FNR is a direct oxygen sensor having a biphasic response curve

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Abstract FNR is a transcription regulator that controls the expression of target genes in response to anoxia. Anaerobiosis is accompanied by the acquisition of two $[4Fe\text{-}4S]^{2+}$ clusters per FNR dimer and the ability to bind DNA site-specifically. Oxidation of the $[4Fe\text{-}4S]^{2+}$ form of FNR by O_2 produced a non-DNA-binding, transcriptionally inactive form which also contains an iron-sulfur cluster, recently identified by Mossbauer spectroscopy as a [2Fe-2S] cluster (Khoroshilova et al., 1997, PNAS. 94, 6078). Complete conversion needed at least 2.5–3.0 molecules of O_2 per $[4Fe\text{-}4S]^{2+}$ cluster. Using sub-stoicheiometric amounts of air-saturated buffer, stable equilibria were established in which the $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ forms co-exist and no EPR detectable free ferric ions were released. In contrast, a 20-fold molar excess $K_3Fe(CN)_6$ was required to oxidise the $[4Fe\text{-}4S]^{2+}$ cluster and in this case, ferric ions were released. FNR is therefore a sensitive O_2 sensor.

Key words: FNR; Oxygen sensing; Iron-sulfur cluster; *Escherichia coli*

1. Introduction

FNR, the anaerobic transcription regulator of *Escherichia coli*, responds to anoxia and controls the expression of a modulon of genes that are mostly concerned with anaerobic energy generation [1]. In its active, anaerobic form, FNR is a dimer containing two oxygen-labile $[4Fe\text{-}4S]^{2+}$ clusters [2–5]. The anaerobic acquisition of the $[4Fe\text{-}4S]^{2+}$ clusters promotes dimerisation and enhances site-specific DNA binding [3,5]. Aerobic purification of FNR from aerobically grown cultures yields a monomeric protein which contains a variable amount of iron (0.02–1.7 atoms per monomer) [4,6]. Transcriptionally active $[4Fe\text{-}4S]^{2+}$-containing FNR can be generated in vitro by anaerobic incubation of apo-FNR with ferrous ions, dithiothreitol, cysteine and the NifS protein of *Azotobacter vinelandii* (which liberates S° from cysteine) [2,5]. Anaerobic purification of FNR also yields a dimeric form containing about 1.5 clusters per dimer [3]. Therefore, although the active form of FNR has been characterised, the exact nature of the aerobic-anaerobic switch has still to be defined. Oxidation of FNR leads to loss of the $[4Fe\text{-}4S]^{2+}$ clusters, monomerisation, reduced DNA binding, and an inability to activate or repress transcription from FNR-regulated promoters [3,5,6]. Under reducing conditions the addition of ferrous ions to apo-FNR increases the iron content to 1.1–2.7 atoms per monomer and confers the ability to activate transcription in vitro [4,7]. Furthermore, exposure of NifS-reconstituted FNR to excess air yielded trace amounts of a $[3Fe\text{-}4S]^{+}$-containing form of the protein [2,5]. Treatment of anaerobically purified FNR with excess air followed by freezing at intervals produced a $[2Fe\text{-}2S]^{2+}$ form with a maximum yield of 60% [8]. This form of FNR is deep red, stable for at least 12 h in air, and lacks the ability to bind DNA [8]. As oxidation proceeds complete destruction of the $[4Fe\text{-}4S]^{2+}$ cluster occurs, but with no further generation of $[2Fe\text{-}2S]^{2+}$ clusters. Mossbauer spectroscopy also shows an undefined ferric species which may include iron sulphides.

The nature of the primary signal is not fully established because expression of FNR-dependent genes can be switched in vivo with $K_3Fe(CN)_6$ [9] as well as with O_2. In the present work the oxidation of reconstituted FNR by O_2 and $K_3Fe(CN)_6$ reveals that FNR responds differently toward the two oxidants. Absorption spectra obtained during the titration of reconstituted FNR with air-saturated buffer indicate that only two species are present, the $[4Fe\text{-}4S]^{2+}$ form and a new cluster taken to be $[2Fe\text{-}2S]^{2+}$. The absorption spectra have an isosbestic point suggesting that only two coloured species are in equilibrium. No monomeric ferric ions can be detected by EPR spectroscopy. Hence the reaction with sub-stoicheiometric O_2 is quantitative in its oxidation of $[4Fe\text{-}4S]^{2+}$. On the other hand the oxidation with $K_3Fe(CN)_6$ requires at least a 20-fold excess and appears to lead to general cluster destruction and the release of free ferric ions. Thus, it is proposed that the FNR protein of *E. coli* is a direct O_2 sensor, and that exposure to O_2 converts the active $[4Fe\text{-}4S]^{2+}$ form of FNR to a transcriptionally inactive $[2Fe\text{-}2S]^{2+}$ form.

2. Materials and methods

2.1. Protein purification and FNR reconstitution

FNR was purified [4] from a GST-FNR fusion protein expressed in *E. coli* BL21 (pGS572). It was reconstituted in stoppered cuvettes by anaerobic incubation with ferrous ions, dithiothreitol, L-cysteine and the NifS protein of *A. vinelandii* [5]. The NifS protein was purified from *E. coli* BL21(λDE3) containing the *nifS* expression plasmid pDB551 [10]. The FNR reconstitution reactions were monitored spectrophotometrically and once complete, FNR was separated from low molecular weight reactants and products by size exclusion chromatography on Sephadex G25 or PD10 columns (Pharmacia) equilibrated with TCB (Tris-HCl, 50 mM; NaCl, 150 mM and $CaCl_2$, 2.5 mM). The assays for iron [5], acid-labile sulphur [11] and protein [5], have been described previously.

2.2. Spectroscopy and oxidation of FNR

A Pye-Unicam UV4 series spectrophotometer with Prism software, or a Hitachi U-4000 spectrometer, were used for optical spectroscopy.

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Abbreviations: TCB, 50 mM Tris-HCl buffer (pH 7.5) containing NaCl (150 mM) and $CaCl_2$ (2.5 mM)

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FNR samples were transferred from an anaerobic cabinet (Don Whitley Mk3 or Faircrest anaerobic workstations) in sealed cuvettes. Air-saturated TCB (approximately 240 μM O_2 at 25°C [12]) or anaerobic $K_3Fe(CN)_6$ was added by injection. For EPR spectroscopy samples of reconstituted FNR were transferred anaerobically from the titration cuvette to sealed EPR tubes and frozen in liquid nitrogen. All EPR spectra were obtained at 10K with a microwave power of 2 mW.

2.3. FNR:DNA interactions

Anaerobic gel retardation analyses were as in [5] except that the *FFmelR* promoter was replaced by an end-labeled *Eco*RI-*Bam*HI fragment of pGS1036 [13] containing the *yfiD* promoter (0.05 pmol in 10 μl). Reconstituted FNR protein (11, 22 and 150 nM dimer, final concentration) was tested for the ability to bind DNA after titration with air-saturated TCB, in the range 0–240 μM O_2. After electrophoresis the fraction (%) of DNA retarded was estimated by quantitative densitometry using a Vilber-Lourmat Bioprofil imaging system. The template for in vitro transcription [5] was *yfiD* DNA.

3. Results

After reconstitution and desalting, the FNR protein released from the GST-FNR fusion was substantially dimeric (≥ 50% as judged by gel filtration A_{280} profiles) and it contained two $[4Fe\text{-}4S]^{2+}$ clusters per dimer, based on the iron and acid-labile sulphur contents. The anaerobic, reconstituted FNR protein has a characteristic broad absorbance around 420 nm [3,5] with an ε_{420} of 13 300 M^{-1} cm^{-1} (expressed per mole of monomer; Fig. 1). The GST-FNR fusion could also be reconstituted and the product was chemically and spectroscopically identical to reconstituted FNR (not shown).

3.1. FNR is a biphasic O_2 sensor

Sequential addition of air-saturated TCB to reconstituted FNR (each addition corresponding 5–10 times less O_2 than FNR) caused a progressive decrease in absorbance at 420 nm (Fig. 1a). This was accompanied by an increase in absorbance in the 500 and 700 nm region (Fig. 1a). The isosbestic point at ca. 490 nm indicated that the $[4Fe\text{-}4S]^{2+}$ cluster is converted into a species which absorbs both at 420 nm (ε_{420} 8400 M^{-1} cm^{-1} at an O_2:FNR ratio of 1) and in the 500–700 nm region. After the initial decrease in 420 nm absorbance that accompanied each O_2 addition, the spectra remained stable for at least 48 h provided no further O_2 was admitted. During the titration samples of partially oxidised FNR were examined using EPR spectroscopy. No EPR signals were detected even after the addition of a 2-fold molar excess of O_2 (Fig. 1b). At O_2:FNR ratios greater than 3, no further reduction of A_{420} was observed, indicating that complete cluster conversion had occurred. At this point the iron content of FNR had fallen from 3.7 atoms per monomer to 2.8 atoms per monomer.

Anaerobic titrations with $K_3Fe(CN)_6$ as the oxidant resulted in qualitatively similar lowering of the absorbance at 420 nm (Fig. 1b). However, compared with O_2, at least 10–20 times more $K_3Fe(CN)_6$ (than O_2) was required to produce the same decrease in A_{420} and the increase at 500–700 nm was far less apparent. EPR spectroscopy further indicated that, in contrast to the reaction with O_2, the addition of $K_3Fe(CN)_6$ liberated ferric ions from the $[4Fe\text{-}4S]^{2+}$ cluster of FNR (Fig. 1b). The differing responses to O_2 and $K_3Fe(CN)_6$ are illustrated in Fig. 2. Both are biphasic but become linear at higher oxidant:FNR ratios. However, with O_2 the steepest slope occurred at the lowest O_2:FNR ratios showing the highest sensitivity to oxidant, whereas low ratios of $K_3Fe(CN)_6$:FNR failed to provoke an FNR response (Fig. 2).

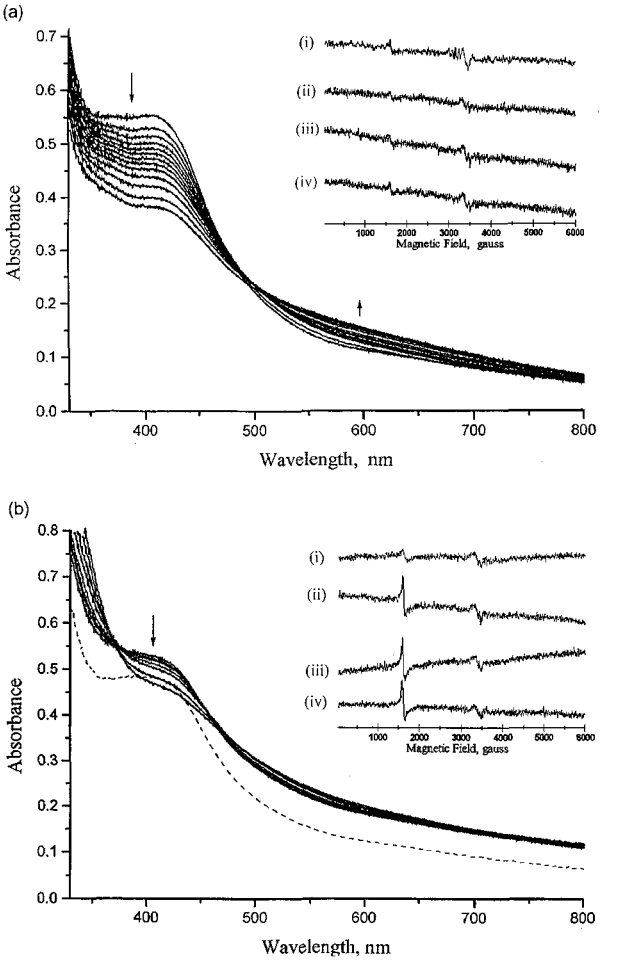

Fig. 1. Oxidation of [4Fe-4S] containing FNR by (a) O_2 or (b) $K_3Fe(CN)_6$ monitored by UV-visible absorbance and EPR spectroscopies. a: Titration of reconstituted FNR (22.8 μM dimer, 7.9 iron atoms per dimer, 32 μM $[4Fe\text{-}4S]^{2+}$ clusters based on ε_{420} of 13 300 M^{-1} cm^{-1}) with air-saturated TCB (equivalent to 0–46 μM O_2). The final O_2 concentrations were (μM): 0, 2.3, 4.6, 7, 9.3, 11.6, 15.6, 19, 22.6, 26.5, 32, 39, 46. Inset are representative EPR spectra obtained during the titration: (i) untreated reconstituted FNR; (ii) plus 4.6 μM O_2; (iii) 22.6 μM O_2; and (iv) 46 μM O_2. b: Titration of reconstituted FNR (30.3 μM dimer, 6.9 iron atoms per dimer, 28 μM $[4Fe\text{-}4S]^{2+}$ clusters) with $K_3Fe(CN)_6$ (0–770 μM). The final $K_3Fe(CN)_6$ concentrations were (μM): 0 (dashed line), 140, 190, 250, 300, 390, 490, 630, 770. The first addition of $K_3Fe(CN)_6$ caused a baseline shift, hence the untreated FNR spectrum appears below the subsequent spectra. Inset are EPR spectra obtained during the titration: (i) untreated reconstituted FNR; (ii) plus 67 μM $K_3Fe(CN)_6$; (iii) 140 μM $K_3Fe(CN)_6$; and (iv) 770 μM $K_3Fe(CN)_6$.

No EPR signals were observed when reconstituted FNR was maintained under anaerobic conditions [3,5], nor did the addition of up to a 2-fold molar excess of O_2 produce any EPR active species, even 48 h after addition of O_2 (Fig. 1a). The $g = 2.01$ signal previously observed, characteristic of a $[3Fe\text{-}4S]^{+}$ cluster, was only observed after the protein had been exposed to excess air followed by rapid freezing. The corresponding amounts of the $[3Fe\text{-}4S]^{+}$ cluster formed were not determined but the signal to noise ratios indicated that they were proportionally very low (not shown).

The responses of FNR to oxidation by O_2 and $K_3Fe(CN)_6$

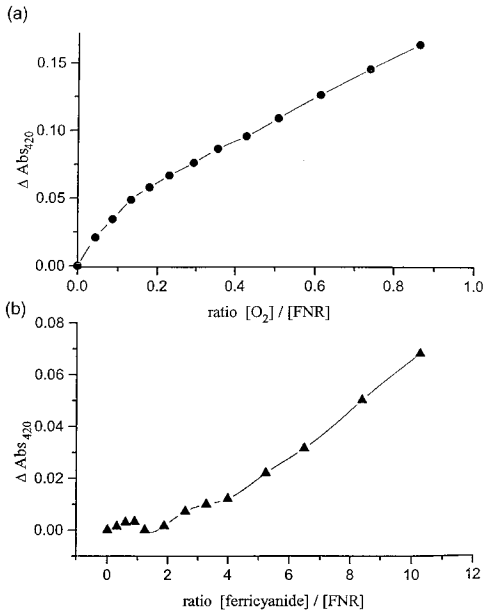

Fig. 2. Comparison of the FNR response to (a) O_2 or (b) $K_3Fe(CN)_6$. Oxidation of FNR was monitored by the stable decrease in absorbance at 420 nm after consecutive additions of the oxidants.

are strikingly different. FNR responds to much lower concentrations of O_2 than of $K_3Fe(CN)_6$ and, whereas oxidation by $K_3Fe(CN)_6$ seems to destroy the $[4Fe\text{-}4S]^{2+}$ cluster (as indicated by the release of ferric ions) O_2 converts the cluster into a new species with a different absorption spectrum. The presence of an isosbestic point in the titration suggests the presence of only two coloured interconverting species. EPR spectroscopy of O_2-treated FNR indicated that the new FNR species is EPR silent, consistent with the presence of a $[2Fe\text{-}2S]^{2+}$ cluster.

3.2. *Titration of FNR with O_2 causes a progressive decrease in DNA binding*

Gel retardation analyses were used to monitor the biological effects of oxidising FNR with O_2 (Fig. 3). Reconstituted FNR was titrated with air-saturated TCB at 25°C, samples being removed once the absorption spectrum had stabilised after each addition. The partially oxidised FNR samples were then tested for their ability to interact with *yfiD* promoter DNA. As the amount of added O_2 was increased, the DNA-binding activity was lost (Fig. 3). A 2–3-fold stoicheiometric excess of O_2 was sufficient to abolish DNA binding and to reduce A_{420} to a basal value that could not be lowered by further O_2 additions. When FNR was added at ≥ 150 nM, retardation was observed even after O_2 treatment, suggesting that a proportion of the protein retains activity even after exposure to air. Likewise, the large decrease in A_{420} at low O_2:FNR ratios, was correlated with a substantial lowering of transcriptional activity, e.g. 85% of transcriptional activity, 70% of DNA-binding ability and 50% of A_{420}, were lost with an O_2:FNR ratio of 0.8.

4. Discussion

Our understanding of the mechanism by which FNR senses and responds to anoxia has advanced rapidly in recent years [2–5]. Studies with mutant proteins (FNR*) that retain some ability to regulate transcription aerobically [2,14] and with wild-type FNR [3–5,8], have shown that the anaerobically active form of FNR is a dimer containing two oxygen-labile $[4Fe\text{-}4S]^{2+}$ clusters. Anaerobic incorporation of $[4Fe\text{-}4S]^{2+}$ clusters enhances both the dimerisation and DNA-binding activity of FNR. Comparing the effects of O_2 and $K_3Fe(CN)_6$ on reconstituted FNR now shows that FNR is sensitive to O_2, responding most readily to the lowest (sub-stoicheiometric) concentrations of O_2. These observations correlate well with the in vivo effects of O_2 on the *frdA::lacZ* reporter fusion [15]. Approximately 60% of the β-galactosidase activity (*frdA* expression) was lost when 2 μM O_2 was introduced into the system. This corresponds to an O_2:FNR ratio of 2.5 based on an average cell size of 1 μm×2 μm and an FNR content of 700 monomers per cell [16]. Such a ratio of O_2:FNR caused a 96% loss of transcriptional activity in vitro, an 83% loss of DNA binding and an 81% loss of absorbance at 420 nm in the

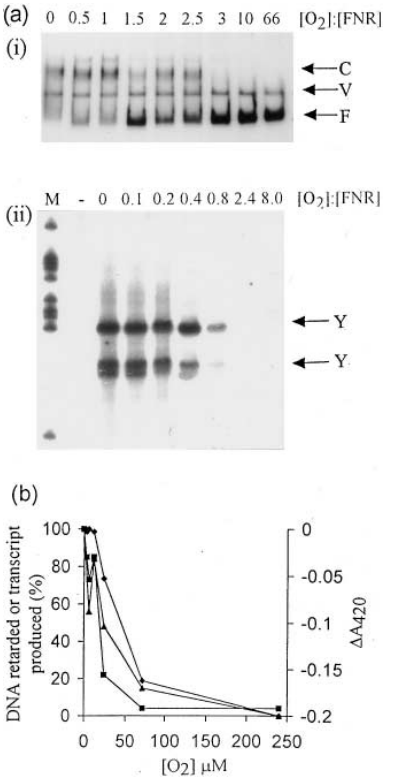

Fig. 3. Functional consequences of the oxidation of FNR by O_2. a: Gel retardation analysis (i) and in vitro transcription assays (ii) were done with *yfiD* promoter DNA and samples of FNR protein treated with aerobic TCB equivalent to the indicated O_2:FNR ratios. (i) An FNR concentration of 3.6 μM (monomer) was used in the initial titration and the final concentration of FNR in the reactions was 11 nM. The positions of free DNA (F), vector DNA (V) and the FNR:*yfiD* complex (C) are indicated. (ii) The FNR concentration used in the initial titration was 30 μM (monomer) and the final concentration of FNR in the reactions was 30 nM. Lane M is a Maxam and Gilbert G track to calibrate the gel; lane -, reaction contains no FNR; the FNR dependent *yfiD* transcripts are indicated (Y). b: Correlation of loss of absorbance at 420 nm (◆, A_{420} of untreated FNR was 0.298) with loss of DNA binding (▲, % of total DNA retarded) and transcriptional activity (■, 100% is the amount of transcript produced by unoxidised FNR). All three parameters were measured during the course of a titration of FNR (30 μM) with aerobic TCB. The amount of DNA retarded or transcript formed was estimated by quantitative densitometry.

experiments reported here. In contrast, at least 20-fold excess $K_3Fe(CN)_6$ was necessary to effect any response from FNR. Furthermore, oxidation by $K_3Fe(CN)_6$ was accompanied by the liberation of ferric ions, indicating that the $[4Fe\text{-}4S]^{2+}$ cluster is destroyed, whereas O_2 converted the $[4Fe\text{-}4S]^{2+}$ clusters into $[2Fe\text{-}2S]^{2+}$ clusters without liberating ferric ions. The absorption spectra show an apparently stoicheiometric conversion of one $[4Fe\text{-}4S]^{2+}$ cluster into a $[2Fe\text{-}2S]^{2+}$ cluster. The ε values of typical $[4Fe\text{-}4S]^{2+}$ clusters at ~420 nm is ~16000 M^{-1} cm^{-1} whereas that of a $[2Fe\text{-}2S]^{2+}$ cluster at similar wavelengths is ~9000–11000 M^{-1} cm^{-1} [17]. Hence the drop in the absorption intensity around 420 nm is consistent with a stoicheiometric conversion of one $[4Fe\text{-}4S]^{2+}$ cluster into one $[2Fe\text{-}2S]^{2+}$ cluster. Minor side reactions that might result in an EPR silent form of iron cannot be excluded, although they cannot be extensive otherwise the isosbestic nature of the absorption spectra would be lost. The previously identified $[3Fe\text{-}4S]^{+}$ cluster was only observed after treatment with excess O_2 and thus is likely to be either a transient intermediate in the oxidation of $[4Fe\text{-}4S]^{2+}$ clusters to $[2Fe\text{-}2S]^{2+}$ clusters or represents a relatively minor oxidation product. Therefore, it seems likely that FNR is inactivated directly by stoicheiometric O_2 via an oxidation in which the $[4Fe\text{-}4S]^{2+}$ clusters are converted into $[2Fe\text{-}2S]^{2+}$ clusters.

Site-directed mutagenesis strongly suggests that residues C20, C23, C29 and C122 are involved in cluster binding. Each monomer is able to bind a single $[4Fe\text{-}4S]^{2+}$ cluster after reconstitution [5] indicating that the essential cysteines act as ligands for the $[4Fe\text{-}4S]^{2+}$ clusters. If one $[4Fe\text{-}4S]^{2+}$ cluster yields only a single $[2Fe\text{-}2S]^{2+}$ cluster the same cysteine ligands could be used but the different spatial requirement of the cluster cores would provide substantial conformational rearrangements of these cysteine ligands. The N-terminal region of FNR contains other residues which are potential cluster ligands such as H19. Thus the switching mechanism for FNR may not require the retention of all 4 cysteine ligands in both aerobic and anaerobic forms of FNR. Indeed, in vivo, the cysteine sulphydryls of FNR are more accessible in aerobic than anaerobic cultures [18]. This suggests either that the $[4Fe\text{-}4S]^{2+}$ clusters are more stable under anaerobic conditions than are the $[2Fe\text{-}2S]^{2+}$ clusters under aerobic conditions, or that some of the cysteine ligands are replaced aerobically, or that the $[2Fe\text{-}2S]^{2+}$ form is only an intermediate in the formation of apo-FNR in vivo.

It is clear that the interconversion of a $[4Fe\text{-}4S]^{2+}$ cluster and a $[2Fe\text{-}2S]^{2+}$ cluster would provide a large conformational drive (requiring the repositioning of two cysteine ligands or the acquisition of new ligands), to switch FNR between transcriptionally active and inactive states.

The data presented here clearly show that FNR is very sensitive to O_2 and that by treating it with O_2 it is converted from the anaerobic $[4Fe\text{-}4S]^{2+}$-containing active form to a $[2Fe\text{-}2S]^{2+}$ form that lacks the ability to bind DNA and is consequently transcriptionally inactive.

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