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Mössbauer spectroscopy as a tool for the study of activation/inactivation of the transcription regulator FNR in whole cells of *Escherichia coli*

(transcriptional control/oxygen sensing/Mössbauer spectra/iron–sulfur cluster)

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ABSTRACT **The global regulator FNR (for fumarate nitrate reduction) controls the transcription of >100 genes whose products facilitate adaptation of *Escherichia coli* to growth under O_2-limiting conditions. Previous Mössbauer studies have shown that anaerobically purified FNR contains a $[4Fe\text{-}4S]^{2+}$ cluster that, on exposure to oxygen, is converted into a $[2Fe\text{-}2S]^{2+}$ cluster, a process that decreases DNA binding by FNR. Using ^{57}Fe Mössbauer spectroscopy of *E. coli* cells containing overexpressed FNR, we show here that the same cluster conversion also occurs *in vivo* on exposure to O_2. Furthermore, the data show that a significant amount of the $[4Fe\text{-}4S]^{2+}$ cluster is regenerated when the cells are shifted back to an anaerobic environment. The present study also demonstrates that ^{57}Fe Mössbauer spectroscopy can be employed to study the *in vivo* behavior of (overexpressed) proteins. The use of this technique to study other iron-containing cell components is discussed.**

Iron–sulfur proteins have been found to be involved in a wide range of vital biological processes. This range extends from intra- and intermolecular electron transfer, to binding of substrates and catalysis, stabilization of protein structures, as well as signaling and sensing of environmental conditions such as changes of Fe or O_2 concentrations (1, 2). A well-studied example of O_2 sensing has been described for *Escherichia coli* where the transcription factor FNR (for fumarate nitrate reduction) plays a major role in altering gene expression in response to oxygen deprivation. FNR is active as a transcription factor only under anaerobic conditions, where it controls the expression of more than 100 genes, particularly those that function in anaerobic respiration (reviewed in ref. 3). Previous studies have shown that anaerobically purified FNR contains a $[4Fe\text{-}4S]^{2+}$ cluster (this form being referred to as 4Fe-FNR; see refs. 4 and 5). The presence of this cluster is required for dimerization and DNA binding of FNR (4) as well as for its function as a transcription factor (6). Apparently the $[4Fe\text{-}4S]^{2+}$ cluster stabilizes the dimer conformation that functions in site-specific DNA binding, resulting in transcriptional regulation.

How the $[4Fe\text{-}4S]^{2+}$ cluster of FNR functions as an oxygen sensor has been the subject of recent studies. Using Mössbauer spectroscopy, we have demonstrated that on exposure of anaerobically purified FNR to oxygen, 4Fe-FNR is converted to a $[2Fe\text{-}2S]^{2+}$ form, 2Fe-FNR, that exhibits substantially decreased DNA binding (5). In addition, Jordan *et al.* (7) demonstrated, by using optical spectroscopy, that oxygen reacts nearly stoichiometrically with 4Fe-FNR during its inactivation. These studies led to the proposal that the $[4Fe\text{-}4S]^{2+}$ cluster is acting directly as an oxygen sensor, i.e., that the $[4Fe\text{-}4S]^{2+}$ cluster is oxidatively decomposed into the inactive $[2Fe\text{-}2S]^{2+}$ form. However, inactive FNR, as purified from aerobically grown bacteria, contains no significant amount of the $[2Fe\text{-}2S]^{2+}$ cluster (4, 8) suggesting that 2Fe-FNR may be a transient state formed during short-term changes in oxygen tension. To test whether cluster conversion also occurs upon oxygen sensing by FNR *in vivo*, we have used ^{57}Fe Mössbauer spectroscopy on whole cells. To enhance the FNR concentration to levels readily detectable with this technique, we have overexpressed FNR in cells cultured in ^{57}Fe-enriched media (5).

MATERIALS AND METHODS

Strains. All strains used in this study were derived from the *E. coli* B strain, PK22 (BL21 Δ*fnr* Δ*crp*), containing either pPK823 (*fnr*$^+$; ref. 8), pPK1868 (*fnr*L28H), or the vector plasmid, pet11a (Novagen). Plasmid pPK1868 (*fnr*L28H) was constructed by cloning a 0.4-kb *Nde*I–*Bst*BI fragment from pPK2012 (9) into the same sites of pPK823 (8).

Growth of Cells for Mössbauer Studies. Strains containing plasmid-encoded *fnr* or *fnr*L28H under the control of an isopropyl β-D-thiogalactoside-inducible T7 RNA polymerase expression system or the vector pet11a were grown by using conditions that previously were observed to provide maximal FNR production for protein purification and where 75–90% of the protein contained a 4Fe cluster (5). Briefly, cells were grown aerobically in ^{57}Fe-enriched minimal medium (5) to mid-logarithmic phase at 37°C and FNR synthesis was induced for 1 h by the addition of isopropyl-β-D-thiogalactoside (400 μM). After continuous sparging with argon at 4°C for 14 h, 2 liters of the culture were harvested by centrifugation at 10,000 × *g* for 20 min, washed with ice-cold anaerobic 10 mM potassium phosphate, pH 6.8/10% glycerol/0.1 M KCl, and centrifuged at 40,000 rpm in a 45Ti rotor (Beckman) for 15 min to form a dense cell pellet. Approximately 800 mg of cell paste was transferred to a Mössbauer cup and frozen on dry ice. Cells prepared in this way were termed "anaerobic" because they were maintained under anaerobic conditions after isopropyl-β-D-thiogalactoside induction, and FNR purified from such cultures was indistinguishable from that obtained from the same strain grown under continuous anaerobic conditions at 37°C, except that the yield of 4Fe-FNR is greater (data not shown). The remaining culture was sparged with air for 15 min,

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Abbreviations: FNR, fumarate nitrate reduction; FNR$^-$, cells lacking FNR; FNR$^+$, cells containing overexpressed FNR.

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and an additional 2 liters were collected for Mössbauer analysis as described for the anaerobic samples (referred to as the "air-exposed" samples). We estimate that the amount of FNR present in these cells is ≈100 μM and thus the conversion was done with a slight excess of O_2 because air-saturated media would be ≈800 μM O_2 at 4°C. For the reconversion experiments, a portion (2 liters) of the air-exposed culture was sparged with argon for an additional 3 h before cell collection as above.

RESULTS

In solution, anaerobically purified FNR is a dimer that contains two $[4Fe\text{-}4S]^{2+}$ clusters (4, 5). We showed previously that the Mössbauer spectrum of the cluster consists of one doublet (Fig. 1*A*) with characteristic quadrupole splitting, $\Delta E_Q = 1.22$ mm/s, and isomer shift, $\delta = 0.45$ mm/s (5). When purified 4Fe-FNR is exposed to air, the $[4Fe\text{-}4S]^{2+}$ cluster is converted (within minutes at 18°C) into a $[2Fe\text{-}2S]^{2+}$ cluster that, at 4.2 K, yields one somewhat broadened doublet with $\Delta E_Q = 0.58$ mm/s and $\delta = 0.28$ mm/s (Fig. 1*B*). The 2+ core oxidation state of both cluster forms is diamagnetic according to the spectra obtained at 4.2 K and, moreover, each cluster yields *one* well resolved doublet with distinct parameters. These favorable spectral attributes, together with signal enhancement obtained by expression of FNR to ≈5% of cell protein, enabled us to determine by Mössbauer spectroscopy whether the oxygen sensor FNR undergoes the same cluster conversion *in vivo* as observed for the isolated protein.

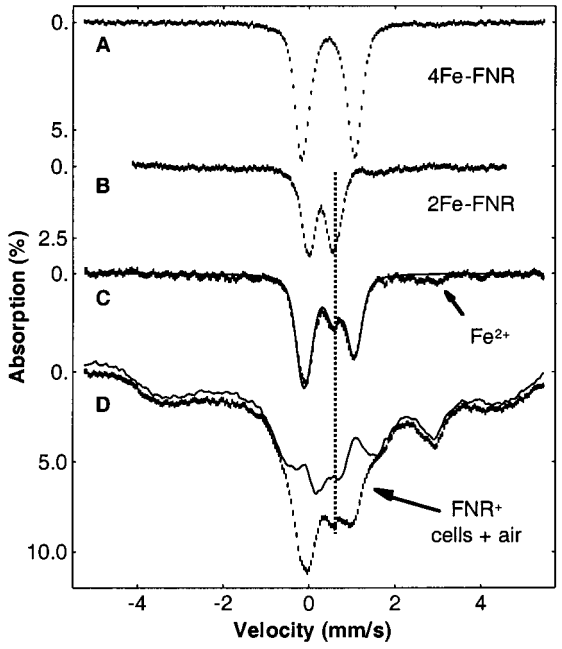

FIG. 1. Mössbauer spectra at 4.2 K of purified 4Fe-FNR (*A*), of 2Fe-FNR (*B*), and of *E. coli* whole cells containing overexpressed FNR (FNR^+ sample), exposed for 15 min to air (*D*, dash marks). Spectra of *A* and *B* were adapted from figure 1 of ref. 5. The solid line in *D* is a "background" spectrum (mostly because of ferritin) obtained by subtracting the doublet of *A* from the anaerobic FNR^+ sample (sample of Fig. 2*B*, spectrum not shown), assuming that 4Fe-FNR represents 14% of total Fe. The difference spectrum (*C*, dash marks), which represents the FNR species in the air-exposed cells, was obtained by subtracting the "background" spectrum (*D*, solid line) from that of the air-exposed sample (*D*, dash marks). The solid line in *C* is a theoretical curve obtained by adding the doublets of 4Fe-FNR and 2Fe-FNR according to a 1:1 cluster ratio, i.e., 2:1 in Fe. The arrow indicates the Fe^{2+} released during cluster conversion; the vertical dotted line marks the high-energy line of the $[2Fe\text{-}2S]^{2+}$ cluster. The spectra shown in *D* are from the same samples as those of Fig. 2*C*.

Before presenting the data obtained from whole *E. coli* cells, we want to mention briefly some features of ferritin Mössbauer spectra because ferritin is one of the major storage forms of Fe in the cell. The iron cores of ferritin (mainly hydrous ferric oxide associated with phosphate) consist of magnetically ordered microcrystals that behave like superparamagnetic particles (10). Such particles exhibit magnetically split Mössbauer spectra below a certain temperature, the so-called blocking temperature, T_B. The magnetization of the particles may undergo spontaneous transitions between energetically equivalent easy axes. These transitions cause the magnetic hyperfine field, sensed by the ^{57}Fe nucleus, to fluctuate. In the fast fluctuation limit (for $T \gg T_B$) the spectra consist of quadrupole doublets, whereas six-line patterns characteristic of a magnetically ordered system are observed for $T \ll T_B$. At intermediate temperatures magnetically split patterns and doublets coexist and, moreover, because T_B depends sensitively on the volume of the ferritin core and thus on the degree of iron loading of the ferritin molecule, the Mössbauer spectra reflect heterogeneous mixtures of species that critically depend on the temperature of measurement (11).

Fig. 2*A* shows Mössbauer spectra of *E. coli* cells that lack FNR. These cells were cultured under the same conditions previously observed to be optimal for obtaining FNR with 70–90% cluster occupancy (see *Materials and Methods*). The spectrum outlined by the dash marks was obtained from an anaerobic sample of *E. coli* cells lacking FNR (referred to as FNR^-) whereas that represented by the solid line was recorded on air-exposed FNR^- cells. The broad spectra displayed in Fig. 2*A* are indicative of a heterogeneous distribution of magnetically ordered species. The shapes of these spectra were found to depend on the temperature in a way that suggests that most of the absorption is associated with superparamagnets such as ferritin (data not shown). For these particular samples, the T_B

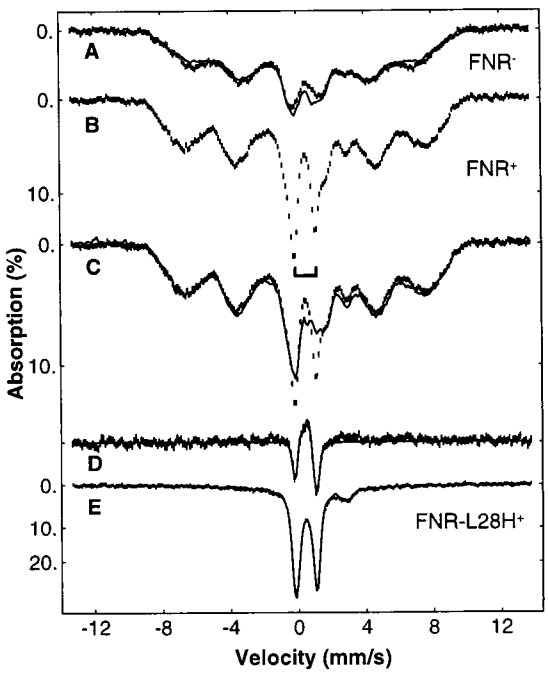

FIG. 2. Mössbauer spectra of *E. coli* cells recorded at 1.5 K (*A–C*) and 4.2 K (*E*). (*A*) FNR^- cells, anaerobic (dash marks) and after exposure to air (solid line). (*B*) Anaerobic FNR^+ cells. Bracket marks 4Fe-FNR. (*C*) Anaerobic FNR^+ cells from *B* (dash marks) and FNR^+ cells exposed for 15 min to air (solid line). (*D*) Difference spectrum of the anaerobic FNR^+ sample minus the spectrum of the air-exposed FNR^+ sample, by using the spectra shown in *C*. The solid line in *D* is a theoretical difference spectrum assuming 4Fe-FNR and 2Fe-FNR in 1:1 cluster ratio. (*E*) Whole-cell spectra of FNR-L28H mutant, anaerobic (dash marks), and exposed to air for 20 min (solid line).

was around 4 K. Because quadrupole doublets of ferritin would partially overlap with the doublets of 4Fe-FNR and 2Fe-FNR, we have minimized such spectral interference by recording all spectra at 1.5 K, where ferritin exhibits magnetically split spectra, thus spreading its absorption over a wide velocity range (Fig. 2*A*). These spectra did not reveal $[4Fe\text{-}4S]^{2+}$ or $[2Fe\text{-}2S]^{2+}$ species in detectable amounts; nor did spectra recorded in applied fields of 8.0 T indicate such species (data not shown). Thus the major iron-containing species in cells that lack FNR are the ferritins and not $[4Fe\text{-}4S]^{2+}$ or $[2Fe\text{-}2S]^{2+}$ clusters. Furthermore, comparison of the spectra of the air-exposed cells to those of the anaerobic samples showed minimal differences (Fig. 2*A*).

FNR from Cells Maintained under Anaerobic Conditions Contains a $[4Fe\text{-}4S]^{2+}$ Cluster. To determine whether FNR in whole cells exhibits properties similar to the purified protein, we analyzed the Mössbauer spectrum of cells containing FNR. Fig. 2*B* shows a 1.5 K spectrum of anaerobic cells containing overexpressed FNR (referred to as FNR^+); this spectrum contains an intense quadrupole doublet (accounting for ≈14% of Fe) that is absent in FNR^- cells. This doublet, indicated by the bracket, has the same ΔE_Q and δ values as purified 4Fe-FNR. Moreover, as revealed by studies in strong applied fields (data not shown), the doublet belongs to a diamagnetic species. We thus conclude that ≈14% of the Fe of the anaerobic FNR^+ cells belongs to 4Fe-FNR.

Exposure of FNR^+ Cells to Air Results in Conversion of the $[4Fe\text{-}4S]^{2+}$ Cluster to a $[2Fe\text{-}2S]^{2+}$ Cluster. We have tested whether exposure of cells to O_2 causes changes in 4Fe-FNR similar to those observed for the purified protein. Fig. 2*C* shows the spectrum of the anaerobic FNR^+ sample (dashed line) together with a spectrum of the same batch of cells that was exposed to air (solid line). Comparison of the spectra shows that exposure of *E. coli* cells to air did not affect the broad (ferritin) background components. However, the intensity of the 4Fe-FNR doublet in the air-exposed sample has decreased to half of the value observed in the anaerobic sample. This decrease is accompanied by the appearance of a doublet (≈4% of Fe) that has the same ΔE_Q and δ values as FNR containing a $[2Fe\text{-}2S]^{2+}$ cluster. This change is best recognized by comparing the two spectra of Fig. 1*D*, which were recorded over a smaller energy range. Because the background absorption of the anaerobic and air-exposed samples is the same, we have subtracted the doublet of purified 4Fe-FNR (using a fit to the spectrum of Fig. 1*A*) from the data of the anaerobic FNR^+ sample to obtain a representation of the non-FNR absorption (solid line in Fig. 1*D*). By subtracting this "background" from the spectrum of the air-exposed FNR^+ sample (Fig. 1*D*, dash marks), we obtained the spectrum of Fig. 1*C* (dash marks) that represents those species containing the iron originally present in the $[4Fe\text{-}4S]^{2+}$ cluster of FNR. The solid line in Fig. 1*C* is a theoretical spectrum obtained by adding the doublets of 4Fe-FNR and 2Fe-FNR according to a 1:1 cluster ratio, i.e., 2:1 in Fe. Thus, at least 50% of the original $[4Fe\text{-}4S]^{2+}$ clusters have been converted into $[2Fe\text{-}2S]^{2+}$ clusters. [Because only 4% of the total iron in the FNR^+ air-exposed sample belongs to 2Fe-FNR, its contribution in the 8.0-T spectrum of this sample (data not shown) is not resolved from that belonging to the 4Fe-FNR. However, by using the fractions of 2Fe-FNR (4% of ^{57}Fe) and 4Fe-FNR (8% of ^{57}Fe) determined from the spectra shown in Fig. 1 *C* and *D* and subtracting a theoretical spectrum of the $[4Fe\text{-}4S]^{2+}$ cluster from the obtained data, we recognized that the spectrum assigned to the $[2Fe\text{-}2S]^{2+}$ cluster must belong to a diamagnetic species.] Part of the iron released during the conversion is observed as high-spin Fe^{2+} (arrow in Fig. 1*C*, see also Fig. 3*A*), and some additional Fe^{2+} may have been oxidized to Fe^{3+}, contributing now to the broad background absorption.

The stated conclusions regarding the 4Fe → 2Fe cluster conversion are reinforced by considering the difference spectrum ("anaerobic" − "air-exposed") of Fig. 2*D*. This spectrum forcefully demonstrates that the background absorption of all other Fe-containing cell components was little affected by the exposure to air. A good fit to the difference spectrum of Fig. 2*D* (solid line) was obtained by assuming that the $[4Fe\text{-}4S]^{2+}$ clusters that disappear on exposure to air are converted quantitatively into $[2Fe\text{-}2S]^{2+}$ clusters (one 4Fe-cluster generates one 2Fe-cluster). To assess whether the changes observed on air exposure could be due to cell components other than FNR, we have exposed an FNR^- sample to air (solid line in Fig. 2*A*). Analysis of the two spectra of Fig. 2*A* suggests that, within the uncertainties, none of the minor changes observed upon air exposure of the FNR^- cells lead to the appearance of a species with the spectral properties of a $[2Fe\text{-}2S]^{2+}$ cluster. We conclude that the cluster conversion observed for isolated FNR (5) also occurs in whole cells.

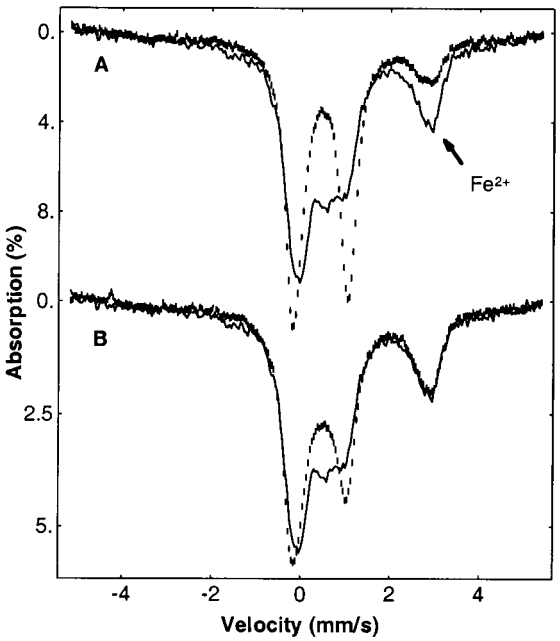

FIG. 3. Mössbauer spectra at 4.2 K of FNR^+ cells (batch different from that in Figs. 1 and 2). Spectra of anaerobic cells (*A*, dash marks), air-exposed cells (*A* and *B*, solid line) and cells after removal of air and incubation under argon at 4°C (*B*, dash marks). The arrow marks the high-energy absorption feature of Fe^{2+}, released by cluster conversion.

During this study we have recorded spectra from four independently grown batches of cells prepared by using the growth regimen described in *Materials and Methods*. Although the total fraction of iron contained in FNR varied from 10% to 25% of total cell iron, all samples exhibited the 4Fe → 2Fe conversion upon exposure to air. The ^{57}Fe incorporated into the cluster may originate from internal storage pools as well as from the growth medium because the majority of the [4Fe-4S] cluster was incorporated into FNR during sparging of the cells with argon (for 14 h at 4°C) after aerobic growth and induction by isopropyl-β-D-thiogalactoside. Because ferritin spectra depend on the degree of iron loading of the molecules and because the iron uptake kinetics under our experimental conditions depend on factors not well understood, it also is not surprising that the "background" absorption reflecting all cell iron other than that contained in FNR was somewhat variable from batch to batch.

The $[4Fe\text{-}4S]^{2+}$ Cluster of the Oxygen-Stable FNR Mutant, L28H, Is Not Converted to a $[2Fe\text{-}2S]^{2+}$ Cluster *in Vivo* upon Exposure to Air. To provide independent evidence for assigning the doublet of Fig. 2*B* to 4Fe-FNR, we also have performed whole-cell Mössbauer studies using a strain that overexpresses an oxygen-stable FNR mutant protein. FNR-L28H has a

substitution of leucine to histidine at position 28 adjacent to one of the cysteine cluster ligands and, unlike the wild-type protein, is functional *in vivo* in the presence of oxygen (12). From our *in vitro* studies, we have concluded that this protein is active in the presence of oxygen, because its 4Fe cluster is resistant to oxidative degradation (unpublished data). The two Mössbauer spectra, superimposed in Fig. 2*E*, were obtained for anaerobic (dash marks) and air-exposed (solid line) FNR-L28H cells. Three observations are noteworthy. (*i*) The central doublet has parameters identical to those of 4Fe-FNR. (*ii*) This doublet accounts for nearly 60% of the iron of the cell, in accord with our observations that the L28H mutant consistently had the highest yield of 4Fe-FNR upon purification (data not shown). (*iii*) The similarity of the two spectra displayed shows that no cluster conversion occurs when cells containing the FNR-L28H mutant are exposed to air for 20 min. Thus, these results parallel those obtained for the purified FNR-L28H protein and provide further evidence that the [4Fe-4S] species that we have observed by Mössbauer spectroscopy in whole cells can be accounted for by 4Fe-FNR. Finally, it should be noted that in the FNR-L28H cells, the contribution of ferritin to the background absorption is much lower; however, the high-energy line of a doublet belonging to high-spin Fe^{2+} species (≈20% of Fe) is discernible at +3 mm/s Doppler velocity. Further experiments will have to address the question of why there is more ferritin in cells containing wild-type FNR than those of the FNR-L28H mutant.

4Fe-FNR Is Restored When Air-exposed Cells Are Shifted Back to Anaerobic Conditions. To test whether the 4Fe → 2Fe cluster conversion is reversible in whole cells, we shifted the air-exposed cells back to anaerobic conditions and compared the spectra of cells kept under anaerobic conditions (Fig. 3*A*, dash marks) to those exposed to air for 15 min and then either frozen in dry ice (Fig. 3 *A* and *B*, solid lines) or sparged with argon for 3 h at 4°C (Fig. 3*B*, dash marks). For the original anaerobic sample, approximately 25% of the iron of the anaerobic sample can be assigned to 4Fe-FNR. Exposure to air converted ≈50% of the 4Fe-FNR into 2Fe-FNR and—after sparging with argon—60% of the newly formed $[2Fe\text{-}2S]^{2+}$ clusters were reconverted to $[4Fe\text{-}4S]^{2+}$ clusters. Thus, these data show that the cluster conversion is essentially reversible under these conditions. As observed above, the spectra of Fig. 3*A* show that ferrous ions (arrow in Fig. 3*A)* are released from 4Fe-FNR upon exposure to air. Furthermore, because [4Fe-4S] cluster rebuilding can occur at 4°C under anaerobic conditions, we cannot exclude the possibility that a fraction of 2Fe-FNR may have been converted into the [4Fe-4S] form during the time when the air-exposed cells were concentrated by centrifugation, because these cells may become oxygen limited. Thus, for the samples of Figs. 2*C* and 3*A*, the yield of 4Fe → 2Fe cluster conversion may have been underestimated.

DISCUSSION

Using Mössbauer spectroscopy, we have shown that cells maintained under anaerobic conditions contain the $[4Fe\text{-}4S]^{2+}$ cluster form of FNR. This is exactly the form of FNR that is purified from anaerobic cells (5), reinforcing the assertion that the 4Fe-FNR is the active form *in vivo*. Furthermore, we have shown that the process of Fe-S cluster conversion, previously observed *in vitro*, also occurs *in vivo* upon exposure to air. The present study thus provides further evidence that FNR senses oxygen directly *in vivo*.

The 4Fe → 2Fe cluster conversion of FNR occurred *in vivo* over approximately the same time scale as we previously have observed *in vitro*. However, contrary to what we have observed *in vitro*, conversion did not appear to be complete after 15 min. This could be caused by the fact that only a slight excess of oxygen over FNR could be achieved during these experiments and, on the basis of the results of Jordan *et al.* (7) with reconstituted protein, we would expect that a 3-fold excess of oxygen per cluster would be required to inactivate all of the FNR. Alternatively, the requirement for centrifugation of cells for the Mössbauer study may have allowed sufficient time for some cluster rebuilding to take place, thus possibly leading to underestimation of the amount of 4Fe → 2Fe cluster conversion.

The method of cell preparation for the studies described here was influenced by the necessity to have cells containing sufficient 4Fe-FNR for a Mössbauer study. Previously, we noted that induction of FNR synthesis in aerobically grown cells followed by sparging of cells with argon at 4°C resulted in the greatest yield of 4Fe-FNR. This observation prompted us to use this growth regimen for the present study to characterize the properties of FNR in whole cells. Although preliminary Mössbauer analysis of *E. coli* cells grown at 37°C showed 4Fe-FNR, the signal-to-background ratios were lower than those of samples analyzed in the present study. In future experiments it might be possible to reduce the background absorption by using mutant strains that are deficient in ferritins (11). On the other hand, the fact that initiation of protein synthesis is prevented at the temperatures used in these studies (4°C; ref. 13) provided us with the opportunity to study cluster conversion under conditions where protein synthesis would be negligible, yet cluster assembly is functional.

The observation of 4Fe → 2Fe cluster conversion in air-exposed cells followed by rebuilding of the 4Fe cluster under anaerobic conditions is consistent with previous *in vivo* results (14) that suggested that FNR activation/inactivation is a reversible process. This previous conclusion was based on the observation that no new protein synthesis was required to observe FNR activation when aerobic cultures were shifted to anaerobic growth conditions (14). We are aware that in the present study, we did not address the question whether in the reconstitution observed *in vivo*, the 4Fe cluster is rebuilt by *de novo* cluster synthesis or by addition of iron and sulfide to the [2Fe-2S] cluster core. Nonetheless, whether it is the 2Fe form or apoprotein (or some combination of both) that is the substrate *in vivo*, activation of FNR will likely require additional proteins that facilitate Fe-S cluster assembly. Such proteins, namely NifS and NifU from *Azotobacter vinelandii*, have been shown to facilitate the assembly of the Fe-S clusters of nitrogenase (15, 16), and homologs of the *nifS* and *nifU* genes exist in *E. coli* (17).

Analysis of the Mössbauer data shows that the 4Fe-FNR originally present in anaerobic cells exists either as 4Fe-FNR or 2Fe-FNR after the cells were exposed to air for 15 min. Thus it appears that none of the 4Fe-FNR has been degraded into apo-FNR upon exposure of cells to air under the experimental conditions used in this study. However, FNR purified from cells that have been continuously aerated does not contain 2Fe clusters (3, 4), suggesting either that 2Fe-FNR is a transient form *in vivo* or that the 2Fe cluster is unstable under the conditions of protein purification. Thus, additional experiments will be necessary to determine whether the $[2Fe\text{-}2S]^{2+}$ cluster of FNR has the same stability *in vivo* as it has *in vitro*. Such information will be necessary to determine the importance of the 2Fe cluster form of FNR in the cycle of activation/deactivation of FNR *in vivo*. Nevertheless, formation of the 2Fe cluster upon exposure to oxygen may have its advantages: it would prevent unnecessary gene expression in that it would make FNR nonfunctional as a transcription activator without fully dismantling its Fe-S cluster. It is possible that generation of 2Fe-FNR is part of a built-in mechanism that allows *E. coli* to adapt rapidly to changes in oxygen tension in its natural habitats.

The studies described above demonstrate that ^{57}Fe Mössbauer spectroscopy is eminently suitable to study *in vivo* the effect of oxygen on the cluster structure of FNR. While the two cluster forms, namely the 4Fe-FNR and 2Fe-FNR, have spec-

tral properties particularly favorable for whole-cell studies, our work has convinced us that it should be possible to extend whole-cell Mössbauer spectroscopy to a variety of other iron-containing proteins. For such studies it is desirable that the spectroscopic behavior of the protein of interest is well known. We have explored which iron-containing proteins—when overexpressed to represent at least 10–15% of the cellular iron—may be suitable for whole-cell Mössbauer studies. We have simulated the spectra of well-studied proteins and added them to background spectra such as those shown in Fig. 2*A* for FNR^- cells. We found that $[2Fe\text{-}2S]^{1+,\ 2+}$, $[4Fe\text{-}4S]^{2+}$, and $[3Fe\text{-}4S]^0$ ferredoxins, as well as proteins such as oxidized and reduced rubredoxins, are amenable to quantitative studies. On the other hand, the paramagnetic $[4Fe\text{-}4S]^{1+,\ 3+}$ and $[3Fe\text{-}4S]^{1+}$ forms yield spectra that would be difficult to discern against the background of other proteins. However, nearly all diamagnetic systems, for instance oxidized di-iron proteins such as *E. coli* ribonucleotide reductase, would be suitable. Studies in strong applied fields at and below temperatures of 4.2 K will considerably facilitate the interpretation of the spectra. In some instances studies may be aided by suppressing the signals of most iron-containing components by growing cells initially with the Mössbauer-silent ^{56}Fe isotope and adding ^{57}Fe at the time of induction. In the present study the spectral background was essentially caused by ferritin and, therefore, signal/background could be improved by decreasing the amount of ferritin by use of suitable mutants (11). Success in decreasing the amount of ferritin iron in *E. coli* also should allow researchers to study the desired protein with lower levels of overexpression. Thus, by optimizing cell growth and overexpression, and by judiciously choosing the conditions for the Mössbauer experiments, a variety of systems should be amenable to whole-cell Mössbauer spectroscopy.

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