Reconstitution of the [4Fe-4S] cluster in FNR and demonstration of the aerobic–anaerobic transcription switch *in vitro*

Jeffrey GREEN*‡, Brian BENNETT†, Peter JORDAN†, Edward T. RALPH*, Andrew J. THOMSON† and John R. GUEST*

*The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, U.K., and †Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

The FNR protein of *Escherichia coli* is a redox-responsive transcription regulator that activates and represses a family of genes required for anaerobic and aerobic metabolism. Reconstitution of wild-type FNR by anaerobic treatment with ferrous ions, cysteine and the NifS protein of *Azotobacter vinelandii* leads to the incorporation of two $[4Fe\text{-}4S]^{2+}$ clusters per FNR dimer. The UV–visible spectrum of reconstituted FNR has a broad absorbance at 420 nm. The clusters are EPR silent under anaerobic conditions but are degraded to $[3Fe\text{-}4S]^{+}$ by limited oxidation with air, and completely lost on prolonged air exposure. The association of FNR with the iron–sulphur clusters is confirmed by CD spectroscopy. Incorporation of the $[4Fe\text{-}4S]^{2+}$ clusters increases site-specific DNA binding about 7-fold compared with apo-FNR. Anaerobic transcription activation and repression *in vitro* likewise depends on the presence of the iron–sulphur cluster, and its inactivation under aerobic conditions provides a demonstration *in vitro* of the FNR-mediated aerobic–anaerobic transcriptional switch.

INTRODUCTION

The FNR protein of *Escherichia coli* is a redox-responsive transcription regulator that activates or represses the expression of a family of genes concerned with anaerobic and aerobic metabolism [1–3]. FNR is both structurally and functionally related to the cAMP receptor protein (CRP) or the catabolite activator protein (CAP) [1,3]. FNR is predicted to retain all the secondary structural elements of the CRP protein, but it differs from CRP in possessing a unique N-terminal extension with three of the four essential cysteine residues Cys-20, Cys-23, Cys-29 and Cys-122 and in containing up to 2.7 atoms of iron per monomer [4–7]. The reciprocal relationship between iron content and cysteine thiol reactivity further indicated that cysteine residues serve as iron ligands [6,8]. In DNase I footprinting studies, the affinity of purified FNR for target DNA was approximately doubled by the presence of protein-bound iron, whereas iron-containing FNR was found to be essential for demonstrating FNR-mediated transcription activation and repression *in vitro* [3,4,9]. It was also shown that transcriptional activity could be restored to apo-FNR by preincubation with ferrous ions under reducing conditions [10]. Thus it was proposed that FNR contains a redox-sensing domain with a cysteine-bound iron cofactor [1,3,10]. However, no requirement for an anaerobic stimulus was detected in these studies and it was therefore assumed that a fraction of the purified iron-containing protein must retain the active anaerobic conformation under aerobic conditions.

The nature of the iron cofactor has recently been defined in studies with *fnr** mutants, which retain some ability to activate anaerobic gene expression under aerobic conditions *in vivo* [11–14]. The corresponding proteins had amino acid substitutions either in the redox-sensing domain, e.g. FNR*(L28H), or at the dimer interface, e.g. FNR*(D154A). Compared with aerobically prepared wild-type FNR, which is monomeric and has a low DNA-binding affinity [6], FNR*(D154A) is substantially dimeric and has a 10-fold higher affinity for target DNA [13]. Furthermore the protein containing both substitutions (L28H D154A, here designated FNR^{2}*) was found to have a polynuclear iron–sulphur cluster, as well as being dimeric and exhibiting an enhanced affinity for DNA [14]. Absorption and EPR spectroscopy indicated that purified FNR^{2}* contains about one $[3Fe\text{-}4S]^{+}$ cluster per 10 monomers, and the higher iron contents of later preparations (2.6 atoms per monomer) indicated that each subunit of FNR^{2}* is associated with one $[3Fe\text{-}4S]^{+}$ cluster [14]. It was further proposed that the native anaerobic protein contains $[4Fe\text{-}4S]^{2+}$ clusters that suffer oxidative degradation to the $[3Fe\text{-}4S]^{+}$ state, although no direct evidence for the presence of a [4Fe-4S] cluster was reported. Similar absorption spectra were obtained with wild-type FNR that had incorporated up to 2.7 iron atoms per monomer as a result of incubating apo-FNR with ferrous ions under reducing conditions [7]. Anaerobic treatment of iron-depleted FNR^{2}* with ferrous ammonium sulphate, cysteine and the NifS protein of *Azotobacter vinelandii* likewise restored the characteristic absorption spectrum and increased the DNA-binding affinity [14]. Thus it is proposed that anaerobiosis initiates a process in which assembly of an iron–sulphur cluster (or the reduction of a pre-existing cluster), and concomitant dimerization and other conformational changes, enhance site-specific DNA binding and effect regulatory interactions with RNA polymerase in the transcription complex [3,15].

The present work shows that the reconstituted active and dimeric form of wild-type FNR contains two $[4Fe\text{-}4S]^{2+}$ iron–sulphur clusters that rapidly degrade to $[3Fe\text{-}4S]^{+}$ clusters in air. The presence of the $[4Fe\text{-}4S]^{2+}$ clusters induces a 7-fold increase in DNA-binding affinity. Furthermore the reconstituted form of FNR activates and represses transcription of relevant genes *in vitro* under anaerobic but not aerobic conditions, thus providing

Abbreviations used: CRP, cAMP receptor protein; FNR, regulator of fumarate and nitrate reduction; K_D, FNR concentration giving 50% retardation under gel-retardation analysis.

‡ To whom correspondence should be addressed.

the first demonstration *in vitro* of the FNR-mediated anaerobic switch.

EXPERIMENTAL

Protein purification and FNR reconstitution

FNR was purified after thrombin-catalysed release from a glutathione-S-transferase–FNR fusion protein that was amplified in aerobically grown *E. coli* CAG627(pGS572), as described previously [7]. This method provides high yields of FNR protein and allows a convenient, simple and rapid purification procedure. The product (molecular mass 29177 Da) contains an extra 15 amino acids derived from the linker, whose presence was confirmed by the total amino acid analysis. NifS protein was purified from *E. coli* BL21(λDE3) containing the *nifS* expression plasmid pDB551 [16]. Protein concentrations were estimated by the Bio-Rad dye-binding procedure [17]. BSA served as the standard, a correction factor of 0.83 being applied to allow for the difference between this standard and a reference sample of FNR (analysed by Alta Bioscience, University of Birmingham).

The incorporation of iron and sulphur into FNR was achieved by a modification of the procedure of Khoroshilova et al. [14]. FNR solutions (0.1–2.0 ml; protein concentration approx. 1 mg/ml) were incubated under nitrogen with 1.4 mM NifS protein, 1 mM L-cysteine (Sigma), 10 mol of Fe^{2+} (as $[NH_4]_2Fe[SO_4]_2$; A.C.S. grade, Sigma) per mol of FNR monomer and 2.5 mM dithiothreitol (Sigma), either for 3 h at 20 °C in a sealable vessel in an anaerobic glovebox (Faircrest or Whitley Anaerobic Workstation) or in a glass-stoppered cuvette for up to 16 h at 4 °C. Longer incubation times resulted in the appearance of a fine black precipitate of FeS. In some experiments an earlier procedure in which FNR was incubated anaerobically with 0.1 mM $[NH_4]_2Fe[SO_4]_2$ and reducing agent (200 mM 2-mercaptoethanol) but no added sulphur [7,9], was used. Attempts to improve the chemical reconstitution of FNR involved adding 2 mM Na_2S with 7 mM dithiothreitol, 2 mM $FeCl_3$ and 1 mM EDTA [18]. In each case the reaction products were applied to a DEAE-cellulose (DE52, Whatman) ion-exchange column (1.5 ml) that had been equilibrated with anoxic 50 mM Hepes buffer, pH 7.4, containing 1 mM dithiothreitol, in the anaerobic glovebox. The column was washed with five bed volumes of the Hepes buffer and bound protein was eluted by 300 mM NaCl in the same buffer.

Iron contents were determined with bathophenanthroline on wet-ashed samples [19] and acid-labile sulphur of denatured samples of FNR protein was determined by the method of Beinert [20] in an anaerobic glovebox.

Spectroscopy

Samples of reconstituted FNR were removed from the anaerobic glovebox in quartz cuvettes sealed with lightly greased ground-glass stoppers for optical spectroscopy, or in EPR tubes stoppered with Suba Seals, and immediately frozen in liquid nitrogen. EPR spectroscopy was performed as described previously [21]. CD spectra were recorded with a modified Jasco J500-D spectropolarimeter.

FNR–DNA interactions

Gel-retardation assays with the *FFmelR* promoter (0.05 pmol in 20 μl) were essentially as described by Green et al. [6] except that EDTA was omitted from all buffers and all manipulations were performed under anaerobic conditions. After electrophoresis, the percentage of total DNA retarded was estimated from autoradiographs by quantitative densitometry with a Vilber-Lourmat Bioprofil imaging system.

The ability of FNR to activate or repress transcription *in vitro* from the respective semi-synthetic *FFmelR* or natural *ndh* promoters was estimated as described previously [4] except that EDTA was again omitted from the binding buffer and all manipulations were done anaerobically. Template DNA containing the *ori* promoter region of the pUC18 vector was included in all reactions to provide an FNR-independent internal control.

RESULTS

Reconstituted FNR contains two [4Fe-4S] clusters per dimer

Anaerobic incubation of wild-type FNR with ferrous ions and 2-mercaptoethanol resulted in the incorporation of a variable amount of iron (0.3–2.7 atoms per monomer). Preparations with the highest iron contents were straw-brown in colour and had absorbance maxima at 320 and 420–450 nm (Figure 1a). The variable degree of reconstitution achieved by this method, and the need to apply highly reducing conditions, hindered attempts to characterize further the mechanism of redox-sensing by FNR

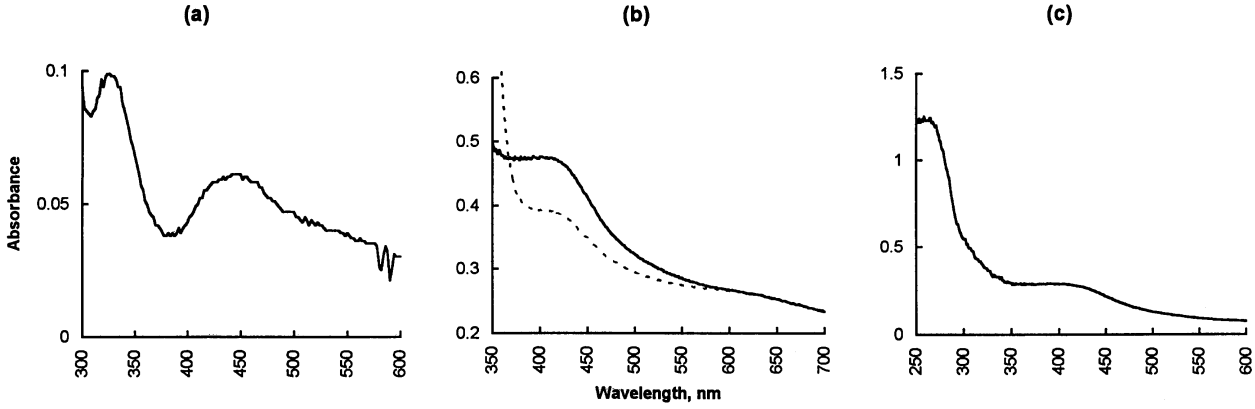

Figure 1 Absorption spectra of FNR

All spectra were obtained with anaerobic samples in sealed cuvettes: (**a**) FNR (1.8 μM dimer) after anaerobic preincubation with 0.1 mM $(NH_4)_2Fe(SO_4)_2$ and 0.4 M 2-mercaptoethanol; (**b**) FNR (11 μM dimer) reconstituted by the NifS-catalysed procedure, before (solid line) and after (dotted line) treatment with dithionite (1 mM for 2 min); (**c**) NifS-reconstituted FNR (9.9 μM dimer).

Table 1 Iron and acid-labile sulphur content of FNR

The amounts of iron and acid-labile sulphur incorporated into samples of reconstituted FNR are recorded, relative to the untreated samples. Protein contents were determined by the Bio-Rad protein assay and related to a reference sample of FNR of known amino acid composition. The results show the range of values obtained from three independent samples.

	Fe and S contents (atoms per mol of FNR monomer)		
FNR	Fe	Acid-labile S	Fe/S ratio
As prepared	0.07	0.08–0.11	0.64–0.88
Reconstituted with NifS, cysteine and ferrous ions	3.40–4.10	3.60–3.90	0.94–1.05
Reconstituted with ferrous ions and reducing agent	0.26–0.36	0.24–0.38	0.95–1.10

[10]. An alternative reconstitution procedure involved the addition of Na_2S as a sulphur source [18] but this produced a fine black precipitate of FeS that prevented re-isolation of the protein. At this stage, the NifS-catalysed procedure, which had been used successfully with FNR²* [14,16], was adopted. Anaerobic incubation of FNR with NifS, ferrous ions and cysteine under reducing conditions again generated a straw-brown product with a broad absorption band at 420 nm (Figures 1b and 1c) that was not present in the starting material, nor did it appear in control experiments lacking FNR. The ratio A_{420}:A_{280} can serve as a useful index of the iron–sulphur cluster content of a protein. In FNR, the aromatic amino acids (11 phenylalanine, 5 tyrosine but no tryptophan) contribute to the absorption at 280 nm ($\epsilon_{280} \approx$ 6000 $M^{-1}\cdot cm^{-1}$). The model compound $[Fe_4S_4(S\text{-}Et)_4]^{2-}$ has an ϵ_{420} of 17200 $M^{-1}\cdot cm^{-1}$ and an ϵ_{420}:ϵ_{280} ratio of approx. 0.7 in dimethylformamide, which show only small variations with changes in solvent or thiol ligand [22]. Hence it can be calculated that the ϵ_{420}:ϵ_{280} ratio for FNR containing one $[4Fe\text{-}4S]^{2+}$ cluster per monomer should be approx. 0.6; note that the contribution from the iron–sulphur cluster is four times that from the protein at 280 nm. Unfortunately, the relatively high absorbance observed at 260 nm suggests that typical FNR preparations contain some residual nucleic acid (approx. 5 nt per monomer) which makes it difficult to obtain useful ϵ_{420}:ϵ_{280} ratios (Figure 1c). Nevertheless the highest ratios obtained with NifS-reconstituted FNR during this work were 0.36, compared with ratios of 0.16 reported for FNR²* [14]. The absorption at 420 nm was partly bleached by treating with dithionite, which is consistent with the reduction of a redox-active iron–sulphur cluster to the $[4Fe\text{-}4S]^{+}$ state (Figure 1b).

Comparison of the iron and acid-labile sulphur contents of wild-type FNR reconstituted by two methods clearly demonstrates the superiority of the NifS-catalysed procedure (Table 1). The stoicheiometries (Fe:S:protein) show that NifS-reconstituted preparations contain one [4Fe-4S] cluster per FNR monomer, whereas reconstitution with ferrous ions and reducing agent alone typically leads to the incorporation of only one-tenth of the amount of iron and sulphur (Table 1). It is possible that in the latter samples iron and sulphur are bound to cysteine thiols or other radicals, to generate functional but non-physiological forms of FNR lacking iron–sulphur clusters, or that clusters are reconstituted in only 10% of the protein. With an ϵ_{420} of 17000 $M^{-1}\cdot cm^{-1}$, the absorbances of typical NifS-reconstituted FNR samples (Figure 1c) correspond to $[4Fe\text{-}4S]^{2+}$ concentrations of 16 μM, compared with protein concentrations of 20 μM (FNR monomers). The stoicheiometric and spectroscopic data strongly suggest that reconstituted FNR contains one iron–sulphur cluster per monomer, or two per dimer.

No EPR signals were observed when NifS-reconstituted FNR was maintained under strictly anaerobic conditions. This is consistent with the presence of an EPR-silent $[4Fe\text{-}4S]^{2+}$ cluster. Although optical bleaching in the presence of dithionite suggested partial reduction of the cluster (Figure 1b), no EPR signals could be detected, even at high power and ultra-low temperature. This indicates that the reduced cluster does not possess the spin $S = 1/2$ ground state typical of reduced $[4Fe\text{-}4S]^{+}$ clusters in reduced ferredoxins, for example. After bubbling NifS-reconstituted FNR with air for 2 min, an EPR signal at $g = 2.01$ was observed (Figure 2). The shape and g value of the signal strongly suggest that it arises from a $[3Fe\text{-}4S]^{+}$ cluster rather than from a $[4Fe\text{-}4S]^{3+}$ cluster of the type detected in the high-potential iron-sulphur protein [23,24]. This EPR signal was lost and no further signals appeared after further bubbling of air through the protein solution at room temperature. A similar signal was detected with aerobically prepared FNR²* [14].

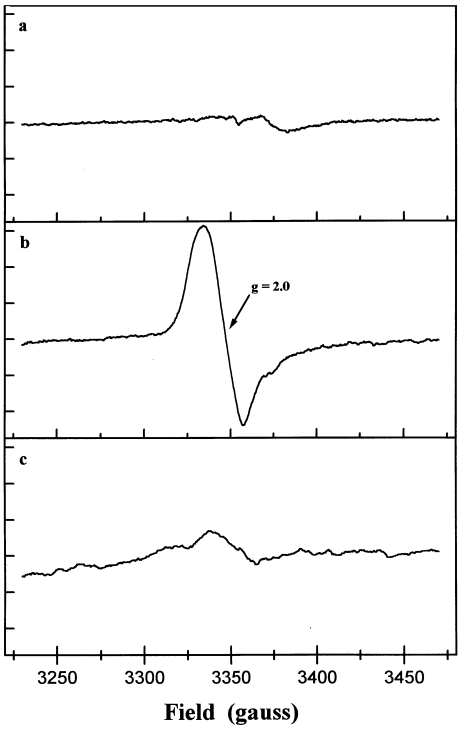

Figure 2 EPR spectrum of partly oxidized FNR

Spectra obtained from samples of NifS-reconstituted FNR (0.25 ml; 11 μM dimer): (**a**) maintained anaerobic; (**b**) exposed to 5 ml of air by slow bubbling for 2 min; (**c**) exposed to air by bubbling for 20 min. Spectra were measured at 16 K, with a microwave power of 20 mW and a field modulation amplitude of 0.2 mT. 1 T = 10^4 gauss.

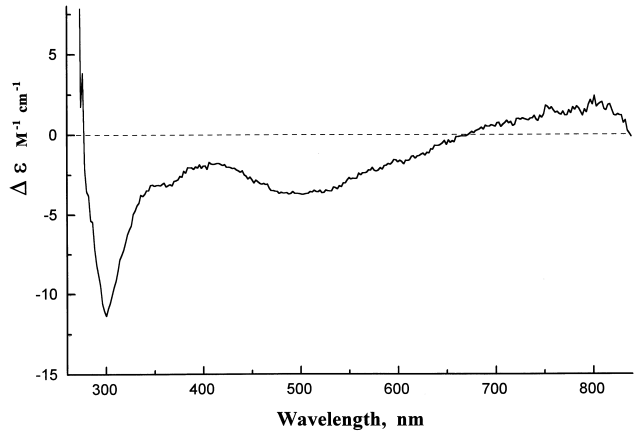

Figure 3 CD spectrum of FNR

The spectrum of NifS-reconstituted FNR (14 μM protein dimer) was recorded at room temperature with 2 nm resolution and a path length of 1 cm.

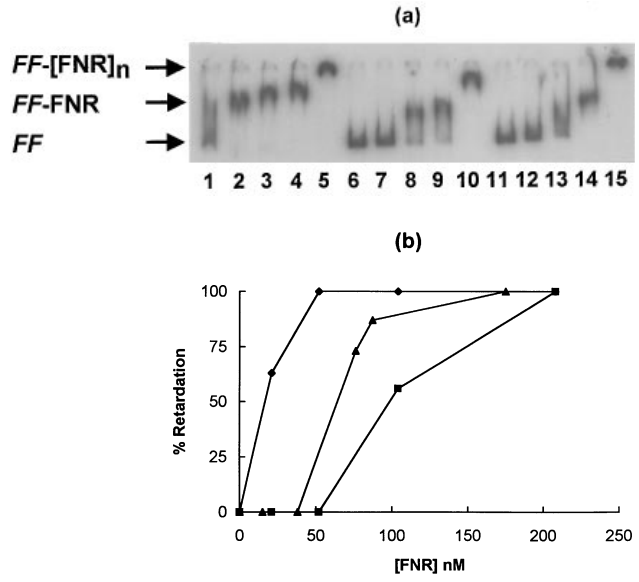

Figure 4 Incorporation of [4Fe-4S] clusters into FNR enhances site-specific DNA binding

(**a**) Gel-retardation assays with radiolabelled *FFmelR* DNA and increasing amounts of FNR. The concentrations of FNR dimers were: lanes 1, 6 and 11, 20 nM; lanes 2, 7 and 12, 50 nM; lanes 3, 8 and 13, 100 nM; lanes 4, 9 and 14, 200 nM; and lanes 5, 10 and 15, 2 μM. The positions of free *FFmelR* DNA (*FF*), FNR–DNA complex (*FF*-FNR) and non–specific FNR-DNA complexes (*FF*-[FNR]$_n$) are arrowed. (**b**) Quantification of site-specific DNA binding. A similar series of experiments to those in (**a**) was analysed by quantitative densitometry: ■, untreated FNR; ▲, FNR treated with ferrous iron and 2-mercaptoethanol; ◆, FNR reconstituted by the NifS-catalysed procedure.

The CD spectrum of NifS-reconstituted FNR held in an anaerobic cuvette revealed weak but reproducible bands between 300 and 850 nm (Figure 3). This suggests that the iron–sulphur cluster is ligated to the protein. The differential extinction coefficient ($\Delta\epsilon$ 0.5 $M^{-1}\cdot cm^{-1}$) is comparable to that of the [4Fe-4S]$^{2+}$ clusters in a typical ferredoxin from *Bacillus stearothermophilus* [25].

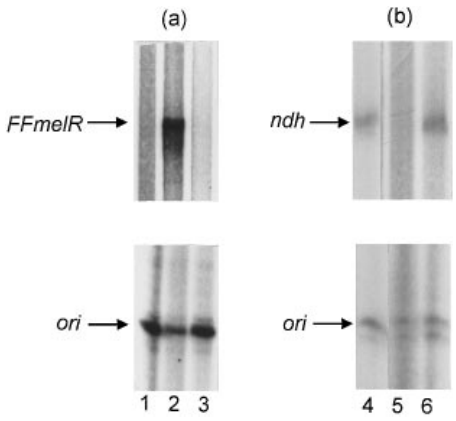

Figure 5 Anaerobic transcription regulation *in vitro* by [4Fe-4S]$^{2+}$-containing FNR

(**a**) Anaerobic activation of transcription from the *FFmelR* promoter with NifS-reconstituted FNR (25 nM dimer): lane 1, no FNR; lane 2, NifS-reconstituted FNR; lane 3, as in lane 2 but with reconstituted FNR exposed to air for 30 min before use. (**b**) Anaerobic repression of transcription from the *ndh* promoter: lane 4, no FNR; lane 5, NifS-reconstituted FNR (50 nM dimer); lane 6, as in lane 5 but with reconstituted FNR exposed to air for 30 min before use. The positions of the respective FNR-dependent *FFmelR* (226 nt) and *ndh* (175 nt) and the FNR-independent *ori* (100 nt) transcripts are indicated.

Presence of the [4Fe-4S]$^{2+}$ cluster enhances the DNA-binding and transcription regulatory activities of FNR

The DNA-binding properties of reconstituted wild-type FNR were investigated with gel-retardation analysis under anaerobic conditions (Figure 4). The presence of the iron–sulphur cluster increased the DNA-binding affinity 7-fold compared with untreated protein: the FNR concentrations giving 50 % retardation (K_D) of *FFmelR* DNA were 14 nM for fully reconstituted FNR dimers and 100 nM for the dimer equivalents of untreated FNR. In comparable tests with FNR reconstituted by the NifS-independent procedure (anaerobic incubation with ferrous ions and 2-mercaptoethanol), 50 % retardation was observed at a concentration of 64 nM dimeric FNR. This is only 1.6-times better than untreated protein, but consistent with the 2-fold increase in DNA-binding observed previously in DNase I footprinting reactions when iron-containing and iron-deficient FNR were compared [4,6]. In all cases, FNR at concentrations of 2 μM or more (dimer) resulted in non-specific DNA binding, as observed previously [6].

Anaerobic transcription studies *in vitro* showed that fully reconstituted FNR activates transcription from the *FFmelR* and represses transcription from the *ndh* promoter (Figure 5). However, the regulatory activities declined progressively until they were completely abolished by prior teatment of reconstituted FNR with air for 30 min (Figure 5). Under the same conditions transcription from the FNR-independent *ori* promoter was unaffected (Figure 5). Thus the combination of aerobic inactivation and NifS-catalysed reactivation of FNR function provides a demonstration *in vitro* of the FNR-mediated aerobic–anaerobic transcriptional switch.

DISCUSSION

Our understanding of the means by which FNR responds to anoxia has been advanced by the recent characterization of the FNR* and FNR^2* proteins (mutant proteins that retain some

activity under aerobic conditions *in vivo*) and the demonstration that FNR²* is associated with a polynuclear iron–sulphur cluster [14]. The present work shows clearly that after reconstitution with ferrous ions and sulphur derived from cysteine by NifS, wild-type FNR contains up to two [4Fe-4S] clusters per dimer under anaerobic conditions. Evidence for the nature of this cluster comes from the analytical data, the absorption spectrum and the absence of an EPR signal. Together with the observation that $[3Fe\text{-}4S]^{+}$ clusters can be generated from the native clusters by oxidation in air, it can be concluded that the native anaerobic protein contains $[4Fe\text{-}4S]^{2+}$ clusters. Exposure to air leads first to the formation of $[3Fe\text{-}4S]^{+}$ clusters followed by a complete loss of clusters. Hence the $[3Fe\text{-}4S]^{+}$ clusters seem to be significant intermediates in the destruction of the native clusters. Such intermediates have been observed during the loss of $[4Fe\text{-}4S]^{2+}$ clusters from both mammalian and bacterial aconitases, which can additionally pass through a $[2Fe\text{-}2S]^{+}$ stage before final cluster destruction [18,26]. Interestingly, an EPR signal ($g = 2$) had been observed previously with one of three samples of unreconstituted FNR that had been purified in the presence of ferrous ions after amplified expression from the *tac* promoter of pGS330 [6] (D. J. Lowe, personal communication). However, its significance was ignored owing to the lack of uniformity between samples.

The $[4Fe\text{-}4S]^{2+}$ clusters in FNR are difficult to reduce fully to the paramagnetic $[4Fe\text{-}4S]^{+}$ state with dithionite, suggesting that they have a low reduction potential. Although most $[4Fe\text{-}4S]^{+}$ clusters have a spin state of $S = 1/2$ with readily observed EPR signals of $g = 2$, some can exist in states of higher spin such as $S = 3/2$ and $5/2$, examples being the $[4Fe\text{-}4S]^{+}$ cluster in the iron protein of nitrogenase [27,28] and in glutamine phosphoribosylpyrophosphate amidotransferase [29]. The EPR signals of these clusters can be difficult to detect given the low transition probabilities and the anisotropy of the g term. It is therefore possible that the clusters in FNR are in the high spin state, which would then account for the lack of EPR signals in the $g = 2$ region. As FNR tends to precipitate at concentrations above 1 mg/ml it could prove difficult to detect the signals of the reduced cluster. Thus anaerobic FNR contains EPR-undetectable but reducible $[4Fe\text{-}4S]^{2+}$ clusters, which, on exposure to air, are degraded via a $[3Fe\text{-}4S]^{+}$ cluster. It is worth noting that the clusters of the other [4Fe-4S]-containing proteins that exhibit $S = 3/2$ spin states are likewise very oxygen-labile. It could be that the steric or electronic factors that bring about the high spin states of reduced four-iron clusters are also those that lead to extreme oxygen sensitivity.

Although previous studies had identified an essential role for an iron cofactor in transcription activation and repression by FNR [3,4,9,10], the affinity of iron-containing FNR for target DNA was only twice that of iron-deficient FNR [4,6]. Even after the incorporation of two [4Fe-4S] clusters per FNR dimer, the increase in specific DNA-binding activity was only 7-fold (K_D 100 nM to 14 nM). This increase is similar to that observed with FNR* and FNR²* [14] but it is significantly lower than the 10^4-fold enhancement observed when CRP is activated by its coeffector, cAMP: K_D 200 nM for CRP with the *lac* promoter; and K_D 0.01 nM for cAMP–CRP with the *lac* promoter [30,31]. These disparate affinity ranges may reflect differences in the signal transduction pathways operating in the two regulators. Dimerization plays an important role in FNR activation, so the concentration-dependent monomer–dimer transition of FNR [13], compared with the stable dimeric organization of CRP with cAMP, could account for the apparent discrepancy.

Two models for the cysteine ligation of the $[4Fe\text{-}4S]^{2+}$ clusters in dimeric FNR are shown in Figure 6. According to one, the clusters form a double cross-link between the FNR subunits to give a cysteine ligation pattern analogous to that of an eight-iron ferredoxin. Here the cross-links could be severed simply by the loss of the Cys-122-bound iron atom from each cluster, giving two $[3Fe\text{-}4S]^{+}$ clusters. The aerobic–anaerobic switch could thus be mediated by the reversible incorporation of the two iron atoms and the simultaneous formation of a covalently linked dimer with enhanced affinity for DNA. In the second model, each $[4Fe\text{-}4S]^{2+}$ cluster is independently co-ordinated to one FNR subunit, and it is envisaged that the functional switch involves allosteric effects on dimer stability and DNA binding that are caused by changes in cluster structure or assembly. In many respects FNR could be regarded as a regulatory counterpart of the oxygen-labile dimeric fumarase A of *E. coli*, which likewise contains two $[4Fe\text{-}4S]^{2+}$ clusters [32]. The possibility that each $[4Fe\text{-}4S]^{2+}$ cluster could be liganded to one or both FNR subunits is being investigated with proteins substituted at each of the four essential cysteine residues. Likewise, questions about the sequence of events leading to the formation of active FNR, e.g. whether incorporation of the iron–sulphur clusters precedes dimerization or vice versa, and whether FNR responds primarily to the redox environment, ferrous ions, or oxygen itself, are now high priorities.

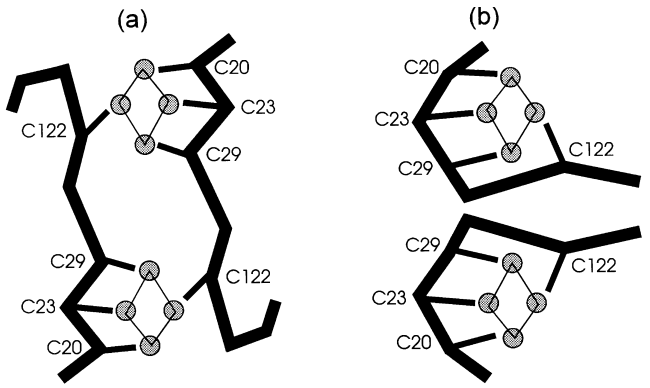

Figure 6 Possible arrangements of the iron–sulphur clusters in dimeric FNR

(**a**) The two iron–sulphur clusters each have ligands to both subunits in the FNR dimer. (**b**) Each FNR subunit contains a discrete iron–sulphur cluster. The essential cysteine residues are shown as iron ligands. It is suggested that dimer stability and DNA binding is weakened by loss of the clusters or by loss of single iron atoms to generate $[3Fe\text{-}4S]^{+}$ clusters.

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