

The O₂ sensitivity of the transcription factor FNR is controlled by Ser24 modulating the kinetics of [4Fe-4S] to [2Fe-2S] conversion

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Fumarate and nitrate reduction regulatory (FNR) proteins are bacterial transcription factors that coordinate the switch between aerobic and anaerobic metabolism. In the absence of O₂, FNR binds a [4Fe-4S]²⁺ cluster (ligated by Cys-20, 23, 29, 122) promoting the formation of a transcriptionally active dimer. In the presence of O₂, FNR is converted into a monomeric, non-DNA-binding form containing a [2Fe-2S]²⁺ cluster. The reaction of the [4Fe-4S]²⁺ cluster with O₂ has been shown to proceed via a 2-step process, an O₂-dependent 1-electron oxidation to yield a [3Fe-4S]⁺ intermediate with release of 1 Fe²⁺ ion, followed by spontaneous rearrangement to the [2Fe-2S]²⁺ form with release of 1 Fe³⁺ and 2 S²⁻ ions. Here, we show that replacement of Ser-24 by Arg, His, Phe, Trp, or Tyr enhances aerobic activity of FNR in vivo. The FNR-S24F protein incorporates a [4Fe-4S]²⁺ cluster with spectroscopic properties similar to those of FNR. However, the substitution enhances the stability of the [4Fe-4S]²⁺ cluster in the presence of O₂. Kinetic analysis shows that both steps 1 and 2 are slower for FNR-S24F than for FNR. A molecular model suggests that step 1 of the FNR-S24F iron-sulfur cluster reaction with O₂ is inhibited by shielding of the iron ligand Cys-23, suggesting that Cys-23 or the cluster iron bound to it is a primary site of O₂ interaction. These data lead to a simple model of the FNR switch with physiological implications for the ability of FNR proteins to operate over different ranges of in vivo O₂ concentrations.

gene regulation | iron-sulfur | oxygen sensing | oxidation | transcriptional regulation

Fumarate and nitrate reduction regulatory (FNR) proteins are members of a superfamily of structurally related bacterial transcriptional factors (1). The structural framework of the founder member of the family, the *Escherichia coli* cAMP receptor protein (CRP), provides a versatile system for sensing and transducing environmental or metabolic signals into a physiological response (2–5). FNR proteins are O₂ sensors that control the switch between aerobic and anaerobic metabolism (5–7). Based on sequence homology with CRP (8), FNR consists of a C-terminal DNA-binding domain, which recognizes specific sequences within FNR-controlled promoters (9), and an N-terminal sensory domain containing 5 cysteine residues, 4 of which, Cys-20, 23, 29, and 122, but not Cys-16, are essential for function (10, 11). The essential cysteine residues bind either a [4Fe-4S]²⁺ or a [2Fe-2S]²⁺ cluster (12–14). Under anaerobic conditions, acquisition of 1 [4Fe-4S]²⁺ cluster per FNR promoter causes dimerization and enhances site-specific DNA binding (15). The [4Fe-4S]²⁺ cluster is converted to a [2Fe-2S]²⁺ cluster by O₂, triggering conformational changes that induce monomerization, preventing DNA binding and hence productive interactions with RNA polymerase (15–21).

Metal-centered oxidation (16) and sulfide-based oxidation (21) mechanisms have been proposed for the reaction of O₂ with the FNR [4Fe-4S]²⁺ cluster. Recently, it was shown that the reaction of O₂ with [4Fe-4S]²⁺ FNR occurs in 2 steps (22, 23).

The first step is a second-order, 1-electron oxidation of the [4Fe-4S]²⁺ cluster that produces a superoxide ion, a [3Fe-4S]⁺ intermediate, and 1 ferrous ion. The second step is a spontaneous (first-order) decay of the [3Fe-4S]⁺ intermediate yielding [2Fe-2S]²⁺ FNR, 2 sulfides, and a ferric ion, consistent with a metal-based oxidation mechanism. The superoxide product from step 1 is thought to be recycled to O₂ via hydrogen peroxide, providing a positive feedback mechanism to amplify the FNR response to O₂ (22). Here, we report the reactivity of the iron-sulfur cluster of an FNR protein with a single amino acid substitution, S24F, adjacent to a Cys cluster ligand and show that the replacement inhibits both the O₂-dependent and O₂-independent steps of cluster conversion. This implies that a serine at position 24 allows the interaction of O₂ with [4Fe-4S]²⁺ FNR and promotes conversion of the [3Fe-4S]⁺ intermediate to the [2Fe-2S]²⁺ product. In vivo transcription experiments indicate that other amino acids with bulky side chains at position 24 also protect the FNR iron-sulfur cluster. Molecular modeling suggests a mechanism by which Phe at position 24 protects the FNR [4Fe-4S]²⁺ cluster and that Cys-23 or the iron bound to it is a primary site of O₂ interaction with the [4Fe-4S]²⁺ cluster.

Results

Replacement of Ser-24 by Phe Enhances FNR Activity Under Aerobic Conditions. Cole and coworkers (24) showed that substitution of Ser-24 by Phe enhanced aerobic activity of FNR in vivo. It was suggested that this was most likely caused by stabilization of the FNR iron-sulfur cluster (24). Here, individual replacement of amino acid residues flanking the essential cysteine residues of FNR by Phe residues confirmed that FNR-S24F has greater aerobic activity than FNR. All other single-phenylalanine substitutions either failed to affect FNR activity significantly or inhibited both aerobic and anaerobic activity to a similar extent (Fig. 1). The amount of FNR-S24F was similar to that of FNR as judged by Western blotting [supporting information (SI) Appendix, Fig. S1]. Thus, these data suggest that the FNR iron-sulfur cluster is stabilized by the presence of Phe at position 24.

Reconstitution of the FNR-S24F Iron-Sulfur Cluster. Isolation of FNR-S24F was achieved as described for wild-type FNR (16). The protein from aerobic cultures was colored, consistent with the presence of an iron-sulfur cluster more stable to O₂. Anaerobic reconstitution of the FNR-S24F iron-sulfur cluster

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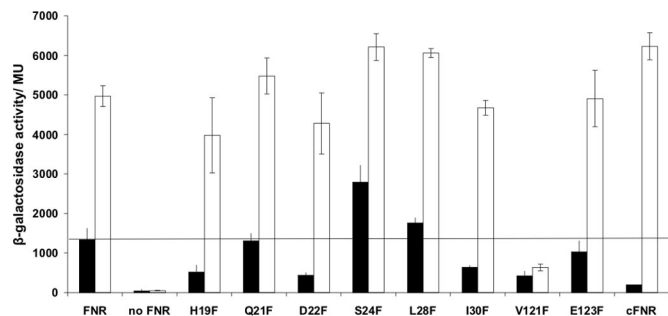


Fig. 1. Activity of FNR proteins with Phe substitutions at residues adjacent to essential Cys ligands. Plasmid pGS24 was subjected to QuikChange site-directed mutagenesis (Stratagene) to create Phe substitutions and introduced to JRG1728 (*fnr lac*) containing pFF-41.5, a plasmid with an FNR-activated promoter fused to *lacZ*. The activity of chromosomally encoded FNR (cFNR) is shown for comparison. Cultures were grown in L broth under aerobic (filled bars) or anaerobic (open bars) conditions. The mean β -galactosidase activities in Miller units (MU) and standard deviations are shown ($n = 9$). The line indicates the amount of aerobic activity associated with wild-type FNR overproduced from pGS24.

resulted in a protein that was EPR-silent, with UV-visible and CD spectra similar to those of anaerobic FNR (Fig. 2), containing 3.4–3.9 Fe per monomer of protein. Thus, the presence of Phe at position 24 does not impair the ability of FNR to bind an iron–sulfur cluster with properties very similar to the [4Fe-4S] cluster acquired by wild-type FNR under the same conditions.

Reaction of FNR-S24F Iron-Sulfur Cluster with O_2 . Titration of the [4Fe-4S] $^{2+}$ cluster of FNR-S24F with air-saturated buffer at

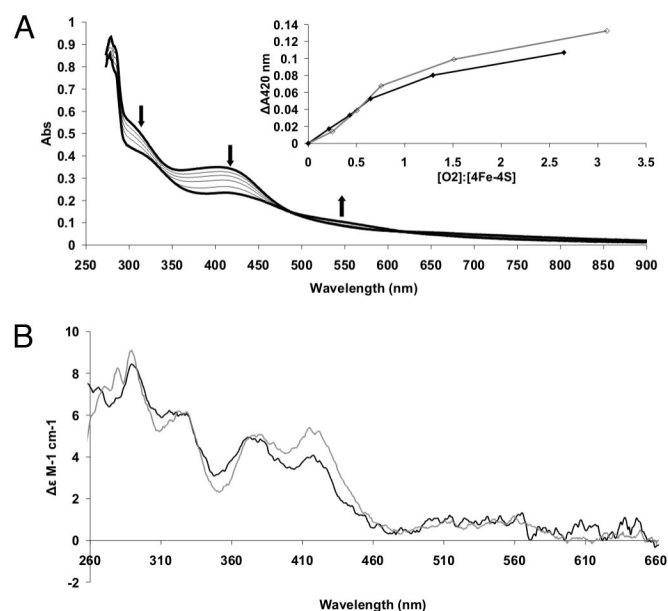


Fig. 2. Spectroscopic properties of reconstituted anaerobic FNR-S24F. (A) UV-visible spectra of 21.9 μ M FNR-S24F cluster during a titration with O_2 as air-saturated buffer. The upper spectrum, with absorbance maxima at 320 and 405 nm, is the anaerobic sample before the addition of O_2 and is indicative of the presence of a [4Fe-4S] cluster. Subsequent spectra show changes upon the addition of 5, 10, 15, 29, and 51 μ M O_2 . After each O_2 addition, the incubations were continued for 90 min before collecting the absorbance spectra to ensure that the reactions were complete. Arrows show direction of absorbance change. The final spectrum, with absorbance maxima at 320, 420, and 500–600 nm, is characteristic of a [2Fe-2S] cluster. (Inset) Relationship between the O_2 :[4Fe-4S] $^{2+}$ ratio and the change in absorbance at 420 nm for FNR (gray line) and FNR-S24F (black line). (B) Comparison of the CD spectra of FNR (gray line) with FNR-S24F (black line).

room temperature caused decreases in absorbance at 420 nm and increases \approx 550 nm, as reported for FNR (Fig. 2A and ref. 16), with the final spectrum indicating that the cluster has undergone conversion to a [2Fe-2S] $^{2+}$ form. Plotting ΔA_{420} against the O_2 :[4Fe-4S] $^{2+}$ ratio for FNR and FNR-S24F resulted in superimposable curves, suggesting that both clusters react in the same way (Fig. 2A). In the data shown, clean end points to the titrations were not obtained because the prolonged incubation period required for reaction of the FNR-S24F [4Fe-4S] cluster with O_2 was sufficient to allow partial degradation of the [2Fe-2S] cluster to contribute to the ΔA_{420} readings; shorter incubations with FNR resulted in titrations entirely consistent with previous data (16).

The first step of the FNR cluster conversion, from [4Fe-4S] $^{2+}$ to [3Fe-4S] $^+$, has been shown to be O_2 -dependent, whereas the second step, to [2Fe-2S] $^{2+}$, is a thermally driven reaction (22). We have tested the FNR-S24F protein to ascertain whether it undergoes a similar 2-step mechanism and, if so, which steps have been altered by the amino acid substitution. Reconstituted FNR-S24F protein was exposed to different O_2 :[4Fe-4S] $^{2+}$ ratios, and the 420-nm-decays were measured (Fig. 3A). The data were best fitted with double exponential functions. With a 10-fold molar excess of O_2 to cluster the observed pseudo-first-order rate constants (k_{obs}) were 0.009 s^{-1} and 0.040 s^{-1} for FNR-S24F and FNR, respectively. A plot of k_{obs} against O_2 concentration reveals a linear relationship, suggesting that the conversion from a [4Fe-4S] $^{2+}$ cluster to the [3Fe-4S] $^+$ intermediate is O_2 -dependent (Fig. 3B) and gives an apparent second-order rate constant, k_1 , of $\approx 80 M^{-1} s^{-1}$ for FNR-S24F (Fig. 3B) and $\approx 270 M^{-1} s^{-1}$ for FNR. The conversion of the FNR-S24F [3Fe-4S] $^+$ to [2Fe-2S] $^{2+}$ was, like that of FNR, O_2 -independent with a rate constant, k_2 , of $\approx 5.0 \times 10^{-4} s^{-1}$, ≈ 3.4 -fold lower than that observed for wild-type FNR ($\approx 1.7 \times 10^{-3} s^{-1}$).

EPR spectroscopy was used to determine whether the conversion of the FNR-S24F cluster proceeds via a [3Fe-4S] $^+$ intermediate. A 10-fold molar excess of O_2 was added to a sample of reconstituted 20 μ M FNR-S24F, and samples were removed and rapidly frozen in EPR tubes. As expected, before the addition of O_2 the cluster was EPR silent, but upon exposure to O_2 a signal centered at $g = \approx 2.02$, characteristic of the [3Fe-4S] $^+$ cluster, increased in intensity up to ≈ 130 s after O_2 exposure, before decreasing again (Fig. 3C and D). These data are consistent with [4Fe-4S] $^{2+}$ to [3Fe-4S] $^+$ and [3Fe-4S] $^+$ to [2Fe-2S] $^{2+}$ conversion rates slower than those observed with the wild-type protein, and with the second step being ≈ 1 order of magnitude slower than the first. Thus, it appears that Phe at position 24 protects the FNR [4Fe-4S] $^{2+}$ cluster from O_2 and also inhibits the O_2 -independent conversion of the [3Fe-4S] $^+$ intermediate to the [2Fe-2S] $^{2+}$ product. This implies that Ser-24 is significant in both steps of cluster conversion.

Bulky Amino Acid Side Chains at Position 24 Stabilize the FNR Iron-Sulfur Cluster. To discover whether other substitutions at position 24 could influence the reactivity of the FNR iron–sulfur cluster, 19 variants were created and tested for the ability to activate an FNR-dependent promoter under aerobic conditions. Replacement of Ser-24 by Arg, His, Phe, Trp, or Tyr enhanced FNR activity >2 -fold under aerobic conditions (Fig. 4). In addition, these variants significantly affected the growth of aerobic *E. coli* cultures, resulting in small colonies on agar plates. These data support the proposal that a bulky amino acid side chain at position 24 protects the FNR iron–sulfur cluster from O_2 and also suggest that enhanced FNR activity is detrimental during aerobic growth of *E. coli*.

Discussion

To function as an O_2 -sensing transcription factor, the sensitivity of the FNR [4Fe-4S] $^{2+}$ cluster must have evolved to operate

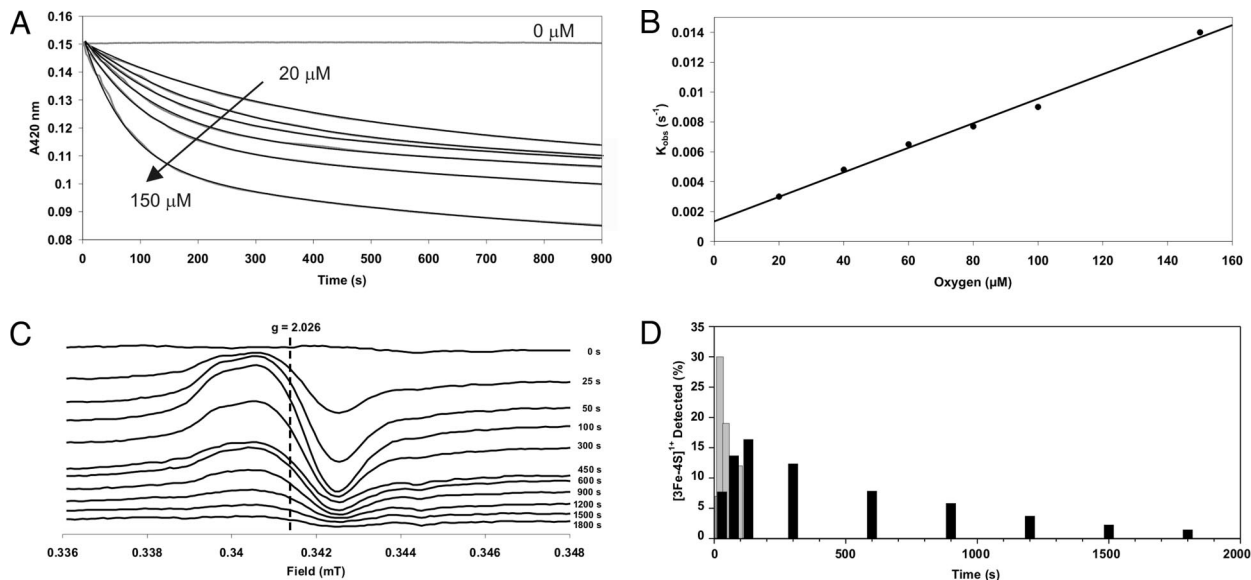


Fig. 3. The O_2 dependence and rate of the FNR-S24F $[4Fe-4S]^{2+}$ cluster conversion upon addition of O_2 . (A) Reaction of $\approx 20 \mu M$ FNR-S24F with different concentrations of O_2 (0, 20, 40, 60, 80, 100, and $150 \mu M$) measured by optical absorbance at 420 nm. Data are shown in gray, and double-exponential function fits are plotted as black lines. Data are the average of at least three experiments. (B) First observed (pseudo-first-order) rate constants, from A, as a function of O_2 concentration. (C) Changes in EPR spectra of $20.4 \mu M$ FNR-S24F cluster during reaction with $200 \mu M$ O_2 . The reaction was performed in a sealed anaerobic cuvette and initiated by the injection of air-saturated buffer. Samples were transferred to EPR tubes and immediately frozen. EPR parameters: temperature, 15 K; microwave power, 2.0 mW; frequency, 9.7 GHz; modulation, 5 G. (D) Signal intensity of the $[3Fe-4S]^+$ intermediate (expressed as a percentage of the original $[4Fe-4S]^{2+}$ cluster concentration) as a function of time for FNR (gray bars) and the FNR-S24F mutant protein (black bars).

within an optimum range of O_2 concentration, and, moreover, this range may well be different for different bacteria. Our current understanding is that FNR is continually supplied with $[4Fe-4S]^{2+}$ clusters by the iron-sulfur cluster biosynthetic machinery (25–27), but O_2 converts the $[4Fe-4S]^{2+}$ cluster to a $[3Fe-4S]^+$ cluster that spontaneously disassembles to yield a $[2Fe-2S]^{2+}$ cluster with concomitant inactivation of FNR (12, 15, 22, 23). The properties of FNR-S24F described here are entirely consistent with this view of the FNR switch. It is implicit in this that the relative rates of cluster incorporation and degradation must be finely balanced; if the rate of degradation in the presence of O_2 is too slow, “anaerobic” genes will be transcribed needlessly. The enhanced aerobic activity associated with FNR-S24F indicates that the amino acid residue adjacent to the iron ligand

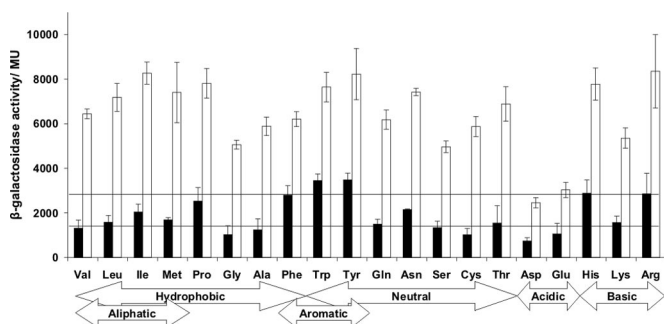


Fig. 4. Activity of *E. coli* FNR proteins with substitutions at residue 24. Plasmid pGS24 was subjected to QuikChange site-directed mutagenesis (Stratagene), and the plasmids expressing the indicated FNR variants were introduced to JRG1728 (*fnr lac*) containing pFF-41.5, a plasmid with an FNR-activated promoter fused to *lacZ*. Cultures were grown in L broth either aerobically (filled bars) or anaerobically (open bars). The mean β -galactosidase activities in MU \pm SD are shown ($n = 9$). The horizontal lines indicate the amount of aerobic activity associated with overproduced wild-type FNR (Ser-24) and FNR-S24F.

Cys-23 influences the reactivity of the iron-sulfur cluster toward O_2 , and the in vitro rate measurements indicate that this is because of a decrease in the rate of O_2 -dependent $[4Fe-4S]^{2+}$ to $[3Fe-4S]^+$ conversion. Thus, the key feature of the FNR switch is the reaction of the $[4Fe-4S]$ cluster with O_2 . Measurement of aerobic activation from pFF-41.5 in the presence of chromosomally encoded FNR suggests that FNR is on average 2.5% active under aerobic conditions [150 ± 8.6 Miller units (MU) under aerobic conditions and $6,177 \pm 343$ MU under anaerobic conditions; Fig. 1, labeled cFNR]. Knowing that the intracellular concentration of FNR is $\approx 6 \mu M$ (21), the concentration of FNR in the OFF state under aerobic conditions is $\approx 5.85 \mu M$. Thus, a model can be constructed (Fig. 5A) that predicts that FNR is 50% active ($3 \mu M$ active FNR) at $6 \mu M$ O_2 , consistent with in vivo measurements (28, 29). The importance of controlling the concentration of FNR by negative autoregulation and proteolysis (31) is evident; the model predicts that a 3-fold increase in FNR concentration results in $3 \mu M$ active FNR at $30 \mu M$ O_2 , an O_2 concentration at which FNR is normally $< 10\%$ active (28). This is supported by the data in Fig. 1 in which increasing FNR concentration by expression from a plasmid (filled bar labeled FNR) leads to an ≈ 10 -fold increase in FNR activity under aerobic conditions compared with chromosomally encoded FNR (filled bar labeled cFNR). The model also emphasizes the importance of regulating iron-sulfur cluster supply, mainly through the action of IscR, for appropriate O_2 sensing (32). Furthermore, the 3.4-fold lower k_1 for FNR-S24F reaction with O_2 means that 50% activity is reached at $20 \mu M$ O_2 (compared with $6 \mu M$ O_2 for FNR); hence, a single amino acid substitution (S24F) is capable of significantly altering the dynamic range of the FNR response.

Alignment of 209 proteins that possess the *E. coli* essential cysteine signature (Cys²⁰XXCys²³XXXXXCys²⁹ and Cys¹²²) reveals that 119 have Ser at the position equivalent to Ser-24 in *E. coli* K-12. However, residues other than Ser (Ala, Arg, Asn, Gln, Gly, His, Leu, Met, Thr, Val, but not aromatic or acidic residues) are found at position 24 (or its equivalent location) in other FNR

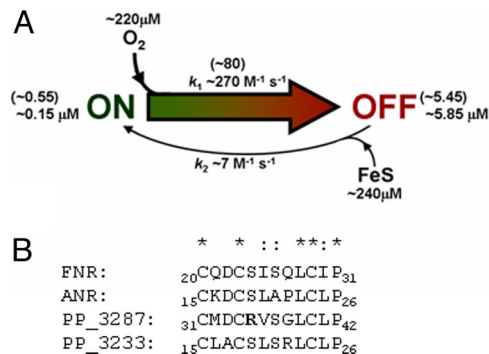


Fig. 5. Model of the reaction of FNR with oxygen and the importance of Ser-24. (A) Simple model of the FNR switch. FNR exists in two functional states: ON, corresponding to the DNA-binding form (i.e., [4Fe-4S] FNR), and OFF, corresponding to the non-DNA-binding forms (i.e., [3Fe-4S], [2Fe-2S], and apo forms). The concentration of FNR in the cell is $\approx 6 \mu\text{M}$ and is relatively constant in the presence and absence of O_2 (21). An FNR-dependent reporter gene suggests that chromosomally encoded FNR is 2.5% active under fully aerobic conditions (Fig. 1, columns labeled cFNR), and thus the concentration of FNR in the OFF state under these conditions is $\approx 5.85 \mu\text{M}$. The ON to OFF switch ($k_1 \approx 270 \text{ M}^{-1} \text{ s}^{-1}$) is O_2 -dependent. At a fully aerobic steady state ($\approx 220 \mu\text{M O}_2$), $[\text{ON}] = k_2/k_1 [\text{OFF}]$, or $(0.15 \times 10^{-6} = k_2/270 \times 5.85 \times 10^{-6})$, hence $k_2 \approx 7 \text{ M}^{-1} \text{ s}^{-1}$. The switch from OFF to ON (k_2) requires the incorporation of a [4Fe-4S] cluster, which depends on the supply of iron-sulfur clusters (FeS) from the biosynthetic machinery. Time-resolved transcriptomic studies suggest that the maximum FNR response during the switch from aerobic to anoxic growth occurs within 5–10 min (30), suggesting that the rate of OFF to ON (v_{ON}) is $\approx 10 \times 10^{-9} \text{ M s}^{-1}$. This information can be used to calculate a nominal concentration of FeS available for incorporation into FNR, $v_{\text{ON}} = k_2 [\text{OFF}] [\text{FeS}]$, giving $[\text{FeS}] \approx 240 \mu\text{M}$. The model can be used to calculate FNR activity at different environmental O_2 concentrations, i.e., 50% FNR activity at $\approx 6 \mu\text{M O}_2$ and 95% activity at $\approx 0.3 \mu\text{M O}_2$. The values in parentheses are those specifically for FNR-S24F. (B) Alignment of amino acid sequences near the N-terminal cysteine residues of *E. coli* FNR and three FNR proteins from *P. putida* (ANR, PP_3287 and PP_3233). *, conserved residues; :, similar residues. The Arg (R) residue proposed to alter the O_2 sensitivity of PP_3387 relative to FNR/ANR is indicated in bold.

proteins. It is notable that for both *E. coli* (see above) and gonococcal (24) FNR proteins, the presence of amino acid residues with bulky side chains (Arg, His, Phe, Trp, or Tyr) results in significant aerobic activity. This suggests that Ser-24 is important for *E. coli*-type FNR proteins to respond within the appropriate physiological range of O_2 concentrations and raises the possibility that evolution has selected other residues in different subclasses of FNR proteins to tune their O_2 response to their environmental niche. It is, therefore, notable that Arg is found in the equivalent of position 24 of some FNR proteins. For example, *Pseudomonas putida* KT2440 has three FNR-type proteins (Fig. 5B); two of these have Ser, whereas the third (PP_3287) has Arg at the equivalent of position 24. The work described here suggests that FNR proteins from *P. putida* operate across different O_2 ranges to optimize gene expression patterns.

The lack of an FNR structure is a barrier to understanding the role of the Ser-24 in modulating the O_2 sensitivity of the FNR [4Fe-4S] cluster. A search of the RCSB protein databank using PSIPRED software (33) yielded two structures with iron-sulfur clusters ligated by an FNR-like CysXXCysXXXXCys motif. These proteins, a thymine-DNA glycosylase (34) and endonuclease III (35, 36), contain redox-inactive, stable [4Fe-4S] clusters that probably fulfill structural roles (37). In both proteins the fourth Cys is close to the others in the primary structure, whereas in FNR the fourth ligand (Cys-122) is more remote, presumably operating across an intramolecular interface between the main body of the protein and the N-terminal region. Comparison of the amino acid sequences equivalent to those linking Cys-23 and

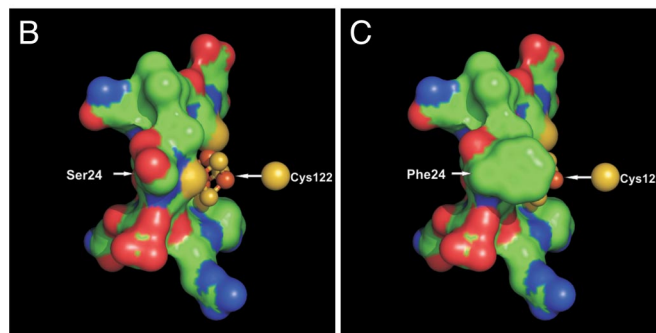
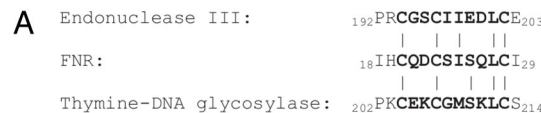


Fig. 6. Model of the [4Fe-4S] binding region of FNR and FNR-S24F. (A) Alignment of amino acid sequences of the modeled region from FNR, endonuclease III, and thymine-DNA glycosylase. Bold type denotes the central Cys motif, connecting lines indicate residue identities, and numbers are the residue number in the full primary sequence of the respective molecules. (B and C) Predicted structure of FNR (B) and FNR-S24F (C) cluster regions produced in SWISS-Model by using the structure of endonuclease III (PDB code 2abk) as a template. Also represented is the sulfur atom of Cys-122 that binds the 4th iron atom of the iron-sulfur cluster. Images were produced by using PyMOL and show identical views. Note that the cluster ligand Cys-23 that is exposed in the FNR model (B) is shielded in the FNR-S24F model (C).

Cys-29 of FNR shows good conservation of side-chain properties (polarity, hydrophobicity) except at the position corresponding to 24 in FNR (Fig. 6A). In endonuclease III and thymine-DNA glycosylase, the position equivalent to Ser-24 in FNR is occupied by nonpolar residues, Ile and Gly, respectively (34, 35). Thus, we modeled residues Cys-20–Cys-29 of FNR with a [4Fe-4S] cluster bound by using SWISS-Model (38–42) and endonuclease III as a template. The model predicts that Ser-24 is positioned such that there is solvent access to the iron-sulfur cluster close to Cys-23 (Fig. 6B). Here, it has been shown that replacement of Ser-24 by the bulky nonpolar amino acid Phe significantly stabilizes the FNR [4Fe-4S] cluster, and the model predicts that the Phe side chain sits in close proximity to the sulfur atom of Cys-23, shielding it and the iron bound by Cys-23 (Fig. 6C). It is also likely that the S24F substitution affects the redox potential of the cluster [4Fe-4S]^{3+/2+} couple and that this in turn contributes to the observed difference in the rate of the initial reaction with O_2 . At present it is unknown exactly how replacement of Ser-24 by Phe inhibits the O_2 -independent [3Fe-4S]⁺ to [2Fe-2S]²⁺ conversion, but a bulky side chain such as Phe could slow the conformational changes required to rearrange the cysteine ligands from a tetrahedral array required to ligate a [4Fe-4S] cluster to a planar one to bind the flat [2Fe-2S]²⁺ cluster.

In summary, we have demonstrated a 2-step mechanism for the FNR [4Fe-4S]²⁺ to [2Fe-2S]²⁺ cluster conversion via a [3Fe-4S]⁺ intermediate, shown that the residue at position 24 in FNR modulates the reaction of the [4Fe-4S]²⁺ cluster with O_2 , and formulated a plausible molecular model that suggests that O_2 interacts with the FNR [4Fe-4S]²⁺ cluster in the vicinity of Cys-23. Thus, our work provides insight into the mechanism of the iron-sulfur cluster rearrangements that switch FNR between active and inactive forms and implies that residues equivalent to position 24 in *E. coli* FNR, inter alia, play a key role in determining the sensitivity of the switch, thereby matching FNR activity to a range of O_2 concentrations that require a response in the environmental niche inhabited by the bacterium.

Materials and Methods

In Vivo Transcription Assays. Cultures of *E. coli* JRG1728 (*fnr lac*) containing the reporter plasmid pFF-41.5 and pBR322 derivatives expressing FNR proteins with single Phe substitutions at positions flanking essential cysteine residues [pGS24 (FNR), pGS2139 (FNR-H19F), pGS2140 (FNR-Q21F), pGS2140A (FNR-D22F), pGS2140B (FNR-S24F), pGS2141 (FNR-L28F), pGS2142 (FNR-I30F), pGS2143 (FNR-V121F), or pGS2144 (FNR-E123F)] or different amino acids at position 24 (pGS2193–2210) were grown in L broth supplemented with appropriate antibiotics (100 $\mu\text{g mL}^{-1}$ ampicillin, 25 $\mu\text{g mL}^{-1}$ tetracycline, 30 $\mu\text{g mL}^{-1}$ chloramphenicol) under aerobic (25-mL universal tubes containing 5 mL of medium shaken at 250 rpm) or anaerobic (sealed bottles filled to the neck) conditions at 37 °C overnight. β -Galactosidase activities were then measured for triplicate cultures (43).

Purification of Reconstituted [4Fe-4S] FNR. GST-FNR and GST-FNR-S24F fusion proteins were overproduced in aerobically grown *E. coli* BL21 λ DE3 cultures containing the expression plasmids pGS572 (FNR) and pGS2120 (FNR-S24F) and were purified under anaerobic conditions in 25 mM Hepes, 2.5 mM CaCl_2 , 100 mM NaCl, 500 mM KCl, 100 mM NaNO_3 (pH 7.5) buffer, as described (16, 44). Under these conditions, O_2 was undetectable in anaerobic buffers (16). The FNR proteins were released by using thrombin, and [4Fe-4S] clusters were reconstituted under anaerobic conditions, as described (16, 44).

Quantitative Procedures. Apo-FNR protein concentrations and iron contents of reconstituted proteins were determined as described in ref. 16; and based on the analyses, [4Fe-4S] FNR samples exhibited $\epsilon_{405\text{ nm}}$ values of $16,220 \pm 135 \text{ M}^{-1} \text{ cm}^{-1}$ (45).

Spectroscopy. Absorbance measurements were made with a Cary UV-visible spectrophotometer. CD measurements were made with a Jasco J-810 spectropolarimeter. EPR measurements were made with an X-band Bruker EMX EPR spectrometer equipped with a TE-102 microwave cavity and an ESR-900 helium flow cryostat (Oxford Instruments). To study the initial products of FNR-S24F reaction with O_2 , an aliquot of [4Fe-4S] FNR-S24F ($\approx 20 \mu\text{M}$ final concentration) was mixed with an aliquot of aerobic buffer (219.5 μM O_2 , 21 °C, final concentration $\approx 200 \mu\text{M}$ O_2), and aliquots were loaded into EPR tubes and frozen rapidly to 77 K at the indicated times.

Kinetic Measurements. Reactions were initiated by injection of air-saturated buffer ($\approx 212 \mu\text{M}$ O_2) into sealed anaerobic cuvettes containing [4Fe-4S] FNR (10 μM final concentration, 1-mL final volume) at 25 °C. The dead time of mixing was ≈ 5 s. Changes in absorbance at 420 nm were used to monitor the conversion of the cluster. The rate constants are the averages of at least three repeats. Measurements by Clark O_2 electrode, Winkler titration (16), and an OxyMicro probe (World Precision Instruments) indicated that O_2 loss to the headspace was insignificant under the conditions used (SI Appendix, Figs. S2 and S3).

Data Analysis. The conversion of [4Fe-4S] FNR to [2Fe-2S] FNR was followed under pseudo-first-order conditions (O_2 in excess) by measuring absorbance changes at 420 nm. Absorbance datasets were fitted by using SigmaPlot 9.0 to the double exponential function $y = y_0 + ae^{-bx} + ce^{-dx}$, where b and d are the 2 first-order rate constants, a and c are the amplitudes, and y_0 is the offset. Observed rate constants (k_{obs}) obtained from the fits (corresponding to b in the above expression, i.e., corresponding to the initial, O_2 -dependent reaction) were plotted against the corresponding initial concentration of O_2 to obtain the apparent second-order rate constant.

3D Structure Modeling. Prediction of the 3D structure of the cluster-containing region of FNR was achieved by using the program SWISS-Model (38–42). The endonuclease III structure was used as a template by submitting amino acid sequence YTCIARKPRCQDC(S/F)ASQLCEYKEKVD, a hybrid sequence of 10 residues of FNR/FNR-S24F from Cys-20 to Cys-29 (in bold), flanked by the 9 N-terminal residues preceding Cys-194 and the 7 C-terminal residues preceding Cys-203 of endonuclease III. The resulting protein database file was edited, removing nonsimilar residues, and images were created by using the program PyMOL (46), resulting in a model of residues PRCQDC(S/F)ASQLCE and the iron-sulfur cluster.

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