsA* transcript was ~ 2.5 -fold more abundant, and the *ndh* transcript was ~ 1.5 -fold less abundant in the *clpX* mutant (Fig. 2b). The abundance of the *tatE* transcript, which does not respond to either FNR or oxygen availability (Jack *et al.*, 2001), was the same in both strains (Fig. 2b). These observations are consistent with the enhanced stability of FNR in the *clpX* mutant.

In the absence of *de novo* FNR synthesis, the protein can be switched between active and inactive forms *in vivo* in response to oxygen availability

The experiments described above supported the hypothesis that FNR can be cycled between active and inactive states. However, the *lac* reporter experiments, and the experiments reported by Engel *et al.* (1991) using the FNR-activated *frdA* promoter, used overproduced FNR synthesized from pGS199. Therefore, the effects of oxygen availability on chromosomally encoded FNR activity were determined by measuring the abundance of the *dmsA*, *ndh* and *tatE* transcripts. Firstly, the half-lives of the *dmsA* and *ndh* transcripts had to be determined. This was achieved by estimating the relative amounts of each transcript using RT-PCR at fixed time points after inhibiting transcription with rifampicin. The half-lives of both transcripts were in the range 2–5 min, which is typical of *E. coli* mRNAs (not shown). The effects of oxygen availability on FNR-mediated regulation of *dmsA* and *ndh* transcription were then investigated by preparing total RNA from cultures treated with chloramphenicol to inhibit protein synthesis. The RNA samples served as the templates in RT-PCR assays, and the amplified cDNA was analysed by agarose gel electrophoresis. Under aerobic conditions, the abundance of the *dmsA* transcript was low (Fig. 3, upper panel, lane 1). However, upon transfer of the culture to anaerobic conditions, even in the absence of *de novo* FNR protein synthesis, the abundance of the *dmsA* transcript was enhanced (Fig. 3, upper panel, lane 2). Moreover, when air was reintroduced to the cultures, the amount of the *dmsA* transcript decreased (Fig. 3, upper panel, lane 3). The opposite pattern was observed with the FNR-repressed transcript *ndh*. In this case the abundance of the *ndh* transcript was greatest when cultures were exposed to air (Fig. 3, middle panel, lanes 1 and 3), and lowest under anaerobic conditions (Fig. 3, middle panel, lane 2). As expected (Jack *et al.*, 2001), the *tatE* transcript did not respond to the changes in oxygen availability (Fig. 3, lower panel). These data suggest that *de novo* FNR protein synthesis is not necessary for FNR activation, and that FNR is cycled between active and inactive states *in vivo*.

DISCUSSION

Bacterial FNR proteins are transcription factors that coordinate the transition between aerobic and anaerobic

Fig. 3. *In vivo* activation and repression of FNR-regulated genes upon transfer from aerobic to anaerobic conditions in the absence of *de novo* FNR synthesis. Cultures of *E. coli* MC1000 were grown aerobically to mid-exponential phase, and then treated with chloramphenicol (30 µg ml⁻¹) to inhibit protein synthesis. After a further 30 min incubation, aliquots (lane 1) were removed, and the cultures were transferred to anaerobic conditions. After 1 h, further samples (lane 2) were taken, and the cultures were then transferred to aerobic conditions, and, after another 1 h incubation, the final samples were taken (lane 3). The panels show the cDNA products generated by RT-PCR of the FNR-activated *dmsA* transcript, the FNR-repressed *ndh* transcript, and the FNR- and oxygen-independent *tatE* transcript using total RNA prepared from the aerobic (O₂) and anaerobic (N₂) samples described above.

metabolic modes (Guest *et al.*, 1996; Sawers, 1999). They use an iron-sulphur-cluster-based switch to respond to changes in oxygen availability (see reviews by Green & Paget, 2004; Kiley & Beinert, 1999, 2003). The intracellular concentration of *E. coli* FNR is similar under aerobic and anaerobic

conditions (Sutton *et al.*, 2004a; Udden & Duchene, 1987), and studies with an FNR variant, FNR-L28H, which possesses a more stable iron-sulphur cluster suggest that [4Fe-4S] clusters are inserted into apo-FNR under both aerobic and anaerobic conditions (Bates *et al.*, 2000). When the [4Fe-4S] form of FNR is exposed to oxygen *in vitro* or *in vivo*, it is first converted to a [2Fe-2S] form, and then to an apo-form (Crack *et al.*, 2004; Engel *et al.*, 1991; Green & Paget, 2004; Kiley & Beinert, 1999, 2003; Popescu *et al.*, 1998; Sutton *et al.*, 2004a, b). Thus, FNR protein isolated from anaerobic cultures is in the transcriptionally active [4Fe-4S] form (Lazazzera *et al.*, 1996), whereas FNR isolated from aerobic cultures is in the inactive apo-form (Green *et al.*, 1991). Early studies suggested that FNR could be recycled by being interconverted between inactive and active forms (Engel *et al.*, 1991). More recently, it has been suggested that the ClpXP protease degrades FNR under aerobic conditions (E. L. Mettert and P. J. Kiley, personal communication reported in Flynn *et al.*, 2003), and that *de novo* FNR synthesis might be necessary for the incorporation of iron-sulphur clusters (Patschkowski *et al.*, 2000). The experiments described here show that FNR is more readily degraded *in vivo* under aerobic conditions compared with anaerobic conditions, and that ClpXP contributes significantly to this degradation, but makes a less pronounced contribution under anaerobic conditions. Nevertheless, it would appear that the FNR protein is sufficiently long lived (half-life ~45 min, under aerobic conditions) to allow cycling between active and inactive forms *in vivo* in the absence of *de novo* protein synthesis. At this point, it is not possible to exclude interconversion of [4Fe-4S] to [2Fe-2S] forms (Popescu *et al.*, 1998). However, the simplest explanation for the data presented here and elsewhere is that in *E. coli*, [4Fe-4S] clusters are continually incorporated

Fig. 4. A simplified scheme illustrating the cycling of FNR between active and inactive states. FNR protein is synthesized under aerobic and anaerobic conditions and [4Fe-4S] clusters (□) are incorporated, promoting the formation of transcriptionally active homodimers. In the presence of oxygen, these [4Fe-4S] clusters are rapidly disassembled generating inactive FNR with [2Fe-2S] clusters (◇). The [2Fe-2S] clusters are further degraded to yield apo-FNR protein, which can then reacquire [4Fe-4S] clusters, and continue around the cycle again. In addition, some [2Fe-2S] containing FNR may be converted to the [4Fe-4S] form. To maintain the intracellular concentration of FNR, *fnr* expression is autoregulated (Spiro & Guest, 1987b), and monomeric apoprotein is more prone to degradation (mediated in part by the ClpXP protease) than the [4Fe-4S]-containing dimer. The width of the arrow is intended to suggest the relative rate of the reaction.

into apo-FNR, but, in the presence of oxygen, these are disassembled forming the transcriptionally inactive [2Fe–2S] form of the protein, which in turn is converted to apo-FNR by prolonged exposure to oxygen and endogenously generated reactive oxygen species (Fig. 4; Achebach *et al.*, 2005; Green & Paget, 2004; Kiley & Beinert, 1999, 2003; Sutton *et al.*, 2004a, b). The apparent enhanced degradation of FNR under aerobic conditions might indicate that the FNR dimer is more stable than the monomer *in vivo*. It has been suggested that FNR possesses both N- and C-terminal sequences that facilitate recognition by the ClpXP protease (Flynn *et al.*, 2003). Clearly the direct involvement of the N-terminal region of FNR in the ligation of the sensory iron–sulphur cluster might cause one of the ClpXP recognition sequences to be masked upon iron–sulphur cluster incorporation, and thereby contribute to the enhanced stability of FNR under anaerobic conditions.

In conclusion, the data presented here and elsewhere suggest that FNR is continually cycling between the inactive and active states. In the absence of oxygen, the cycle is interrupted at the [4Fe–4S] to [2Fe–2S] conversion, and consequently the transcriptionally active [4Fe–4S] form accumulates, and FNR-mediated control of gene expression is exerted. This scheme (Fig. 4) accounts for the isolation of apo-FNR from aerobic cultures of *E. coli* (Green *et al.*, 1991; Achebach *et al.*, 2005), the oxygen-dependent changes in the reactivities of the cysteine thiols that act as ligands for the FNR iron–sulphur cluster (Engel *et al.*, 1991), and the ability to incorporate functional [4Fe–4S] clusters into apo-FNR *in vitro* (Green *et al.*, 1996; Jordan *et al.*, 1997; Khoroshilova *et al.*, 1995). Thus, it seems likely that FNR continually samples its environment by cycling through inactive and active states, providing an economical and rapidly responding mechanism to monitor changes in environmental oxygen.

ACKNOWLEDGEMENTS

We thank the Biotechnology and Biological Sciences Research Council (UK), and the Medical Research Council (UK), for supporting this work, and F. R. Blattner for providing the MG1655 *clpX* mutant.

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