Mechanism of Oxygen Sensing by the Bacterial Transcription Factor Fumarate-Nitrate Reduction (FNR)*

Received for publication, September 5, 2003, and in revised form, November 25, 2003
Published, JBC Papers in Press, November 25, 2003, DOI 10.1074/jbc.M309878200

Jason Crack‡, Jeffrey Green§, and Andrew J. Thomson¶
From the School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, NR4 7TJ and the Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

The facultative anaerobe Escherichia coli adopts different metabolic modes in response to the availability of oxygen. The global transcriptional regulator FNR (fumarate-nitrate reduction) monitors the availability of oxygen in the environment. Binding as a homodimer to palindromic sequences of DNA, FNR carries a sensory domain, remote from the DNA binding helix-turn-helix motif, which responds to oxygen. The sensing mechanism involves the transformation of a [4Fe-4S]2+ cluster into a [2Fe-2S] form in vitro on reaction with oxygen. Evidence is presented to show that this process proceeds by at least two steps, the first, an oxidative one, being the formation, on reaction with O2, of a [3Fe-4S]1+ cluster as an intermediate accompanied by the production of hydrogen peroxide. This is followed by a slower, non-redox, pseudo-first order step in which the [3Fe-4S]1+ form converts to a [2Fe-2S]2+ cluster. This must be accompanied by a substantial protein conformational change since the four cysteine ligands that bind the two forms of the FeS clusters have different spatial disposition. Hydrogen peroxide is also an oxidant of the [4Fe-4S]2+, causing a similar cluster transformation to a [2Fe-2S] form. Either the hydrogen peroxide formed on reaction with oxygen can be recycled by intracellular catalase or it can be used to oxidize further Fe-S clusters. In both cases, the efficacy of oxygen sensing by FNR will be increased.

Escherichia coli is a facultative anaerobe that adopts different metabolic modes in response to the availability of oxygen (1). A hierarchy of metabolism exists in which aerobic respiration is preferred to anaerobic respiration, which in turn is preferred to fermentation (1). In simple terms, the global transcriptional regulator FNR1 (designated due to defects in fumarate-nitrate reduction) provides a large conformational change in the N-terminal region of the protein by four cysteine thiol ligands that sit at the vertices of a tetrahedron, to a planar [2Fe-2S] cluster, also thought to possess four cysteine thiols and capable of ligating an oxygen-sensitive [4Fe-4S]-iron-sulfur cluster (6–11). A variety of studies have revealed that the active form of FNR contains four [4Fe-4S]2+ cluster/FeS clusters, each monomer binding to one half-site (2). They consist of two functionally distinct domains, a DNA binding helix-turn-helix motif and an N-terminal region of antiparallel β-strands forming the sensory domain (3). The sensing regions are adapted to respond to different effectors (2). Thus, CAP reversibly binds cAMP to monitor glucose status (4), and the sensing domain of the CoaA protein of Rhodospirillum rubrum possesses a b-type cytochrome that binds CO (5). The sensory region of FNR has four conserved cysteine residues (Cys-20, -23, -29, and -122) that are essential for in vivo activity and capable of ligating an oxygen-sensitive [4Fe-4S]-iron-sulfur cluster (6–11). A variety of studies have revealed that the active form of FNR contains four [4Fe-4S]2+ clusters, each monomer that is converted to a [2Fe-2S]2+ cluster, together with other, less well-defined iron species, following exposure to oxygen both in vitro and in vivo (10, 12–15). The switch from a cubane [4Fe-4S] cluster, bound to the protein by four cysteine thiol ligands that sit at the vertices of a tetrahedron, to a planar [2Fe-2S] cluster, also thought to possess four cysteine ligands but being the formation, on reaction with oxygen will provide a large conformational change in the N-terminal region of FNR, presumably thereby initiating the switch of the protein from a DNA binding state to one incapable of binding DNA (8). Initial attempts to characterize the process of cluster conversion revealed that FNR requires at least 2.5 molecules O2 per cluster for complete cluster conversion (8). Prolonged exposure to O2 can lead to complete loss of cluster and the formation of apo-FNR (17).

The overall reaction of FNR with O2 can be written as Reaction 1. The redox states of the individual Fe atoms in the cluster are indicated.

$$[\text{2Fe}^{II}/\text{2Fe}^{III}-\text{4S}]^{2+} + x\text{O}_2 \rightarrow \text{y}[\text{2Fe}^{III}]-\text{2S}]^{2+} + \text{other products}$$

Reaction 1

Neither the product(s) of O2 reduction nor the overall stoichiometry of the reaction is known. The chemical state of iron and sulfur released is also unknown. An EPR signal characteristic of a [3Fe(III)-4S]1+ cluster (S = 5/2) has occasionally been observed in FNR samples following brief exposure to O2 but has never accounted for more than about 5% of the original [4Fe-4S]2+ cluster in the wild type protein (18).

Here we investigate the transformation of the [4Fe-4S]2+ cluster of FNR into the [2Fe-2S] form in vitro by the effect of oxygen. Evidence is presented to show that the process proceeds by at least two steps, the first, the oxidative one, being the formation on reaction with O2 of a [3Fe-4S]1+ cluster as an intermediate accompanied by the production of hydrogen peroxide. This is followed by a slower, non-redox, pseudo-first
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EXPERIMENTAL PROCEDURES

Preparation and Purification of FNR—E. coli BL21 ΔΔES3, transformed with the expression vector pGSS72 encoding the fusion protein GST-FNR (19), was grown at 37 °C in LB medium (20) containing 100 mg liter−1 ampicillin. Transcription was induced with isopropyl-1-thio-
β-D-galactopyranoside (1 mM) (19). Cells with pGSS72 were suspended (~30 ml liter−1 culture) in buffer (25 mM HEPES, 2.5 mM CaCl2, 100 mM NaCl, 100 mM NaNO2, 10 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5) disrupted by sonication (on ice), and then cellular debris was removed by centrifugation. The supernatant was frozen in liquid nitrogen and stored at −80 °C until needed.

Protein purification and handling was carried out under strictly anaerobic conditions in a Faircrest anaerobic cabinet, typically operating at <2.0 ppm O2 by volume, and all buffers were sparged with oxygen-free nitrogen gas for a minimum of 2 h. Bacterial extract (10 ml) was applied to buffer without protease inhibitor equilibrated GSH-agarose affinity column (2 ml). Bovine thionin (Sigma), incubated at ambient temperature (~25 °C) for 16 h, released apo-FNR, which was collected and stored at 4 °C until required. Protein concentration was determined using the Bio-Rad protein reagent with bovine serum albumin as the standard. A correction factor of 0.83 was applied to FNR samples according to Ref. 18. Purity of the isolated FNR was checked by SDS-PAGE.

Reconstitution of FNR—Solutions of FeS(1) (50 mM l-cysteine, 125 mM dithiothreitol, 25 mM HEPES, 2.5 mM CaCl2, 100 mM NaCl, 100 mM NaNO2, pH 7.5) and FeS(2) (20 mM (NH4)2Fe(SO4)2·4H2O, 25 mM HEPES, 2.5 mM CaCl2, 100 mM NaCl, 100 mM NaNO2, pH 7.5) were prepared. Recombinant factor of 0.53 was determined. The iron-sulfur cluster was achieved with an aliquot of FeS(1), giving a final concentration of 1 mM l-cysteine and 2.5 mM dithiothreitol, an aliquot of Ni5, l-cystine desulfurase (~225 mM final concentration), purified as reported by Zheng et al. (21), and an appropriate amount of FeS(2), providing a 10 molar excess of Fe2+/FNR monomer. Samples were transferred to a Hewlett Packard 8453 spectrophotometer fitted with a thermostatic cell holder at 37 °C and stirred magnetically throughout the reconstitution. Spectra were recorded every 20 min, and after completion (~4 h), samples were transferred back into the anaerobic cabinet. Reconstituted protein was purified on a PD10 desalting column (Amersham Biosciences) equilibrated with purification buffer.

The concentration of FNR was determined assuming εmax of 13,300 M−1 cm−1 for the [4Fe-4S]2+ cluster (8).

Spectroscopy and Oxidation of FNR—A Hitachi U3200 spectrophotometer, scanning at 120 nm min−1, or a Jasco J-810 spectrophotometer, scanning at 200 nm min−1, were used to measure absorbance or CD spectra of FNR samples in sealed anaerobic cuvettes (1 cm). Temperature dependency for a 1-m1 sample of FNR, containing catalytic amounts of catalase (370 units ml−1), was determined after the sample was injected with an aliquot (4 μl) of a 15.4 mM H2O2 stock solution, equivalent to 30.7 μM O2, and the decrease in A420 was monitored continuously using a Hewlett Packard 8453 spectrophotometer fitted with a thermostatic cell holder set to 25 °C.

The optical spectra of oxidized holo-FNR—a 2-ml sample of FNR, containing catalase (176 units ml−1), was injected with 10 μl of a 30.7 mM H2O2 stock solution, giving a final concentration of 72.7 μM O2, and the decrease in absorbance at 420 nm accompanied an increase in absorbance in the region 500–700 nm (Fig. 1B). A plot of ΔA420 nm against the ratio [O2]:[4Fe-4S] revealed that the reaction was ~64% complete at a ratio of 0.5, ~78% complete at a ratio of 1.0, and ~87% complete at a ratio of 2.0 (Fig. 2). Increasing the [O2]:[4Fe-4S] further caused minimal change in the A420 obtained at an [O2]:[4Fe-4S] > 5.5. The product of the reaction was stable for several hours at room temperature provided no further O2 was introduced. Using values of ε420 of 11,040 M−1 cm−1 for the oxidized form (see above), 28.2 μM oxidized FNR was formed following the addition of 77.3 μM O2, indicating that 95% of the [4Fe-4S]2+ cluster originally present has formed a [2Fe-2S]2+ cluster. Taking the initial slope and the asymptote at high oxygen levels, a binding stoichiometry of 0.58 ± 0.04 O2/[4Fe-4S] cluster is obtained. A complete binding curve cannot validly be fitted since the reaction turns out to be complex (see “Intermediate in the Oxidation of FNR by Oxygen”). However, the initial slope should be a reliable indicator of the stoichiometry at low oxygen levels.

The reaction of O2 with FNR was also monitored using CD spectroscopy (Fig. 3A). Iron-sulfur clusters gain optical activity from the fold of a polypeptide chain. In the absence of O2, the FNR CD spectrum displayed weak bands in the region 280–310 nm. When the oxygen-free nitrogen gas was bubbled through an aliquot (25 ml) of the stock solution, which was then treated with an aliquot (100 ml) of 1 M H2SO4. The addition of 10 ml of 10% (w/v) NaI in 1 M H2SO4 followed by five drops of a 3% (w/v) MoO42−/H2O catalyst solution liberated iodine, which was immediately titrated with a 0.1 M solution of Na2S2O3·5H2O, using 1% (w/v) starch solution as indicator (23). Stock solutions (21.6 ± 2.16 mM H2O2) were made freshly, calibrated, maintained at ±5 °C, and used on the same day.

RESULTS

 Stoichiometry of Oxygen Reaction with FNR—The optical spectrum of FNR in the absence of O2 displays absorbance maxima at 320 and 405 nm, with values of ε320 17,443 M−1 cm−1 and ε405 13,559 M−1 cm−1, respectively, together with the broad shoulder at 420 nm, giving the sample a characteristic straw brown color (Fig. 1A). Trace amounts of catalase were added to the FNR sample. Molecular O2 was introduced by the addition of 145.5 μM H2O2. Catalase reacts with H2O2 at close to the diffusion-controlled limit with a rate constant of −1 × 106 s−1 (24). Therefore, added H2O2 is decomposed by catalase to O2 before H2O2 can react with the [4Fe-4S]2+ cluster (see “Oxidation of FNR by Hydrogen Peroxide”). An [O2]:[4Fe-4S] ratio of 1.8 yielded absorbance maxima at 310 and 420 nm, with ε310 16,855 M−1 cm−1 and ε420 11,040 M−1 cm−1, respectively, together with a broad absorbance shoulder at 430 nm and an increased absorbance in the region 500–600 nm resulting in a solution with a red/brown color.

A titration of FNR with O2 was carried out by sequential additions of aerobic purification buffer (229 μM dissolved O2 at 20.0 °C) into an anaerobic sample of protein. This caused a progressive decrease in the absorbance at 420 nm accompanied by an increase in absorbance in the region 500–700 nm (Fig. 1B). A plot of ΔA420 nm against the ratio [O2]:[4Fe-4S] revealed that the reaction was ~64% complete at a ratio of 0.5, ~78% complete at a ratio of 1.0, and ~87% complete at a ratio of 2.0 (Fig. 2). Increasing the [O2]:[4Fe-4S] further caused minimal change in the A420 obtained at an [O2]:[4Fe-4S] > 5.5. The product of the reaction was stable for several hours at room temperature provided no further O2 was introduced. Using values of ε420 of 11,040 M−1 cm−1 for the oxidized form (see above), 28.2 μM oxidized FNR was formed following the addition of 77.3 μM O2, indicating that 95% of the [4Fe-4S]2+ cluster originally present has formed a [2Fe-2S]2+ cluster. Taking the initial slope and the asymptote at high oxygen levels, a binding stoichiometry of 0.58 ± 0.04 O2/[4Fe-4S] cluster is obtained. A complete binding curve cannot validly be fitted since the reaction turns out to be complex (see “Intermediate in the Oxidation of FNR by Oxygen”). However, the initial slope should be a reliable indicator of the stoichiometry at low oxygen levels.

The reaction of O2 with FNR was also monitored using CD spectroscopy (Fig. 3A). Iron-sulfur clusters gain optical activity from the fold of a polypeptide chain. In the absence of O2, the FNR CD spectrum displayed weak bands in the region 280–800 nm with three positive features at λmax 330, 380, and 420 nm. The Δε values were of the same order of magnitude as those of other proteins containing [4Fe-4S] cluster types (25, 26). The CD of anaerobic FNR was gradually lost as the ratio of [O2]:[4Fe-4S] was increased and eventually replaced by a broader spectrum with two positive features at λmax 310 and 440 nm and a single negative feature at λmax 370 nm. When the [O2]:[4Fe-4S] ratio exceeds 1.1, only minimal further changes to the CD spectrum occurred. The Δε values and forms of the CD spectra of [2Fe-2S] clusters vary widely. However, the FNR CD spectrum after exposure to O2 is reminiscent of the form of CD spectra obtained for Spirulina maxima 2Fe-ferredoxin and the Pseudomonas putida 2Fe-ferredoxin, particularly the broad positive band at 440 nm. Although the Δε values for the FNR [2Fe-2S] cluster are considerably lower than those of other proteins (26), the CD spectrum after O2 treatment can be assigned to the [2Fe-2S]2+ cluster. A plot of the binding isotherm (not shown) showed that
the reaction was ~63% complete at a [O₂]:[4Fe-4S]^{2+} ratio of 0.5 and ~84% complete at a ratio of 1.0, in agreement with the observations made by optical spectroscopy (see above). The CD spectra of FNR, the first to be reported, provide a useful means of monitoring the status of the FNR iron-sulfur cluster.

Product of Oxygen Reduction by FNR—The maximum number of electrons available from complete oxidation of one [4Fe-4S]^{2+} cluster to two Fe(III) ions is two, suggesting that hydrogen peroxide may be a product. To test this, an anaerobic sample of FNR was titrated with O₂ in the presence of Amplex Red (27) and horseradish peroxidase (HRP). Peroxide reacts with Amplex Red and HRP to generate the highly fluorescent dye Resorufin (27). The addition of aliquots of O₂ caused a progressive increase in fluorescence intensity at 587 nm (Fig. 4A). Control samples of Amplex Red and HRP in the presence of FNR and the absence of O₂ did not show any change in fluorescence intensity at 587 nm. A plot of [H₂O₂] formed as a function of the ratio [O₂]:[4Fe-4S] (Fig. 4B) reveals that the reaction was ~65% complete at a ratio of 0.5, ~78% complete at a ratio of 1.0, and ~85% at a ratio of 2.0. Increasing [O₂]:[4Fe-4S] beyond 2.0 gave no further increase in the fluorescence intensity at 587 nm. These observations are in good agreement with those from optical and CD spectroscopy and clearly demonstrate that H₂O₂ is a major product when FNR is exposed to O₂. A maximum value of 13.1 mol of H₂O₂ was formed from 29.2 mol of a [4Fe-4S]^{2+} cluster at an [O₂]:[4Fe-4S] ratio of 2.0. Thus, ~45% of the total amount of [4Fe-4S]^{2+} clusters originally present in the FNR sample-generated H₂O₂.

Oxidation of FNR by Hydrogen Peroxide—Since H₂O₂ is a strong oxidant, it was important to determine whether H₂O₂ itself would react with the [4Fe-4S] cluster of FNR. Anaerobic H₂O₂ was titrated into a sample of anaerobic FNR (Fig. 5A). The optical spectra showed absorbance decreases at 420 nm accompanied by increases in absorbance in the region 460–680 nm and isosbestic points at 450 and 680 nm. A plot of ΔA_{420} against the ratio of H₂O₂:[4Fe-4S], saturates at a ratio of >2.5. A reaction stoichiometry of 1.59 ± 0.03 H₂O₂:[4Fe-4S] is obtained from the asymptotes (Fig. 5B). The reaction with H₂O₂...
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**Fig. 2.** Titration of FNR with air-saturated buffer. Oxidation of FNR (29.6 μM [4Fe-4S]^{2+} cluster) monitored at ΔA_{420} was plotted versus the [O_2]:[4Fe-4S] cluster ratio after reaction with aliquots of air-saturated purification buffer (229 μM O_2 at 20 °C).

**Fig. 3.** Circular dichroism spectra of FNR as a function of oxidation by oxygen and hydrogen peroxide. As shown in A, air-saturated purification buffer (224 μM O_2 at 21.5 °C) was titrated into FNR (30.8 μM [4Fe-4S]^{2+}). The upper spectrum corresponds to FNR in the absence of O_2, and the lowest spectrum corresponds to FNR in the presence of 30.0 μM O_2. The final O_2 concentrations were 0.0, 2.2, 4.4, 6.5, 8.6, 10.7, 14.6, 21.3, 24.9, and 30.0 μM. After each addition, samples were incubated for 5 min prior to measurement. The arrows indicate the direction of movement of spectral features during the titration. As shown in B, anaerobic H_2O_2 (334 μM) was titrated into FNR (22.7 μM [4Fe-4S]^{2+} cluster). Upper spectrum, FNR in the absence of H_2O_2; lower spectrum, FNR in the presence of 59.7 μM H_2O_2. The final H_2O_2 concentrations were 0.0, 6.6, 12.9, 18.9, 24.8, 30.4, 37.1, 51.6, and 59.7 μM. After each addition, samples were incubated for 5 min prior to measurement. The arrows indicate the direction of movement of spectral features during the titration.
has also been followed by CD spectroscopy (Fig. 3B). Increasing the ratio \([\text{H}_2\text{O}_2]:[4\text{Fe-4S}]\) caused the progressive loss of CD features at \(\lambda_{\text{max}}\) 330, 380, and 420 nm, characteristic of the \([4\text{Fe-4S}]\) cluster and the generation of a new CD spectrum that becomes stable at a ratio of \([\text{H}_2\text{O}_2]:[4\text{Fe-4S}]\) 3.0. This spectrum is not identical to that obtained from the reaction of FNR with \(\text{O}_2\) but still shows a broad CD band at 440 nm, characteristic of the FNR \([2\text{Fe-2S}]\) cluster.

**Intermediates in the Oxidation of FNR by Oxygen**—The time course of the reaction of FNR with \(\text{O}_2\) (generated by the reaction of catalase with \(\text{H}_2\text{O}_2\)) was monitored at 420 nm, revealing a 50% decrease in 1 min, 82% decrease after 5 min, and 98% decrease after 10 min (Fig. 6). A semilog plot of the data (not shown) was linear, showing first order kinetics with a rate constant of 5.57 ± 0.09 \(\text{min}^{-1}\), giving a half-life of ~2 min. A rate that is first order in FNR, at equivalent concentrations of FNR and \(\text{O}_2\), suggests a rate-determining step that does not involve oxygen. Previous EPR studies have shown the presence of only small quantities, <5%, of a \([3\text{Fe-4S}]^{1+}\) cluster during the reaction of FNR with \(\text{O}_2\) (12). However, the \([3\text{Fe-4S}]^{1+}\) cluster may be an intermediate in the \([4\text{Fe-4S}]^{2+}\) to \([2\text{Fe-2S}]^{2+}\) cluster conversion (18, 28).

During the course of an \(\text{O}_2\) titration, samples of FNR were taken within ~1 min after \(\text{O}_2\) addition, placed in an EPR tube, and frozen rapidly to 77 K. The EPR spectra of these samples, measured at 10 K, display a signal centered at \(g = 2.014\), characteristic of a \([3\text{Fe-4S}]^{1+}\) cluster which decreased rapidly in intensity on increasing the temperature until it was lost at ~30 K (Fig. 7A). Integration of the signal showed an initial EPR signal intensity, which declined steeply during the course of the titration (Fig. 7B). After the addition of a stoichiometric amount of \(\text{O}_2\), no \([3\text{Fe-4S}]^{1+}\) cluster was detected by EPR. The maximum intensity accounted for 22.8 \(\mu\text{mol}\) of an electron spin, corresponding to 50% of the \([4\text{Fe-4S}]^{2+}\) cluster originally present in the FNR sample, showing that the \([3\text{Fe-4S}]^{1+}\) cluster is formed in significant quantities over the course of an \(\text{O}_2\) titration but is absent when the reaction is driven to completion. This supports the idea that it is an intermediate in the \([4\text{Fe-4S}]^{2+}\) to \([2\text{Fe-2S}]^{2+}\) cluster conversion.

**DISCUSSION**

FNR proteins are global transcription factors that respond to changes in environmental \(\text{O}_2\) through the assembly and disas-
**Fig. 5. Oxidation of FNR by hydrogen peroxide.** A, the reaction of FNR (22.7 μM [4Fe-4S]^{2+} cluster) with a dilute anaerobic solution of H₂O₂ (334 μM H₂O₂ in buffer) monitored by absorbance spectroscopy at A₄₂₀. After the addition of each aliquot, the sample was incubated at an ambient temperature for 5 min prior to measurement. The upper spectrum corresponds to FNR in the absence of H₂O₂, whereas the lowest spectrum corresponds to the presence of 79.6 μM H₂O₂. The final H₂O₂ concentrations were 0.0, 6.6, 12.9, 18.9, 24.8, 30.4, 37.1, 51.6, 59.7, 66.3, 70.6, and 79.6 μM. After each addition, samples were incubated for 5 min prior to measurement. The arrows indicate the direction of movement of spectral features during the titration. Inset, changes occurring in the region 320–520 nm. The arrows show the direction of spectral change during the titration. B, a plot of ΔA₄₂₀ versus [H₂O₂] for the reaction of FNR with aliquots of anaerobic H₂O₂ solution (334 μM H₂O₂).

**Fig. 6. Time course of the reaction of FNR with oxygen.** As shown in A, ΔA₄₂₀ (% of original value) of FNR (24.0 μM [4Fe-4S]^{2+}) was monitored at 1-s intervals in the presence of 30.7 μM O₂ generated from the decomposition of 61.4 μM H₂O₂ by catalase. Inset, changes within the first 60 s of the reaction. First order rate constants of k₁ = 5.57 ± 0.09 × 10⁻³ s⁻¹ and half-life of t₀ of ~2 min were obtained from the fits shown as well as from a log versus t plot (not shown).
Molecular O2 brings about conversion of the [4Fe-4S]2+ cluster of FNR to the [2Fe-2S]2+ form, thereby triggering a conformational change in the protein that prevents sequence-specific DNA binding and interaction with the transcriptional machinery (reviewed in Ref. 2). The interconversion between a [4Fe-4S]2+ and [2Fe-2S]2+ cluster upon exposure to O2 has been observed in proteins that generate a radical species by reductive cleavage of S-adenosyl methionine such as biotin and lipoate synthase (29). This appears to be a very large family with over 600 members (30). However, the reactions between [4Fe-4S]2+ clusters and O2 have not previously been studied quantitatively. Here we have shown that upon exposure to O2, the FNR [4Fe-4S]2+ cluster is converted to the [2Fe-2S]2+ form via a [3Fe-4S]1+ intermediate with the concomitant production of H2O2. H2O2 can also oxidize the FNR [4Fe-4S]2+ cluster, probably to the [2Fe-2S]2+ form.

Reaction of FNR with Oxygen—The spectrophotometric titrations reveal a progressive conversion of the [4Fe-4S]2+ cluster to a [2Fe-2S]2+ cluster in which FNR displayed higher sensitivity at low ratios of O2:[4Fe-4S]. After exposure to a 3-fold molar excess of O2, >95% of the [4Fe-4S]2+ clusters originally present were converted to an anaerobically stable [2Fe-2S]2+ cluster. Thus, the conversion of the [4Fe-4S] to the [2Fe-2S] form of FNR does not result in an autocatalytic, runaway process. The stoichiometry indicates that 0.5 O2 molecule interacts with one [4Fe-4S]2+ cluster.

We have demonstrated the generation of H2O2 following reaction of FNR with O2 with the amount of peroxide produced being dependent on the amount of O2 added. However, quantification of H2O2 yielded only ~45% of that expected for oxidation of one [4Fe-4S]2+ cluster. This suggests either that the reaction with O2 produces products in addition to H2O2, or more likely, that the H2O2 generated can oxidize unreacted [4Fe-4S]2+ clusters. Evidence to support this latter possibility has been obtained. This could account for the observation that more than one iron-sulfur cluster can be disassembled by one O2 (see below). If the overall oxidation process of the FNR by O2 is written as Reaction 2, then the stoichiometry of oxygen to [4Fe-4S] is not as determined.

\[
[4Fe-4S]^{2+} + O_2 + 2H^+ \rightarrow [2Fe-2S]^{2+} + H_2O_2 + 2S^{2-} + 2Fe^{3+}
\]

REACTION 2
The time course for the reaction of equimolar FNR with $O_2$ displayed kinetics that were first order with respect to the [4Fe-4S]$^{2+}$ cluster, implying that the reaction is not the simple bimolecular process shown in Reaction 2. The detection for the first time of significant quantities of the [3Fe-4S]$^{1+}$ form of FNR indicates that it is an intermediate in [4Fe-4S] to [2Fe-2S] conversion, not a product of a side reaction. Thus, the simplest explanation is that exposure of the FNR [4Fe-4S]$^{2+}$ cluster to $O_2$ yields a [3Fe-4S]$^{1+}$ cluster as an early intermediate with concomitant production of $H_2O_2$ (Reaction 3).

$$[4Fe-4S]^{2+} + O_2 + 2H^+ \rightarrow (k_1/k_{-1})[3Fe(III)-4S]^{1+} + H_2O_2 + Fe^{3+}$$

**REACTION 3**

This is followed by the degradation of the [3Fe-4S]$^{1+}$ cluster to generate the [2Fe-2S]$^{2+}$ cluster (Reaction 4).

$$[3Fe-4S]^{1+} \rightarrow (k_3)[2Fe-2S]^{2+} + 2S^{2-} + Fe^{3+}$$

**REACTION 4**

The first in a two-step process is oxidative, involving a two-electron oxidation of the [4Fe-4S]$^{2+}$ cluster, leading to a [3Fe(III)-4S]$^{1+}$ cluster and the loss of one $Fe^{3+}$. No inorganic sulfur would be lost at this stage. The product of this reaction is $H_2O_2$ (Reaction 3). This reaction is too fast to follow accurately by conventional spectrophotometry, although at the shortest time intervals, a fast phase was observed (Fig. 6). The second slower, rate-determining step (Reaction 4) occurs, in which sulfide ions and further $Fe^{3+}$ are lost. These two steps lead to the overall reaction scheme given by Reaction 2, in accord with the kinetic observations provided that $k_1 >> k_2$.

**Reaction of FNR with Hydrogen Peroxide**—The major product of oxygen reduction by FNR, hydrogen peroxide, will itself oxidize the [4Fe-4S]$^{2+}$ cluster in FNR. The CD spectrum suggests the product of the reaction with $H_2O_2$ to be a [2Fe-2S]$^{2+}$ cluster, although further spectroscopic characterization is needed. The reaction scheme, Reaction 5, is also a two-electron process but with water as the product.

$$[4Fe-4S]^{2+} + H_2O_2 + 2H^+ \rightarrow [2Fe-2S]^{2+} + 2H_2O + 2S^{2-} + 2Fe^{3+}$$

**REACTION 5**

A cooperative interaction between a pair of iron sulfur clusters in the dimeric form of FNR would lead to the four-electron reduction of oxygen to water. If the $H_2O_2$ produced from oxidation of one cluster by $O_2$ were subsequently to react with the partner [4Fe-4S] cluster in a co-operative reaction, this would result in the reduction of one $O_2$ molecule to two $H_2O$ molecules by a pair of [4Fe-4S] clusters of dimeric FNR (Reaction 6)

$$2[4Fe-4S]^{2+} + O_2 + 4H^+ \rightarrow [2Fe-2S]^{2+} + 2H_2O + 4S^{2-} + 4Fe^{3+}$$

**REACTION 6**

This leads to an $O_2$:[4Fe-4S] of 0.5, as observed here in the in vitro experiments with FNR.

This raises interesting new issues. Is the reaction of FNR in the absence of DNA the same when FNR is bound to DNA? It will clearly be of great interest to repeat the present studies with FNR-DNA complexes. Is the oxidation of a single [4Fe-4S] cluster of the homodimer sufficient to cause FNR to release DNA, or are both clusters required to undergo oxidation? In vivo catalase may also rapidly decompose $H_2O_2$. This will regenerate $O_2$ to oxidize further FNR, again leading to an $O_2$:[4Fe-4S] stoichiometry of 0.5. Either chain of events would provide amplification of the signal molecule $O_2$ leading to a higher cellular sensitivity.

Attempts to detect the number and the oxidation states of Fe$^{2+}$ or Fe$^{3+}$ ions released from FNR using colorimetric reagents were unsuccessful even in the absence of $O_2$; FNR iron-sulfur clusters disassemble in the presence of strong iron chelators used for such assays. However, in carrying out this reaction with oxygen, the [4Fe-4S]$^{2+}$ cluster is displaying a rather well described chemistry (31). Conversion of protein-bound [4Fe-4S]$^{2+}$ clusters to their [3Fe-4S]$^{1+}$ forms is well known to occur via at least two pathways, first and rather rarely, reversible loss of $Fe^{2+}$ from a labile [4Fe-4S]$^{2+}$ as in ferredoxin III from *Desulfovibrio africanus* (32), and secondly, by oxidatively induced release of $Fe^{3+}$ from the hypervalent state, [4Fe-4S]$^{3+}$, which is unstable in many proteins (33). Armstrong and co-workers (31) have shown, by application of strongly oxidizing electrochemical pulses to protein-bound Fe-S clusters, that sensitivity to oxygen, resulting in cluster degradation, is largely determined by the redox potential between the $2^-$ and the hypervalent state, [4Fe-4S]$^{3+}$. This suggests a possible mechanism for control of the oxygen sensitivity of FNRs, namely, for the surrounding protein to tune the [4Fe-4S]$^{3+/2+}$ cluster redox potential. There is qualitative evidence that FNRs from different species, e.g. CydR (34), have very different oxygen sensitivities, fit for the range of oxygen tension over which the switch is required to operate.

**Conclusions**—The mechanism of FNR uncovered by this work reveals a remarkable oxygen sensor and redox-activated conformational switch. Each [4Fe-4S] cluster has been shown to be a two-electron device in which the sensitivity to the signal molecule, oxygen, can be amplified either by recycling the initial product, hydrogen peroxide, to oxygen via a catalase or by direct interaction of the hydrogen peroxide itself with a cluster. A key intermediate is the [3Fe-4S]$^{1+}$, cluster which, by release of a protein cysteine side chain, initiates a conformational rearrangement, leading subsequently to the reattachment of
cysteine residues to form a [2Fe-2S] cluster. This results in an inability to bind DNA and the switching on of transcription. By tuning the redox potential of the FNR [4Fe-4S]^{2+/-1+} cluster, the range of oxygen levels sensed could be altered to fit the purpose. Note that two sequential one-electron oxidations of the cluster [4Fe-4S]^{2+} would not occur at the same potential, a higher oxidizing potential being required to remove the second electron. However, by loss of an Fe(III) ion from the [4Fe-4S]^{3+} state to form [3Fe-4S]^{2+}, a concerted two-electron loss can take place at a single potential.

Although the crystal structure of FNR has not been determined, it is possible to model its structure and response to cluster interconversion now that structures are available for the analogues of FNR, CAP, and CooA, the CO-sensing protein of R. rubrum. The structure of CooA shows that, in the CO-off form, the DNA binding domain of the protein has swung almost through 180 degrees, lengthening the long helix that forms the dimer interface (5). We have generated structures using CAP as a template for the DNA binding state via the Swiss model and the structure of CooA as a template for the form that fails to bind DNA. Fig. 8 shows our results. In the DNA binding state, the DNA recognition helices lie exposed at the top of the structure, whereas our earlier studies of the reaction of FNR with hydrogen peroxide are both oxidants that lead to a [2Fe-2S] specific toward oxidants. This work has shown that oxygen and nitric oxide (35) revealed nitrosylation of the [4Fe-4S] cluster, whereas our earlier studies of the reaction of FNR with Fe(III) ion from the [4Fe-4S]^{3+} state to form [3Fe-4S]^{2+}, a concerted two-electron loss can take place at a single potential.

Acknowledgments—We thank Dr. D. R. Dean (University of Virginia) for the kind gift of NifS (pDB551) and Dr. S. Spiro for discussion.

REFERENCES

1. Guest, J. R. (1995) Philos. Trans. R. Soc. Lond. B Biol. Sci. 350, 189–202
2. Green, J., Scott, C. & Guest, J. R. (2001) Adv. Microb. Physiol. 44, 1–34
3. Schultz, S. C., Shields, G. C. & Steitz, T. A. (1991) Science 253, 1001–1007
4. Busby, S. & Kolb, A. (1996) in Regulation and Gene Expression in Escherichia coli (Lin, E. C. C. & Lynch, A., eds) pp. 255–260, Landes, Austin, TX
5. Lanzilotta, W. N., Schuller, D. J., Thorsteinsson, M. V., Kerby, R. L., Roberts, N. P. & Paulus, W. (1997) J. Biol. Chem. 272, 25275–25280.
6. Sharrocks, A., Green, J. & Guest, J. R. (1990) FEMS Lett. 70, 119–122
7. Melville, S. B. & Gunsalus, R. P. (1990) J. Biol. Chem. 265, 18733–18736
8. Jordan, P. A., Thomson, A. J., Ralph, E. T., Guest, J. R. & Green, J. (1997) FEMS Lett. 146, 349–356
9. Kiley, P. J. & Beinert, H. (1999) FEMS Microbiol. Rev. 22, 341–352
10. Lazazzera, B. A., Beinert, H., Khoroshilova, N., Kennedy, M. C. & Kiley, P. J. (1999) J. Biol. Chem. 274, 21012–21015
11. Beinert, H. & Kiley, P. (1996) FEBS Lett. 382, 218–219
12. Khoroshilova, N., Popescu, C., Munck, E., Beinert, H. & Kiley, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6087–6092
13. Popescu, C. V., Bates, D. M., Beinert, H., Munck, E. & Kiley, P. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13431–13435
14. Becker, S., Hulighaus, G., Gabrielytzky, T. & Unden, G. (1996) J. Bacteriol. 178, 4515–4521
15. Unden, G. & Schirawski, J. (1997) Mol. Microbiol. 25, 205–210
16. Deleted in proof
17. Green, J., Trageres, M., Six, S., Unden, G. & Guest, J. R. (1991) Proc. R. Soc. Lond. B Biol. Sci. 244, 137–144
18. Green, J., Bennett, B., Jordan, P., Ralph, E. T., Thomson, A. J. & Guest, J. R. (1996) Biochem. J. 316, 887–892
19. Green, J., Irvine, A. S., Meng, W. & Guest, J. R. (1996) Mol. Microbiol. 19, 125–137
20. Miller, J. H. (1972) Experiments in Molecular Genetics, pp 431–435, Cold Spring Harbor Press, Cold Spring Harbor, NY
21. Zheng, L., White, R. M., Cash, V. L., Jack, R. F. & Deamn D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5754–5758
22. Vogel, A. I. (1989) in Vogel’s Textbook of Quantitative Chemical Analysis (Jeffery, G. H., Bassett, J., Mendham, J. & Denney, R. C., eds) 5th Ed., pp 395–396, Longman Scientific & Technical, Harlow, UK
23. Vogel, A. I. (1989) in Vogel’s Textbook of Quantitative Chemical Analysis (Jeffery, G. H., Bassett, J., Mendham, J. & Denney, R. C., eds) 5th Ed., pp 344–345, Longman Scientific & Technical, Harlow, UK
24. Aebi, H. (1984) Methods Enzymol. 155, 121–126
25. Stephens, P. J., Jensen, G. M., Devlin, F. J., Morgan, T. V., Stout, C. D., Rao, K. R. & Hall, D. O. (1978) in Experiments in Molecular Genetics, pp 210–219, Cold Spring Harbor, Cold Spring Harbor, NY
26. Stephens, P. J., Irvine, A. S., Meng, W. & Guest, J. R. (1996) Biochem. J. 233, 276–284
27. Zhou, M., Diwu, Z., Panchuk-Voloshina, N. & Haugeand, R. P. (1997) Anal. Biochem. 253, 162–168
28. Khoroshilova, N., Beinert, H. & Kiley, P. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2499–2503
29. Ollagnier-de Choudens, S., Sanakis, Y., Hewitsen, S., Roach, P., Baldwin, J., Munck, E. & Fontecave, M. (2000) Biochemistry 39, 4165–4173
30. Sofianou, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F. & Miller, N. E. (2001) Nucleic Acids Res. 29, 1097–1106
31. Tilley, G. J., Camba, R., Burgess, B. K. & Armstrong, F. A. (2001) Biochem. J. 360, 717–726
32. Flint, D. H., Tuminello, J. F. & Emptage, M. K. (1993) J. Biol. Chem. 268, 22369–22376
33. Aggarwal, A., Li, D. & Cowan, J. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9440–9444
34. Wu, G., Cruz-Ramos, H., Hill, S., Green, J., Sawers, G. & Poole, R. K. (2000) J. Biol. Chem. 275, 4679–4686
35. Cruz-Ramos, H., Crick, J., Wu, G., Hughes, M. N., Scott, C., Thomson, A. J., Green, J. & Poole, R. K. (2002) EMBO J. 21, 3235–3244
36. Guz, N. & Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
37. Shaw, D. J., Rice, D. W. & Guest, J. R. (1983) Adv. Microb. Physiol. 166, 241–247
38. Guz, N., Niemand, A. & Peitsch, M. C. (1999) Trends Biochem. Sci. 24, 364–367
39. Bes, M. T., Paraini, E., Inda, I. A., Saraiwa, L. M., Pelato, M. L. & Sheldrick, G. M. (1999) Structure Fold. Des. 7, 1201–1211