# Superoxide Destroys the [2Fe-2S]$^{2+}$ Cluster of FNR from *Escherichia coli*†

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ABSTRACT: The oxygen sensing ability of the transcription factor FNR depends on the presence of a [4Fe-4S]$^{2+}$ cluster. In the presence of $O_2$, conversion of the [4Fe-4S]$^{2+}$ cluster to a [2Fe-2S]$^{2+}$ cluster inactivates FNR, but the fate of the [2Fe-2S]$^{2+}$ cluster in cells grown under aerobic conditions is unknown. The present study shows that the predominant form of FNR in aerobic cells is apo-FNR (cluster-less FNR) indicating that the [2Fe-2S]$^{2+}$ cluster, like the [4Fe-4S]$^{2+}$ cluster, is not stable under these conditions. By quantifying the amount of [2Fe-2S]$^{2+}$ cluster in 2Fe-FNR in vitro in the presence of various reductants and oxidants (GSH, DTT, cysteine, $O_2$, hydrogen peroxide, and superoxide), we found that superoxide, a byproduct of aerobic metabolism, significantly destabilized the [2Fe-2S]$^{2+}$ cluster. Mössbauer spectroscopy was used to monitor the effects of superoxide on 2Fe-FNR in vivo; under cellular conditions that favored superoxide production, we observed the disappearance of the signal representative of the [2Fe-2S]$^{2+}$ cluster. We conclude that the [2Fe-2S]$^{2+}$ cluster of FNR is labile to superoxide both in vitro and in vivo. This lability may explain the absence of the [2Fe-2S]$^{2+}$ cluster form of FNR under aerobic growth conditions.

Fe-S clusters are among the most versatile cofactors in biology. Not only do these metal centers serve as efficient catalysts for electron-transfer reactions, they also have structural, substrate binding, and sensing roles (*1*, *2*). The utility of Fe-S clusters for $O_2$ sensing has been established for FNR, a global transcription regulator in *Escherichia coli*. Under anaerobic conditions, FNR exists in a transcriptionally active form that consists of a homodimeric complex containing one [4Fe-4S]$^{2+}$ cluster per subunit (herein called 4Fe-FNR)[1] (*3*, *4*). Upon exposure to $O_2$, FNR is inactivated by conversion of the [4Fe-4S]$^{2+}$ cluster to a [2Fe-2S]$^{2+}$ cluster, which produces the 2Fe-FNR form. Since the two cluster types of FNR utilize the same four cysteinyl ligands (*4*), studies of FNR provide an unusual opportunity to compare the properties of two types of Fe-S clusters within the same protein environment.

The [4Fe-4S]$^{2+}$ cluster has been shown to be necessary for FNR function because it enhances dimerization, which in turn promotes site-specific DNA binding and transcriptional regulation (*4*−*7*). While the biochemical properties of 4Fe-FNR have been well studied, little is known about the properties of 2Fe-FNR. Studies of 2Fe-FNR have been limited mainly to preparations obtained upon air exposure of isolated 4Fe-FNR, which also produces other uncharacterized reaction products. Nevertheless, this [2Fe-2S]$^{2+}$ cluster species of FNR was inactive for site-specific DNA binding and appeared to be in a monomeric form (*3*).

While the $O_2$-induced conversion of 4Fe-FNR to 2Fe-FNR is sufficient to explain how FNR in anaerobically grown *E. coli* is inactivated upon $O_2$ exposure, less is known about how FNR in aerobically grown cells is kept in an inactive state. FNR protein is present in similar amounts in cells grown under aerobic and anaerobic conditions (*8*), yet only the anaerobically isolated protein was found to contain an Fe-S cluster (*3*, *9*). Nevertheless, a variant of FNR (FNR-L28H) with an $O_2$-stable [4Fe-4S]$^{2+}$ cluster accumulates intracellularly as the [4Fe-4S]$^{2+}$ cluster form under both aerobic and anaerobic conditions, suggesting that de novo assembly of clusters into wild-type FNR can occur under aerobic conditions (*10*). Therefore, we proposed that 4Fe-FNR is synthesized under aerobic growth conditions but is then rapidly inactivated by $O_2$-induced cluster conversion (*4*). One prediction of this hypothesis is that the [2Fe-2S]$^{2+}$ cluster form of FNR should accumulate under aerobic growth conditions. Yet, previous studies indicated that FNR isolated from aerobically grown cells was in a cluster-less form. However, these results were obtained prior to the discovery of 2Fe-FNR, which raised the question of whether 2Fe-FNR was stable under the isolation conditions used in these early studies.

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[1] Abbreviations: 2Fe-FNR, [2Fe-2S]$^{2+}$ cluster containing FNR; 4Fe-FNR, [4Fe-4S]$^{2+}$ cluster containing FNR; apo-FNR, cluster-less FNR; DTT, dithiothreitol; FNR, fumarate nitrate reduction; GSH, glutathione; IPTG, isopropyl-thio-$\beta$-D-galactoside.

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The goal of this study was to investigate the fate of the $[2Fe\text{-}2S]^{2+}$ cluster form of FNR in *E. coli*. To address this question, we have developed a method for purifying 2Fe-FNR from whole cells, which has allowed us to examine whether significant amounts of 2Fe-FNR are present in aerobically grown cells and whether the $[2Fe\text{-}2S]^{2+}$ cluster is stable to oxidants found under aerobic growth conditions. Our results indicate that the properties of the $[2Fe\text{-}2S]^{2+}$ cluster of FNR are significantly different from those of the $[4Fe\text{-}4S]^{2+}$ cluster. These data also provide additional evidence for the versatile reactivity of Fe-S clusters.

## EXPERIMENTAL PROCEDURES

*Isolation of 2Fe-FNR. E. coli* B strain PK872 (containing *fnr* under control of the T7 promoter on pET11a) was grown as previously described (*10*) to allow the overproduction and accumulation of 4Fe-FNR. All subsequent steps were performed under aerobic conditions at 4 °C. The $O_2$-dependent conversion of 4Fe-FNR to 2Fe-FNR was induced in these cell cultures by rapid stirring with air for 15 min (*11*). Cells containing 2Fe-FNR were lysed and clarified by centrifugation as previously described (*10*), except that dithionite was omitted from the cell resuspension buffer. 2Fe-FNR was purified using a Hi-Trap Heparin column (Amersham) on a Pharmacia FPLC with a 15 mL linear gradient of buffer (50 mM $KPO_4$ buffer, pH 6.8, 10% glycerol) from 0.1 to 1.0 M KCl at 1.0 mL/min, while monitoring the absorbance of the eluate at 405 nm. For Mössbauer analysis, 2Fe-FNR was purified from cells grown in $^{57}$Fe-enriched glucose minimal medium, and was then concentrated with a 1-mL Biorex 70 column as described previously (*3*). Aliquots (1 $\mu$L) of isolated protein fractions were then run in a 12% SDS−PAGE gel along with samples from cell cultures grown to an $OD_{600}$ of 0.3 and induced with 0.4 mM IPTG (from which 0.75 mL aliquots were pelleted and resuspended in 15 $\mu$L of SDS loading buffer).

*Iron/Sulfide Quantitation.* The concentrations of protein, iron, and sulfide were measured in duplicate as described in Khoroshilova et al. (*4*), Kennedy et al. (*12*) and Beinert (*13*), respectively. Throughout the paper, FNR concentrations are expressed in terms of cluster-occupied FNR, based on the determination of sulfide and protein in each preparation. In the 2Fe-FNR preparations used in this study, 50−80% of the FNR molecules contained $[2Fe\text{-}2S]^{2+}$ clusters and the average $Fe/S^{2-}$ ratio was 1.07 ± 0.06.

*Molecular Sieve Chromatography.* Purified preparations of 4Fe-FNR (*10*) and 2Fe-FNR were analyzed under anaerobic conditions as described previously (*5*) except that the elution buffer contained no glycerol. For each analysis, 200 $\mu$L of 20 $\mu$M FNR was loaded onto an Amersham Pharmacia Biotech HR-12 Superose molecular sieve column at a flow rate of 0.5 mL/min. The column was calibrated with the following molecular weight standards: BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome-*c* (12.4 kDa).

*In Vitro Assays of [2Fe-2S] Cluster Stability.* The stability of the [2Fe-2S] cluster of FNR was monitored at 550 nm in 50 mM $KPO_4$ buffer (pH 6.8), 10% glycerol, and 0.4 M KCl at a final concentration of 20 $\mu$M 2Fe-FNR. $H_2O_2$, DTT, cysteine, or GSH was added to a final concentration of 1 mM where indicated. Superoxide was produced with a xanthine oxidase system composed of 22 milliunits/mL xanthine oxidase (Sigma) and 1 mM hypoxanthine (Sigma). Where indicated, 500 units of catalase (Roche) and/or 100 units of superoxide dismutase (SOD) (Roche) were added. The absorption spectra (250−700 nm) of the reaction mixtures were collected at 25 °C on a Perkin-Elmer $\lambda 2$ spectrophotometer. For anaerobic analyses, screw top quartz cuvettes (Starna) were filled and sealed within an anaerobic chamber (Coy).

*Measurement of $Fe^{2+}$ Release In Vitro.* The release of $Fe^{2+}$ from the $[2Fe\text{-}2S]^{2+}$ cluster was measured in triplicate by monitoring $A_{593}$ during the reaction of 3 $\mu$M 2Fe-FNR with the xanthine oxidase system in the presence of 1 mM ferene (Sigma). The extinction coefficient ($\epsilon$), determined using ferrous ethylene diammonium sulfate (GFS Chemicals) as a standard, of the $Fe^{2+}$-ferene complex in 50 mM $KPO_4$ buffer (pH 6.8, 10% glycerol, 0.4 M KCl) is 39.6 $mM^{-1}$ $cm^{-1}$ at 593 nm. Kinsim software (1997) was used to determine the half-life of the [2Fe-2S] cluster in the presence of superoxide.

*Measurement of Superoxide In Vitro.* Superoxide (produced by the xanthine oxidase system) was measured spectrophotometrically by monitoring the increase in the $A_{550}$ indicative of the reduction of cytochrome-*c*. The extinction coefficient ($\epsilon$) of reduced cytochrome-*c* is 21.0 $mM^{-1}$ $cm^{-1}$ at 550 nm (*14*). In the presence of 100 $\mu$M cytochrome-*c*, xanthine oxidase (22 milliunits/mL) reacted with 1 mM hypoxanthine to produce ~45 $\mu$M $O_2^-$ over a period of ~20 min.

*Examination of 2Fe-FNR Stability by Whole Cell Mössbauer Spectroscopy. E. coli* B strains PK872 (containing *fnr* under control of the T7 promoter on pET11a) or PK879 (containing pET11a) (*15*) were grown in glucose minimal medium (*4*) containing 10 $\mu$M $^{57}$Fe-ferric citrate (94.69% $^{57}$Fe enriched; Pennwood Chemicals). Cell cultures (6 L) for Mössbauer analysis were prepared as described previously (*11*), except that the time for sparging cell cultures with argon after IPTG induction (to allow for accumulation of 4Fe-FNR) was increased to 17 h and the initial centrifugation step was decreased to 15 min by using a Beckman JLA 10.500 rotor, run at 8000 rpm in a Beckman Avanti J-25 centrifuge. All steps following the IPTG induction were carried out at 4 °C. Following centrifugation, the cells containing 4Fe-FNR were resuspended in 30 mL of buffer M (10 mM $KPO_4$ buffer, pH 6.8, 10% glycerol, 0.1 M KCl). A total of 10 mL of cells were processed under anaerobic conditions as previously described (*11*). To induce the conversion of 4Fe-FNR to 2Fe-FNR, 10 mL of cells were diluted in 390 mL of buffer M and sparged with 100% $O_2$ for 15 min. To induce the production of superoxide, the remaining 10 mL of cells were similarly treated with $O_2$ except that 250 $\mu$M paraquat and 0.2% glucose were also present during the sparging step. The cells were then concentrated for transfer to Mössbauer cups as previously described (*11*).

## RESULTS

*Isolation of 2Fe-FNR.* Our earlier studies had suggested to us that the $[2Fe\text{-}2S]^{2+}$ cluster form of FNR might accumulate under aerobic growth conditions. Yet, previous efforts to isolate FNR from aerobically grown cells yielded only an apo-form (*3, 9*). However, since this apo-protein had been isolated prior to the discovery of 2Fe-FNR, it was unknown whether 2Fe-FNR was stable under the purification

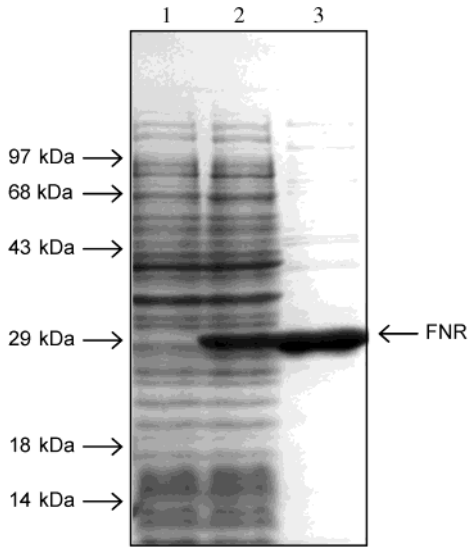


FIGURE 1: SDS−PAGE analysis of 2Fe-FNR purification. Lane 1: PK872 cells grown without FNR induction; lane 2: PK872 cells induced at an $OD_{600}$ of 0.3 with 0.4 mM IPTG for 1 h; lane 3: 2Fe-FNR-enriched fractions from the heparin agarose column.

conditions employed in these earlier studies. To address this question, we developed a method to efficiently purify 2Fe-FNR, capitalizing on our previous observation that cells containing 2Fe-FNR can be obtained by briefly (∼15 min) exposing anaerobic cells containing 4Fe-FNR to $O_2$ (*11*).

In our previous studies, we found that we could convert approximately 50% of 4Fe-FNR to 2Fe-FNR by exposing cells to $O_2$. Since both 4Fe-FNR and 2Fe-FNR are efficiently detected by Mössbauer spectroscopy of whole cells (*11*), we have used this technique to optimize conditions for this conversion, and below we show that we achieve essentially 100% conversion to 2Fe-FNR. We have studied a variety of whole cell preparations in which we have varied growth conditions, especially the $^{57}$Fe concentration in the growth medium, and conditions of harvesting. We have obtained cells containing as much as 40% of the $^{57}$Fe as 4Fe-FNR (data not shown). After determining how to efficiently convert 4Fe-FNR into 2Fe-FNR in whole cells, we developed a new protocol to purify 2Fe-FNR from cells thus treated. As estimated by SDS−PAGE analysis (Figure 1), >95% of the protein is FNR in the 2Fe-FNR preparations produced with the improved methods. Typically, 50−80% of the FNR molecules contained [2Fe-2S]$^{2+}$ clusters.

*Isolated 2Fe-FNR Contains a [2Fe-2S]$^{2+}$ Cluster and Is Monomeric in Solution.* As reported previously (*4*), the [4Fe-4S]$^{2+}$ cluster of 4Fe-FNR exhibits a quadrupole splitting $\Delta E_Q$ = 1.22 mm/s and isomer shift (at 4.2 K relative to Fe metal at 300 K) $\delta$ = 0.45 mm/s. Figure 2 shows a 4.2 K Mössbauer spectrum of 2Fe-FNR isolated by the new method. The spectrum exhibits one doublet with $\Delta E_Q = 0.53 \pm 0.02$ mm/s and $\delta = 0.27 \pm 0.01$ mm/s. The parameters observed are typical for [2Fe-2S]$^{2+}$ clusters, and they are in agreement with our previous results, $\Delta E_Q$ = 0.58 mm/s and $\delta$ = 0.28 mm/s (*4*).

Previous studies (*3*) of isolated 4Fe-FNR had shown that 1 min of air-exposure was sufficient to convert dimeric 4Fe-FNR to a monomeric form. However, in these earlier experiments, it was not possible to assess how much [2Fe-2S]$^{2+}$ cluster was associated with the monomeric form.

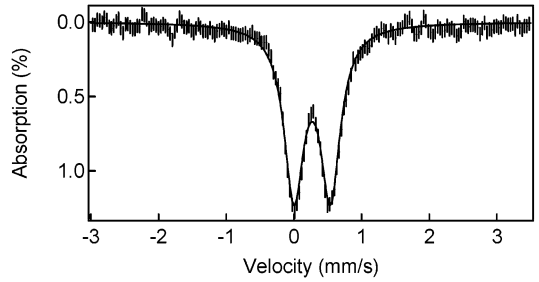


FIGURE 2: 4.2 K Mössbauer spectrum of purified 2Fe-FNR. The solid line is a least-squares fit to one quadrupole doublet with $\Delta E_Q$ = 0.53 mm/s and $\delta$ = 0.27 mm/s. The 2Fe-FNR preparation used contained 0.192 mM [2Fe-2S] clusters.

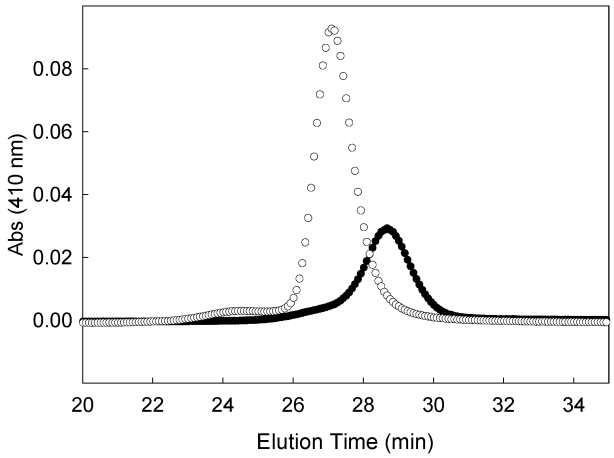


FIGURE 3: Molecular sieve analysis of isolated 2Fe-FNR. Purified preparations of 2Fe-FNR (filled circles) and 4Fe-FNR (hollow circles) were analyzed by molecular sieve chromatography using an Amersham Pharmacia Biotech HR-12 Superose molecular sieve column at a flow rate of 0.5 mL/min. 54% of the FNR molecules contained [2Fe-2S]$^{2+}$ clusters, whereas 76% of the FNR molecules in the 4Fe-FNR preparation contained [4Fe-4S]$^{2+}$ clusters. The absorption at 410 nm that is common to both [4Fe-4S]$^{2+}$ and [2Fe-2S]$^{2+}$ clusters is plotted although unique visible spectral features of the visible spectrum from 350 to 600 nm were used to distinguish the two cluster types (data not shown).

Therefore, we have examined here the oligomeric state of 2Fe-FNR of known [2Fe-2S]$^{2+}$ stoichiometry by monitoring the absorption spectrum of the eluate from the molecular sieve column on line from 200 to 600 nm (Figure 3). All of the FNR that had the absorption properties indicative of the 2Fe-2S cluster (data not shown, and Figure 3) had the elution time (28.5 min) expected of a FNR monomer, in contrast to that of a dimer represented by the elution time of the 4Fe-FNR species (26.9 min) (*3*, *5*). Taken together, the properties observed for 2Fe-FNR isolated with this method match the characteristics observed for the FNR species produced by oxidizing 4Fe-FNR in vitro.

*FNR Purified from Aerobically Grown Cells Has No Cluster.* In the course of developing the purification scheme for 2Fe-FNR, we realized that the conditions previously used to isolate FNR from cells grown under aerobic conditions were not optimal for the recovery of 2Fe-FNR (*9*, *15*−*18*). In particular, we found that sufficient salt, low temperature, and relatively rapid purification were important for the successful purification of 2Fe-FNR. Therefore, we used the new method of isolation to determine whether 2Fe-FNR was the predominant species of FNR under aerobic growth conditions.

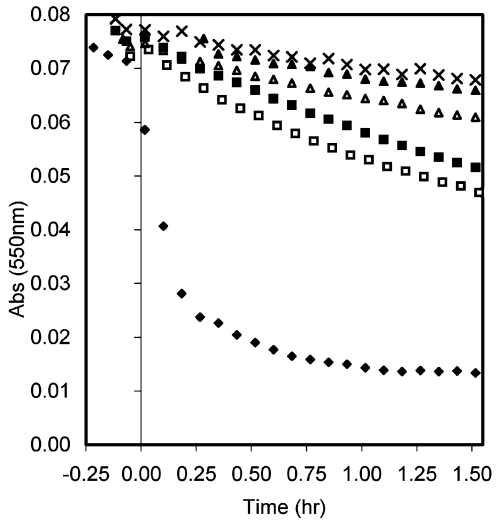


FIGURE 4: Effect of redox reagents on the $[2Fe\text{-}2S]^{2+}$ cluster of FNR. 2Fe-FNR (20 $\mu$M final concentration) was exposed under aerobic conditions (▲) to redox reagents (1 mM final concentration): hydrogen peroxide (□), GSH (△), cysteine (■), or DTT (×). Superoxide (◆) was produced by the reaction of xanthine oxidase (22 milliunits/mL) with hypoxanthine (1 mM) under aerobic conditions. For all conditions, 2Fe-FNR was incubated until time 0, when the appropriate reagent was introduced.

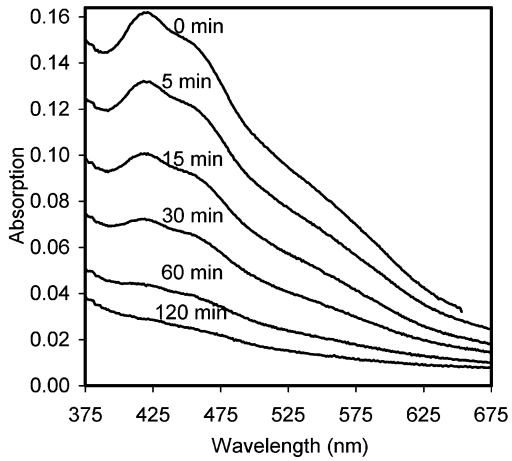


FIGURE 5: Effect of superoxide on the $[2Fe\text{-}2S]^{2+}$ cluster of FNR. The spectral properties of the $[2Fe\text{-}2S]^{2+}$ cluster were monitored for the reaction of 20 $\mu$M 2Fe-FNR with the xanthine oxidase system. Spectra shown represent the absorption observed during the reaction at 0, 5, 15, 30, 60, and 120 min after the addition of the xanthine oxidase system.

We would have preferred to analyze the FNR species present in aerobically grown cells containing normal FNR levels, but all of the available techniques that can distinguish the species by cluster content require the overexpression of FNR. Therefore, we grew PK872 cells at 37 °C under aerobic conditions in a minimal glucose medium and induced overexpression of FNR with IPTG for 1 h, after which the cells were immediately processed for purification. Since the overnight sparging step was omitted, we overproduced FNR under analogous anaerobic growth conditions to establish that, during the 1 h induction with IPTG, sufficient 4Fe-FNR was formed to allow detection of cluster from the purified protein. We found that 4Fe-FNR was successfully produced and purified from the *anaerobically* grown cultures. However, there was no measurable iron or acid-labile sulfide in FNR protein isolated from *aerobically* grown cells, even though the yield of total FNR protein was similar under both conditions (data not shown). Therefore, we conclude that the predominant species of FNR in aerobic cells is apo-FNR. These results imply that the [2Fe-2S] cluster of FNR, like the [4Fe-4S] cluster of FNR (*4*, *10*, *11*), is unstable under aerobic growth conditions.

*2Fe-FNR Is Stable in Both the Presence and Absence of $O_2$ In Vitro.* We used an in vitro system to examine the stability of the 2Fe-2S cluster of FNR under a variety of conditions. The loss of the [2Fe-2S] cluster from purified 2Fe-FNR was monitored as a decrease in absorption at 550 nm. We found that the [2Fe-2S] cluster was relatively stable in both the presence (Figure 4) and absence of air (data not shown), with a half-life of several hours. Thus, the lack of 2Fe-FNR in aerobic cells is difficult to explain by lability of the [2Fe-2S] cluster to $O_2$ as has been previously observed with the [4Fe-4S] cluster (*4*, *19*).

*Superoxide Destabilizes the $[2Fe\text{-}2S]^{2+}$ Cluster of FNR In Vitro.* The same assay was used to monitor the effect of various redox reagents on the stability of the [2Fe-2S] cluster of FNR (Figure 4). A very slow rate of destabilization was observed under aerobic conditions in the presence of $H_2O_2$, GSH, DTT, or cysteine. In contrast, when xanthine oxidase/hypoxanthine were tested, the [2Fe-2S] cluster was rapidly lost (Figure 4), presumably as a result of the production of superoxide.

Two lines of evidence indicate that the destabilizing effect was due specifically to superoxide. First, when the reaction was performed under anaerobic conditions, no loss of the $[2Fe\text{-}2S]^{2+}$ cluster was observed until air was introduced (data not shown). Since the production of superoxide by xanthine oxidase is $O_2$-dependent (*20*), these data suggest that superoxide is responsible for destabilizing the $[2Fe\text{-}2S]^{2+}$ cluster of FNR. Second, when superoxide dismutase was added prior to the addition of the xanthine oxidase system (data not shown), the resulting cluster decay matched the slow decay produced by hydrogen peroxide (see Figure 4), the other product of the xanthine oxidase reaction. When both catalase and superoxide dismutase were added, the [2Fe-2S] cluster was stable (data not shown) (*21*). These findings indicate that among the reagents tested, only superoxide led to the rapid destruction of the $[2Fe\text{-}2S]^{2+}$ cluster of FNR.

The analysis of absorption spectra produced during the reaction of 20 $\mu$M 2Fe-FNR with the xanthine oxidase system showed the loss of all observable spectral features characteristic of the $[2Fe\text{-}2S]^{2+}$ cluster (Figure 5). This demonstrates the effectiveness of superoxide in the destruction of the [2Fe-2S] cluster of FNR. Additional experiments showed that the spectral changes observed in the very slow reaction of the [2Fe-2S] cluster with hydrogen peroxide correspond well with the changes induced by superoxide (data not shown), suggesting that the $[2Fe\text{-}2S]^{2+}$ cluster is destroyed in both cases.

*Superoxide-Induced Destruction of the $[2Fe\text{-}2S]^{2+}$ Cluster Releases Iron and Sulfur.* To address the question of how superoxide led to the loss of the $[2Fe\text{-}2S]^{2+}$ cluster, we assayed the oxidation state of the Fe released during the reaction. The release of $Fe^{2+}$ from 2Fe-FNR upon reaction with superoxide was monitored in the presence of ferene, an $Fe^{2+}$-specific iron chelator (Figure 6A). From the reaction of 3 $\mu$M 2Fe-FNR (i.e., protein containing 3 $\mu$M $[2Fe\text{-}2S]^{2+}$

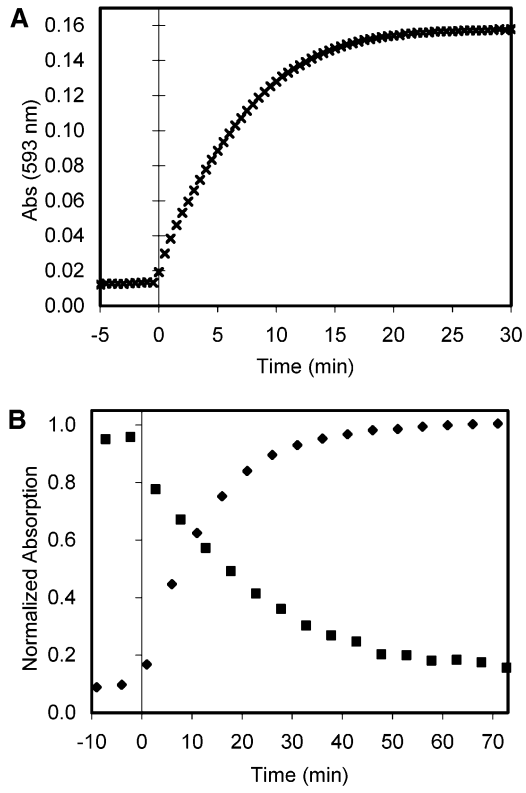


FIGURE 6: (A) Release of $Fe^{2+}$ from the [2Fe-2S]$^{2+}$ cluster of FNR upon reaction with superoxide. 3 $\mu$M 2Fe-FNR was exposed to the xanthine oxidase system at time 0. $Fe^{2+}$ release was monitored by formation of the $Fe^{2+}$-ferene complex by measuring $A_{593}$. (B) Comparison of cluster destruction with $Fe^{2+}$ release. The reaction of 5 $\mu$M 2Fe-FNR with the xanthine oxidase system was monitored in the presence and absence of ferene. Cluster loss is shown as a decrease in absorption at 550 nm (■), and formation of the $Fe^{2+}$-ferene complex increases the absorption at 593 nm (◆). The absorption values were normalized for presentation on a single scale.

clusters) with the xanthine oxidase system, 3.75 ± 0.14 $\mu$M $Fe^{2+}$ ions were released. Thus, our results show that about 1.25 $Fe^{2+}$ ions (63%) are released per [2Fe-2S]$^{2+}$ cluster. These data (Figure 6A) were used to analyze the kinetics of $Fe^{2+}$ release since, under these conditions, superoxide was being produced in excess relative to 2Fe-FNR (data not shown). However, because superoxide was produced concomitant with its reaction with 2Fe-FNR, our experiments can suggest only the lower limit of the rate at which superoxide reacts in vitro with the [2Fe-2S]$^{2+}$ cluster of FNR: $t_{1/2} \leq 4.2$ min. The addition of catalase produced only a very minute decrease in the rate and quantity of $Fe^{2+}$ released (data not shown), further supporting the hypothesis that the rate of cluster destruction by superoxide is very fast relative to that produced by hydrogen peroxide.

To compare the loss of the visible absorption features of 2Fe-FNR with the release of $Fe^{2+}$ under the reaction conditions used, 5 $\mu$M 2Fe-FNR was exposed to the xanthine oxidase system in the presence and absence of ferene, and the absorption from 350 to 700 nm was monitored over time. The absorption peaks indicative of the [2Fe-2S] cluster were lost (represented in Figure 6B as absorption at 550 nm), roughly in parallel with the release of $Fe^{2+}$ from the cluster (Figure 6B; measured as absorption at 593 nm). However, due to the lack of sensitivity of the spectrophotometric measurement at the 4-fold lower 2Fe-FNR concentration used in this assay, a quantitative comparison of the kinetics of the loss of all of the 2Fe-FNR spectral features and of the release of chelatable iron was not possible.

To further characterize the destruction of the [2Fe-2S] cluster of FNR, the $Fe^{3+}$-specific chelator Tiron was used to monitor release of $Fe^{3+}$ from the [2Fe-2S]$^{2+}$ cluster during the reaction with superoxide. No $Fe^{3+}$ was detected (data not shown), which suggests that the remaining Fe (following the release of 1.25 $Fe^{2+}$ per monomer) is not released as $Fe^{3+}$.

According to the analysis for sulfide, after superoxide treatment only one-third of the sulfide originally present in 2Fe-FNR (data not shown) remained in a form that was responsive to the *p*-phenylenediamine/ferric chloride reagent that is generally used for determination of sulfide (*13*). The contribution of the Fe-S clusters of xanthine oxidase to the total sulfide content of the reaction mixtures was negligible (data not shown). The undetectable sulfur (two-thirds) was most likely present in some form of iron sulfide or in one of its higher oxidation states. Therefore, our data suggest that during the reaction with superoxide, the [2Fe-2S] cluster is destroyed and that the majority of the iron and sulfur is lost from the cluster.

*Superoxide Destabilizes the [2Fe-2S]$^{2+}$ Cluster of FNR In Vivo.* To investigate the effect of superoxide on 2Fe-FNR turnover in vivo, we were limited to whole-cell methods that monitor the status of the Fe-S cluster (since 2Fe-FNR has no known in vivo activity), and all such available methods require the overexpression of FNR. Consequently, we used whole cell Mössbauer spectroscopy (*11*) (Figure 7) to determine if conditions that favored production of superoxide (paraquat and glucose at 4 °C) would decrease the level of 2Fe-FNR in cells. We reasoned that low temperatures would retard overall metabolism, thus slowing the use of $O_2$ by the electron transport chain and ensuring the availability of $O_2$ for superoxide production. Glucose was added as an electron source for the production of NAD(P)H, which is the electron source for diaphorase-mediated reduction of paraquat; superoxide is produced by the reduction of $O_2$ by reduced paraquat. For comparison, culture aliquots were also prepared under conditions known to accumulate only 4Fe-FNR (anaerobic) or 2Fe-FNR ($O_2$ treated).

Figure 7A−D shows Mössbauer spectra of whole-cell samples derived from a batch of anaerobically prepared cells that was divided in three equal fractions. The spectrum of the anaerobically harvested fraction is shown in Figure 7A. The second fraction was exposed to $O_2$ for 15 min; its Mössbauer spectrum is shown in Figure 7B. Figure 7C shows the difference spectrum (A) minus (B). The third fraction was exposed to $O_2$, glucose, and paraquat (the superoxide generating system) for 15 min; the spectrum of this sample is shown in Figure 7D. To properly quantify the spectra of 2Fe-FNR and 4Fe-FNR, we have to know reasonably well the background absorption produced by other cell components. Various high-spin ferrous complexes, present in all samples, produce a quadrupole doublet ($\Delta E_Q \approx 3.1$ mm/s and $\delta \approx 1.3$ mm/s) with a shape outlined above the spectrum of 7D; since its high-energy line, at 2.9 mm/s, is well resolved, this component can readily be quantified. All spectra contain a broad magnetic component with absorption stretching from $\sim -8$ to $+8$ mm/s. This component represents ferric ions in magnetically ordered (perhaps imper-

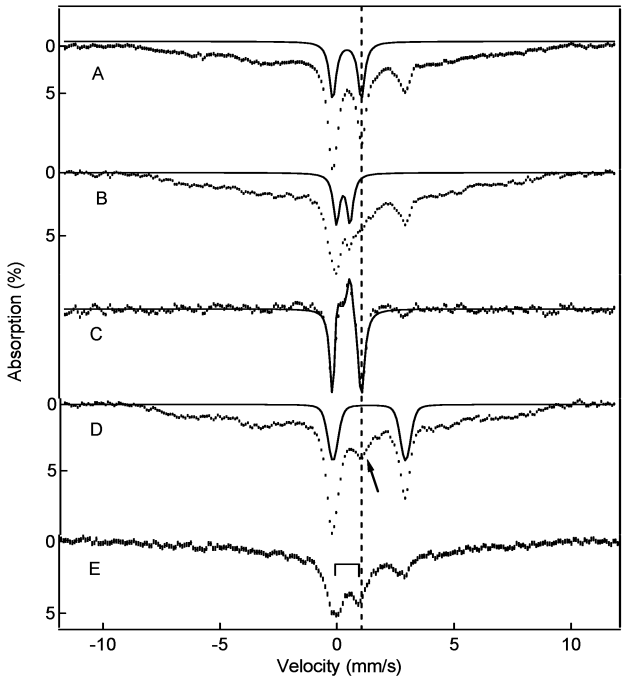


FIGURE 7: 1.5 K Mössbauer spectra of whole *E. coli* cells. (A) Spectrum of anaerobically grown cells. The central doublet, outlined by the solid line, represents 4Fe-FNR; $\Delta E_Q = 1.22$ mm/s and $\delta = 0.45$ mm/s. (B) Spectrum after exposing the cells for 15 min to $O_2$. The solid line outlines the contribution of the $[2Fe\text{-}2S]^{2+}$ cluster; $\Delta E_Q = 0.53$ mm/s and $\delta = 0.27$ mm/s. (C) Difference spectrum (A) minus (B). Note that the high-spin ferrous doublets cancel, i.e., there is no additional accumulation of ferrous ions after cluster conversion. The solid line is a spectral simulation of the difference spectrum assuming that all the $[4Fe\text{-}4S]^{2+}$ clusters (17% of Fe) have been converted to $[2Fe\text{-}2S]^{2+}$ clusters (8.5% of Fe). (D) Spectrum of cells after 15 min exposure to $O_2$, glucose, and paraquat (the superoxide generating system). The $[2Fe\text{-}2S]^{2+}$ cluster is absent. The solid line outlines the contribution of high-spin ferrous material and the arrow marks the high-energy line of the "background doublet" discussed in the text. (E) Spectrum of a control sample of cells grown on $^{57}$Fe without overexpression of FNR. The vertical line at +1.05 mm/s marks the position of the high-energy line of the $[4Fe\text{-}4S]^{2+}$ cluster of FNR.

fectly) phases at sufficiently low temperature. Unfortunately, the major portion of this spectral component appears as a broad quadrupole doublet at $T \geq 4.2$ K, obscuring the features of 2Fe-FNR and 4Fe-FNR. By lowering the temperature from 4.2 to 1.5 K a major portion, but not all, of the doublet feature changes into the broad magnetic component, removing most of its absorption from the velocity range where it would interfere with the detection of the FNR spectra. The magnetic component is not homogeneous, and, moreover, its spectral features depend on the growth conditions of the bacterial culture. As controls, we have studied whole cells grown on $^{57}$Fe without overexpressing FNR. These samples also exhibit the broad component at 1.5 K together with a "remnant doublet feature". An example is shown in Figure 7E; this particular sample exhibits a stronger doublet feature (bracket) than the samples of Figure 7A,B,D. In Figure 7 we have marked the position of the high-energy line of the doublet of 4Fe-FNR with the dashed vertical line. It can be seen that the high-energy line of the "background doublet" occurs at the same velocity. Our analysis suggests that the absorption peak marked by the arrow in Figure 7D belongs to the "background doublet" rather than to a remnant 4Fe-FNR, and in the following we assume that this "background doublet" is present in the same amount in the spectra of Figure 7A,B,D.

The predominant absorption (17% of total Fe and outlined by the solid line) of the central doublet in Figure 7A belongs to the $[4Fe\text{-}4S]^{2+}$ cluster of 4Fe-FNR. After exposure of the cells to $O_2$, the 4Fe-FNR spectrum disappears and a new component (solid line in Figure 7B) representing 2Fe-FNR appears. The difference spectrum (A) minus (B) shown in Figure 7C reveals that the two irons released during cluster conversion end up in the pool of ferric ions; no additional ferrous ions have accumulated. The solid line drawn through the difference spectrum is a spectral simulation assuming that all $[4Fe\text{-}4S]^{2+}$ clusters (17% of total Fe) are converted into $[2Fe\text{-}2S]^{2+}$ clusters (8.5% of total Fe). This assumption explains the difference spectrum reasonably well. The spectrum shown in Figure 7D was obtained from cells prepared using conditions that favor superoxide production. It can be seen that the doublet representing 2Fe-FNR has essentially disappeared, i.e., the $[2Fe\text{-}2S]^{2+}$ cluster has been destroyed by the formation of superoxide. A difference spectrum (D) minus (B) (data not shown) revealed that the iron released in the destruction of the [2Fe-2S] cluster ends up as ferrous iron. In addition, the superoxide-treated sample acquired more ferrous ions (11% of the total Fe), suggesting that some iron from the ferric pool became reduced.

The spectra shown in Figure 7 were from a batch obtained by using a growth medium containing 10 $\mu$M $^{57}$Fe. We have repeated this experiment with cells grown with 5 $\mu$M $^{57}$Fe in the medium. While the second batch had a better 4Fe-FNR/background ratio ($\sim$30% of the Fe belonged to FNR), a larger fraction of the background produced a "background doublet" at 1.5 K. However, the results showing FNR cluster conversions and cluster destruction by superoxide were essentially the same as described above.

## DISCUSSION

*Different Responses of [2Fe-2S] Cluster Proteins to Superoxide.* While destabilization of protein-bound [4Fe-4S] clusters by superoxide is well-known, to our knowledge 2Fe-FNR is the first identified example of a protein-bound [2Fe-2S] cluster that is destroyed by superoxide. While this cluster is reactive with superoxide, we have no evidence that FNR plays any role in the well-known adaptive response to superoxide stress, since 2Fe-FNR has no known transcription activity. Rather, the main transcriptional regulator of this response in *E. coli* is another [2Fe-2S] containing transcription factor, SoxR (*22*). However, the destabilization of the [2Fe-2S] cluster of FNR with superoxide is not a general property of [2Fe-2S] proteins since imposing a superoxide stress on SoxR results only in a change in cluster oxidation state (from $[2Fe\text{-}2S]^{1+}$ to $[2Fe\text{-}2S]^{2+}$), which converts SoxR into an active transcription factor (*23*). Nonetheless, the [2Fe-2S] cluster of SoxR can be destroyed, but only with conditions that promote formation of glutathione radicals (*24*). As found with SoxR, spinach dihydroxy-acid dehydratase contains a $[2Fe\text{-}2S]^{2+}$ cluster that is also stable to the presence of superoxide (*25*). These results demonstrate the versatile yet selective reactivity of Fe-S clusters.

*Lability of Fe−S Clusters to Superoxide.* Among the [4Fe-4S] proteins, superoxide-sensitive $[4Fe\text{-}4S]^{2+}$ clusters have been mainly observed in hydrolyases, of which aconi-

tase is the best studied example (*25*). Within these proteins, three of the four iron atoms of the [4Fe-4S]$^{2+}$ cluster have cysteinyl ligands, while the fourth iron has a water or hydroxide ligand and is thereby accessible for substrate binding and catalysis. A mechanism for the superoxide-induced destabilization of the [4Fe-4S]$^{2+}$ cluster has been proposed in which superoxide initially binds the iron atom that lacks the cysteinyl ligand. Superoxide is then protonated by a nearby amino acid residue and oxidizes the [4Fe-4S]$^{2+}$ cluster to a [4Fe-4S]$^{3+}$ cluster, releasing hydrogen peroxide. The [4Fe-4S]$^{3+}$ cluster is unstable and decays to a [3Fe-4S]$^{1+}$ cluster (*25*).

The mechanism by which the [2Fe-2S]$^{2+}$ cluster of FNR is destabilized by superoxide is not yet understood. However, the [2Fe-2S]$^{2+}$ cluster of FNR does not contain any iron lacking a cysteinyl ligand like that of the hydrolyase clusters, and thus the decomposition of 2Fe-FNR is likely to show some differences from that of the hydrolyase clusters. While all cluster-specific absorption bands were lost during the reaction of 2Fe-FNR with the xanthine oxidase system, only ~63% of the Fe was released as $Fe^{2+}$ ion. Therefore, ~37% of the Fe originally present in the cluster is either not released from the protein or is released in a form that is not chelatable by either ferene (specific for $Fe^{2+}$) or Tiron (specific for $Fe^{3+}$). However, our finding that 33% of the sulfur was detectable as labile sulfide indicates that Fe-S remnants are still associated with the protein, which would suggest that the majority of the "nondetectable iron" is in these protein-bound Fe-S remnants. The remaining sulfur (67%), which was no longer responsive to the reagent commonly used in determining sulfide (*13*), is most likely present in some form of Fe-S or in one of sulfur's higher oxidation states such as $S^0$, sulfinic, or sulfenic acid. Thus, since both sulfur and Fe are released from the cluster upon reaction with superoxide, we conclude that the attack by superoxide on the [2Fe-2S]$^{2+}$ cluster of FNR is likely to be more complex than that of the hydrolyase clusters from which no sulfur is released.

*2Fe-FNR Is a Transient Intermediate Under Aerobic Growth Conditions.* The lability of the Fe-S clusters of FNR to $O_2$ (*4*) and superoxide (observed in this study) could explain the inability to isolate FNR containing any Fe-S clusters from aerobically grown cells, since both oxidants are present during aerobic metabolism. The absence of any measurable amount of Fe-S cluster within FNR in aerobically grown cells implies that both 4Fe-FNR and 2Fe-FNR are relatively unstable under aerobic growth conditions. In accordance with this notion, 4Fe-FNR has been shown to react rapidly with oxygen to produce 2Fe-FNR both in vitro and in vivo (*4*, *11*). Similarly, the finding that superoxide rapidly destroys the [2Fe-2S]$^{2+}$ cluster of FNR in vitro may be relevant to the conversion of 2Fe-FNR to apo-FNR in vivo, even though the in vitro reaction produced residual Fe-S remnants. The fact that such Fe-S remnants were found associated with the FNR protein following the reaction of 2Fe-FNR with superoxide in vitro is not inconsistent with the existence of a superoxide-mediated cluster destruction pathway for 2Fe-FNR in vivo, since we predict that Fe-S remnants formed in vivo are unlikely to remain associated with the FNR protein.

*Is Superoxide Responsible for the Instability of 2Fe-FNR In Vivo?* Our Mössbauer results indicate that the [2Fe-2S] cluster of FNR is destroyed in cells using conditions that favor superoxide production. A limitation of these experiments is that FNR was overexpressed to produce sufficient amounts of 2Fe-FNR to detect a Mössbauer signal, and the cells were manipulated at 4 °C. Accordingly, these results may not reflect the status of 2Fe-FNR when produced at normal cellular concentrations and at an optimal growth temperature. However, the concentration of FNR produced normally in cells is ~1 $\mu$M and cells grown aerobically in Luria broth with glucose produce superoxide at the rate of ~5 $\mu$M/s (*26*). Therefore, we propose that the rate of superoxide production under aerobic growth conditions is sufficient to explain the formation of apo-FNR from the 2Fe-FNR that is produced by the $O_2$-dependent conversion of 4Fe-FNR.

Superoxide is normally produced in cells through the adventitious reduction of $O_2$ by auto-oxidizing flavoenzymes including sulfite reductase, succinate dehydrogenase, NADH dehydrogenase II, and fumarate reductase (*21*, *26*−*28*). However, to protect against cellular oxidative damage, superoxide is decreased to ~$10^{-10}$ M by reaction with a variety of cellular molecules, including Fe-S cluster-containing proteins, glutathione (GSH), and superoxide dismutase (*26*, *29*). Therefore, since the amount of superoxide that is available in cells to react with 2Fe-FNR cannot be presently determined, we cannot rule out that other as yet unknown factors may also play a role in the formation of apo-FNR.

*A Model to Explain Superoxide-Mediated 2Fe-FNR Destruction.* Nevertheless, our current working model is that the 4Fe-FNR formed de novo under aerobic growth conditions (*10*) is initially converted in an oxygen-dependent step to 2Fe-FNR (*4*, *11*) and then to apo-protein in a superoxide-dependent manner. However, it is not obvious why superoxide-mediated destruction of the [2Fe-2S]$^{2+}$ cluster of FNR is necessary since 2Fe-FNR is already inactive for transcriptional regulation. A rationale for this observation can be provided if one assumes that the apo-FNR produced from 2Fe-FNR is not as readily reactivated to 4Fe-FNR as is 2Fe-FNR. If this is the case, then superoxide-mediated conversion of 2Fe-FNR to apo-FNR may make the inactivation of 4Fe-FNR irreversible under aerobic growth conditions, preventing any transcriptional regulation by FNR. Moreover, if superoxide were the major contributor to the destruction of the [2Fe-2S]$^{2+}$ cluster of FNR under aerobic growth conditions, the superoxide concentration would determine the levels of 2Fe-FNR that would be available for reactivation. While superoxide production is rapid under steady-state aerobic growth conditions, its production is proportional to cellular respiration and therefore to $O_2$ availability (*26*). Thus, at lower $O_2$ concentrations that are still sufficient to inactivate 4Fe-FNR (*30*), we propose that superoxide is unlikely to accumulate to levels sufficient to destroy 2Fe-FNR. Consequently, we predict that there will be a buildup of 2Fe-FNR under such conditions. Since previous Mössbauer studies (*4*) showed that 4Fe-FNR is restored after $O_2$ has been removed from cells containing 2Fe-FNR, we hypothesize that accumulated 2Fe-FNR may be rapidly converted to 4Fe-FNR, thus preparing cells for anaerobic growth conditions. In this way, 2Fe-FNR could serve an important adaptive function as a superoxide-sensitive intermediate in FNR regulation, by responding to fluctuating $O_2$ levels in the environment. However, since the in vivo rate of the interconversion of the various FNR forms has not been studied, additional

experiments are needed to test this hypothesis. Studies to examine this and other aspects of FNR regulation are ongoing.

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