Substitution of Leucine 28 with Histidine in the *Escherichia coli* Transcription Factor FNR Results in Increased Stability of the [4Fe-4S]^{2+} Cluster to Oxygen*

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To understand the role of the [4Fe-4S]^{2+} cluster in controlling the activity of the *Escherichia coli* transcription factor FNR (fumarate nitrate reduction) during changes in O\textsubscript{2} availability, we have characterized a mutant FNR protein containing a substitution of Leu-28 with His (FNR-L28H) which, unlike its wild type (WT) counterpart, is functional under aerobic growth conditions. The His-28 substitution appears to stabilize the [4Fe-4S]^{2+} cluster of FNR-L28H in the presence of O\textsubscript{2} because air-exposed FNR-L28H did not undergo the rapid [4Fe-4S]^{2+} to [2Fe-2S]^{2+} cluster conversion or concomitant loss in site-specific DNA binding and dimerization, which are characteristic of WT-FNR under these conditions. This increased cluster stability was not a result of His-28 replacing the WT-FNR cluster ligands because substitution of any of these four Cys residues (cysteine 20, 23, 29, or 122) with Ser resulted in [4Fe-4S]^{2+} cluster-deficient preparations of FNR-L28H. The Mössbauer spectra of FNR-L28H indicated that the coordination environment of the [4Fe-4S]^{2+} cluster did not differ from that of WT-FNR. Whole cell Mössbauer spectroscopy showed that aerobically grown cells overexpressing FNR-L28H had levels of the FNR species containing the [4Fe-4S]^{2+} cluster similar to those of cells grown under anaerobic conditions. Thus, the increase in cluster stability is sufficient to allow accumulation of the [4Fe-4S]^{2+} cluster form of FNR-L28H under aerobic conditions and provides a reasonable explanation for why this mutant protein is functional under aerobic growth conditions. From these results, we present a model to explain how WT-FNR is normally inactivated under aerobic growth conditions.

In recent years, considerable progress has been made in identifying the global regulatory proteins and cofactors that function in biological O\textsubscript{2} sensing pathways. One emerging feature of such systems is that cofactors with well known roles in either O\textsubscript{2} binding (heme and flavins) or in electron transfer (heme, flavins, and Fe-S clusters) also have a regulatory function in O\textsubscript{2} sensing (1–4). To understand further the mechanism of Fe-S cluster-based O\textsubscript{2} sensing, we have been studying the *Escherichia coli* global anaerobic regulator, FNR (5), its function largely in anaerobic respiratory pathways and by repressing expression of genes that are involved in aerobic energy generation (for review, see Refs. 6–9). Our studies have focused on the role of the [4Fe-4S]^{2+} cluster in regulating the activity of FNR in response to changes in O\textsubscript{2} availability (for review, see Ref. 10). The [4Fe-4S]^{2+} cluster is essential for the function of FNR as a transcription factor because through its specific ligation, the [4Fe-4S]^{2+} cluster increases dimerization and DNA binding (11, 12). In addition, the [4Fe-4S]^{2+} cluster is O\textsubscript{2}-labile and, upon exposure of anaerobically purified wild-type WT FNR to air, the [4Fe-4S]^{2+} cluster is converted rapidly to a more O\textsubscript{2}-stable [2Fe-2S]^{2+} cluster (13). Although the [2Fe-2S]^{2+} cluster is the first stable intermediate detected on a minute time scale, the exact pathway for conversion from the [4Fe-4S]^{2+} cluster to the [2Fe-2S]^{2+} cluster in the presence of O\textsubscript{2} remains to be elucidated (10). Nevertheless, the decrease in site-specific DNA binding and in the extent of dimerization that occurs concomitant with cluster conversion provides a reasonable explanation for why FNR is only active as a transcription factor under anaerobic conditions in *vivo* (11, 13, 14). Furthermore, [4Fe-4S]^{2+} to [2Fe-2S]^{2+} cluster conversion in FNR has also been observed in *vivo* following exposure of anaerobically maintained cells to air (15). Thus, inactivation of FNR by O\textsubscript{2}, appears to be mediated by an O\textsubscript{2}-sensitive [4Fe-4S]^{2+} cluster both in *vivo* and in *vivo*. Although these investigations have provided clear insights into how FNR from anaerobic cells is inactivated by O\textsubscript{2}, much less is known about how FNR is maintained in an inactive state under aerobic growth conditions. Although WT-FNR purified from aerobically grown cells has previously been shown to lack any Fe-S cluster (apo-FNR; apo-FNR), it is not known if this apo-FNR arises from the continuous destruction of [4Fe-4S]^{2+} cluster-FNR (4Fe-FNR) or from the lack of FNR [4Fe-4S]^{2+} cluster assembly under these growth conditions. To distinguish between these two possibilities for WT-FNR are not
currently available. Therefore, as an alternative approach to address this question, we have studied a FNR mutant protein that can activate transcription under aerobic growth conditions in contrast to WT-FNR.

In this study, we report the characterization of this FNR mutant protein, which contains an amino acid substitution of Leu-28 with His (FNR-L28H, (18)). Leu-28 is adjacent to Cys-29, one of the four Cys residues generally accepted to be the ligands of the [4Fe-4S]²⁺ cluster (19, 20), although this assignment awaits structural confirmation. Previous studies have shown that FNR-L28H is active in vivo in the presence of air only when all of these four cysteine residues are present (21), suggesting that the L28H substitution might increase the stability of the Fe-S cluster to O₂. To test this hypothesis, we purified the FNR-L28H protein under anaerobic conditions and found that the L28H substitution stabilizes the [4Fe-4S]²⁺ cluster in the presence of O₂. This property of FNR-L28H also allowed us to show that assembly of the [4Fe-4S]²⁺ cluster of FNR-L28H occurs under aerobic growth conditions, suggesting that formation of the O₂ sensitive [4Fe-4S]²⁺ cluster in WT-FNR can also occur under aerobic growth conditions.

**EXPERIMENTAL PROCEDURES**

**Plasmid and Strain Construction—**Cysteine to serine substitutions at amino acid positions 20, 23, and 29 were created by site-directed mutagenesis (22). Single-stranded, uracil-containing DNA of pPK1887 (p-fnr-L28H, Ref. 15) was obtained from the dut− ung− strain, K1032 (22). Oligonucleotides were synthesized which change base 57, 66, and 84 (base 1 is the A of the initiation codon of p-fnr-L28H) from T to A, creating C20S, C23S, and C29S, respectively. A NdeI BamHI fragment from each of the resulting plasmids was then cloned into the same sites of the expression vector pET-11a (23) to generate pPK2024 (p-fnr-L28H-C20S), pPK2025 (p-fnr-L28H-C23S), and pPK2022 (p-fnr-L28H-C29S). NdeI-BamHI digests of pPK1868 (Ref. 16) (p-fnr-L28H) were cloned into the same sites of the expression vector pET-11a, creating pPK2011 and pPK1868, respectively. An expression vector harboring the allele fnr-L28H-C20S, pPK2024 (p-fnr-L28H-C23S), and pPK2013 (p-fnr-L28H-C29S). Cells were grown aerobically at 37 °C in 4 liters of glucose-M9 minimal medium (24) that also contained 20 m M KCl, and the appropriate fractions were pooled.

**Growth Conditions and Protein Purification—**FNR proteins were purified from E. coli B strain PK22 (BL21 Δrep-1 lacZΔ proAB) harboring pPK823 (p-fnr-L28H, Ref. 15) (p-fnr-L28H), pPK1868 (p-fnr-L28H, Ref. 16) (p-fnr-L28H-C20S), pPK2024 (p-fnr-L28H-C23S), pPK2025 (p-fnr-L28H-C29S), and pPK2013 (p-fnr-L28H-C23S). Cells were grown aerobically at 37 °C in 4 liters of glucose-M9 minimal medium (24) that also contained 20 µM ferric ammonium citrate. When the culture reached an A₆₅₀ of 0.3−0.5, FNR synthesis was induced by the addition of isopropyl-thio-galactoside (final concentration of 400 µM) for 1 h, and then the culture was sparged with argon for 4 °C to deplete O₂ from the growth medium and promote Fe-S cluster assembly (13, 15). To minimize exposure of 4Fe-FNR to O₂, all subsequent manipulations were carried out either under a stream of copper-scraped argon gas or in an anaerobic chamber (Coy) which had an atmosphere of 5% CO₂, 5% H₂, and 90% N₂.

Harvesting of the cells and preparation of anaerobic cell extracts were carried out as described previously (11) except that the resuspension buffer also contained 1 mM dithiothreitol, and the extract was centrifuged in a Beckman 70.1 Ti rotor for 1 h at 45,000 rpm. The supernatant was then passed through a filter (0.2-µm pore size, Nalgene) and loaded at a flow rate of 0.5 ml/min onto a PolyCAT A HPLC column (9.4 × 200 mm, Polylc) that was connected to a Beckman Nouveau Gold system and maintained at 5 °C. The column was washed with 2 column volumes of anaerobic buffer A (50 mM KPO₄, pH 6.8, 10% glycerol, 0.1 mM KCl) containing 1 mM dithiothreitol, 1.7 mM dithionite, and 0.1 mM phenylmethanesulfonyl fluoride, followed by anaerobic buffer A alone (~3 column volumes). FNR was eluted from the column in a linear gradient from 1:1 to 10 mM KCl in anaerobic buffer A at a flow rate of 1.5 ml/min. Fractions containing 4Fe-FNR were identified by the characteristic absorption spectrum using an on-line multichannel scanning array detector (190–600 nm); the peak of 4Fe-FNR typically was eluted at ~300 mM KCl, and the appropriate fractions were pooled. HPLC-purified FNR was estimated to be >95% pure as judged by SDS-polyacrylamide gel electrophoresis. For Mossbauer studies on purified FNR-L28H, ⁵⁷Fe-enriched FNR-L28H was obtained in the same way except PK22 cells containing pPK1886 were grown aerobically in ⁵⁷Fe-enriched glucose minimal medium (13), and the pooled fractions were concentrated over a 1-ml Biorex 70 column as described previously (13).

For purification of FNR-L28H from aerobic cells, PK22 cells containing pPK1886 were grown in glucose minimal medium as described above except that the cells were harvested immediately after the 1-h period of FNR synthesis. The cell pellet was resuspended in anaerobic buffer A, containing phenylmethanesulfonyl fluoride and dithiothreitol but lacking dithionite, and the subsequent purification steps were carried out under anaerobic conditions as described above to prevent any potential loss of the [4Fe-4S]²⁺ cluster from FNR-L28H during purification.

**Whole Cell Mossbauer Spectroscopy—**PK22 strains containing plasmid pPK823 (WT-FNR; Ref. 11) or pPK1868 (FNR-L28H) were grown in ⁵⁷Fe-enriched glucose minimal medium (15) under either aerobic or anaerobic growth conditions, and FNR synthesis was induced by the addition of isopropyl-thio-galactoside for 1 h. The whole cell samples were prepared for Mossbauer analysis as described previously (15) with the following modifications. The cells were harvested at 4 °C directly after the 1-h period of FNR synthesis, the buffer for washing the cells was prechilled on ice, and for the anaerobically grown cultures, all sample handling was done in air. In contrast to our previous experiments where cells were sparged with argon for 14 h at 37 °C to increase the yield of ⁴Fe-FNR (15), the induction conditions used here were designed to assay the amount of Fe-S cluster assembly under better defined growth conditions.

**O₂-induced Degradation of the Fe-S Cluster—**A 1-ml sample of anaerobically purified FNR-L28H protein (242 µg) was placed in a 1.7-ml glass conical tube and stirred slowly in air at 18 °C as described previously (13) and then assayed for sulfide and DNA binding as indicated below. UV-visible spectra were recorded on protein samples at 25 °C by employing a Lambda 2 UV-visible spectrophotometer (Perkin-Elmer). Samples were kept anaerobic by placing them in a cuvette sealed with a Teflon cap. To expose FNR to air, the Teflon cap on the anaerobic cuvette was removed temporarily, and the sample (35 µl) was mixed with air by gently inverting the cuvette two or three times at periodic intervals.

**Iron, Sulfide, Protein, and Optical Determinations—**Iron (25) and sulfide (26) were analyzed in duplicate. Protein concentrations (reported here for monomers) were determined by a biuret procedure as described previously (13). To determine extinction coefficients at 406 nm (ε₄₀₆) and 278 nm (ε₂₇₈), anaerobically purified FNR-L28H, containing 2.84 and 4.42 mol of Fe/mol of FNR, was hydrolyzed by acid, and the concentration of amino acids was measured by HPLC (Protein Facility, Medical College of Wisconsin). The UV-visible spectrum of anaerobically purified FNR-L28H was recorded, and the calculated values for ε₄₀₆ and ε₂₇₈ were 17.3 ± 1 µM⁻¹ cm⁻¹ and 39 ± 2 µM⁻¹ cm⁻¹, respectively.

**DNA Binding Assay—**Site-specific DNA binding by FNR-L28H to a 48-bp target fragment containing the consensus FNR DNA binding sequence (27) was measured using a gel retardation assay as described previously (13). The fraction of the total amount of radioactively labeled DNA (57 µs) bound over a range of protein concentrations was quantified by use of a PhosphorImager (Molecular Dynamics). The amount of protein required to bind 50% of the maximally bound target DNA was calculated as described (13).

**Gel Exclusion Chromatography—**To determine the apparent molecular weight of FNR-L28H, 200 µl of either anaerobic or air-exposed FNR-L28H (20 µg) was passed over a Superose 12 HR (Amersham Pharmacia Biotech) column (10 × 50 mm) at a flow rate of 0.5 ml/min. Air-exposed FNR-L28H was obtained by slowly stirring ~400 µl of protein (150 µg FNR-L28H) in a 1.7-ml conical glass in air at 18 °C for 1 h and then diluted prior to gel filtration analysis. The column was maintained within the anaerobic chamber and was equilibrated with anaerobic buffer (50 mM KPO₄, pH 6.8, 10% glycerol, and 0.4 mM KCl), and the absorbance of the column effluent was monitored between 190 and 600 nm. Bovine serum albumin, carbonic anhydrase, and cytochrome c protein standards (Sigma) were used to calibrate the column.

**RESULTS**

The [4Fe-4S]^{2-} Cluster of FNR-L28H Shows Increased Stability to O₂ in Vitro—The observation that FNR-L28H, unlike WT FNR, is able to activate transcription under aerobic growth conditions (18, 21) suggested that the [4Fe-4S]²⁻ cluster in FNR-L28H might be less sensitive to O₂ than WT-FNR. To test...
this possibility directly, we examined the stability of the [4Fe-4S]$^{2+}$ cluster of purified FNR-L28H by comparing the UV-visible spectrum before and after exposure to air. Like WT-FNR (13), the UV-visible spectrum of anaerobically purified FNR-L28H had an absorption maximum centered at 278 nm, a shoulder at 315 nm, and a broad absorbance centered at 409 nm, characteristic of a protein containing a [4Fe-4S]$^{2+}$ cluster (Fig. 1). Upon exposure of the anaerobically purified FNR-L28H protein to air, no changes in the absorbance spectrum were observed until 60 min, when a slight (~5%) decrease in the absorbance at 409 nm was observed (Fig. 1). This minor effect on the absorption spectrum is in sharp contrast to what was observed previously for WT-FNR where a much larger decrease in absorbance at 409 nm (~50%) was observed after only 10 min of air exposure (13), suggesting that the [4Fe-4S]$^{2+}$ cluster of FNR-L28H is more stable to air than WT-FNR.

Exposure of FNR-L28H to air for 60 min did not yield a red color, nor did it have any of the visible absorption features at 315 nm, nor did it have any of the visible absorption features at 278 nm (Fig. 1). Upon exposure of the anaerobically purified FNR-L28H protein to air, no changes in the absorbance spectrum were observed until 60 min, when a slight (~5%) decrease in the absorbance at 409 nm was observed (Fig. 1). This minor effect on the absorption spectrum is in sharp contrast to what was observed previously for WT-FNR where a much larger decrease in absorbance at 409 nm (~50%) was observed after only 10 min of air exposure (13), suggesting that the [4Fe-4S]$^{2+}$ cluster of FNR-L28H is more stable to air than WT-FNR.

Comparison of the Fe:S$^{2-}$:protein ratios from many independent preparations of WT-FNR and FNR-L28H also revealed a higher average Fe-S cluster occupancy for the FNR-L28H protein preparations. Anaerobically purified FNR-L28H contained an average of 3.59 ± 0.49 mol of Fe ($n = 32$) and 3.03 ± 0.49 mol of S$^{2-}$ ($n = 33$) of FNR monomer, indicating that, on average, 76% of the protein was occupied with a [4Fe-4S]$^{2+}$ cluster on the basis of the S$^{2-}$ concentration. Anaerobically purified WT-FNR contained an average of 3.12 ± 0.52 mol of Fe ($n = 10$) and 2.69 ± 0.49 mol of S$^{2-}$ ($n = 13$) of FNR, indicating that on average, 67% of the protein is occupied with a [4Fe-4S]$^{2+}$ cluster, on the basis of the S$^{2-}$ concentration. The higher average occupancy of FNR-L28H suggests that there may be a relationship between cluster stability and occupancy in these purified preparations.

**DNA Binding by FNR-L28H Is Retained upon Exposure to O$_2$**—To analyze the effects of increasing the stability of the [4Fe-4S]$^{2+}$ cluster on FNR function, the amount of site-specific DNA binding by FNR-L28H was assayed in *vitro*, using a gel retardation assay, before and after air exposure. In the absence of O$_2$, FNR-L28H formed a DNA-protein complex of the same apparent electrophoretic mobility, as did WT-FNR (11, 13). The concentration of FNR-L28H required to bind 50% of the DNA was 4–8 nM, similar to that observed for WT-FNR (Fig. 3) (13). However, exposure of FNR-L28H to air did not result in a rapid decrease in site-specific DNA binding as seen with WT-FNR (13). Even after 60 min of air exposure, only a small (2-fold) reduction in the apparent affinity for the DNA target site was observed compared with a >100-fold decrease observed with WT-FNR after 8 min (Fig. 3) (13). Thus, the increased stability of the [4Fe-4S]$^{2+}$ cluster to O$_2$ allows FNR-L28H to retain site-specific DNA binding activity in the presence of air.

**The Stability of the [4Fe-4S]$^{2+}$ Cluster Affects Dimerization of FNR**—Because O$_2$-induced dimer dissociation of WT-FNR appears to be mediated by the disruption of the [4Fe-4S]$^{2+}$ cluster (11), the increase in cluster stability of FNR-L28H predicts that its size should be largely unaffected by air. Molecular sieve chromatography of anaerobically purified FNR-L28H showed that the fraction of FNR which contained a [4Fe-4S]$^{2+}$ cluster (monitored at 410 nm) was eluted from the gel filtration column with a retention time of 26.9 ± 0.2 min, corresponding to an apparent molecular mass of 66,000 (Fig. 4, *dashed line*). This is approximately the size expected for an FNR dimer (60,000 Da). After a 1-h exposure of FNR-L28H to air, the relative mobility of the protein did not change significantly (retention time equal to 26.8 ± 0.2 min), indicating very little dissociation to monomers (Fig. 4, *solid line*). In contrast, the majority of WT-FNR was eluted as a monomer after just 1 min of O$_2$ exposure (11). Thus, these data demonstrate that stabilization of the [4Fe-4S]$^{2+}$ cluster, as in FNR-L28H, results in a significant decrease in the O$_2$-induced dimer dissociation, confirming that the [4Fe-4S]$^{2+}$ cluster is a key element in the dimerization of FNR.

**Substitution of Histidine for Leucine at Position 28 Does Not Replace Any of the Ligands for the [4Fe-4S]$^{2+}$ Cluster**—Because [4Fe-4S] clusters of some proteins, such as the iron-only hydrogenase from *Clostridium pasteurianum* (28), are ligated by a His residue, we considered the possibility that His-28 in FNR-L28H may alter the stability of the [4Fe-4S]$^{2+}$ cluster by providing an alternate ligand for the iron in the cluster. However, two lines of evidence suggest that the [4Fe-4S] cluster of FNR-
LH28 has the same four ligands as WT-FNR, indicating that ligand replacement is not likely.

First, Fig. 5B shows the 4.2 K Mössbauer spectra of the WT-FNR protein (hatch marks) and L28H variant (solid line). It can be seen that the spectra are essentially identical, suggesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 suggesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 indicating that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 gesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 gesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 gesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 gesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 gesting that the clusters in both proteins have the same coordination, most probably (Cys)4. In Fig. 5 Cysteines (cysteines 20, 23, 29, and 122) that are generally accepted to be the cluster ligands in the WT protein (19, 20) were individually replaced by a serine residue. If His-28 replaced any of the cysteine ligands, then one of these cysteine residues should be dispensable for association of the [4Fe-4S]2+ cluster in FNR-L28H. However, after anaerobic purification, none of the FNR-L28H mutant proteins containing a serine substitution at position 20, 23, 29, or 122 had significant amounts of inorganic sulfide or iron, absorption in the visible range, or DNA binding activity in vitro (data not shown), indicating that all of the cysteine residues are required for [4Fe-4S]2+ cluster association in FNR-L28H. Thus, it is unlikely that His-28 replaces a cluster ligand. These data also provide additional support for the role of cysteines 20, 23, 29, and 122 in ligation of the [4Fe-4S]2+ cluster as was suggested previously by the in vivo phenotype of the single substitutions (19–21) and are consistent with equivalent coordination of the iron sites as described above.

A [4Fe-4S]2+ Cluster Can Be Assembled into FNR-L28H under Aerobic Conditions—Our data indicate that the [4Fe-4S]2+ cluster of FNR-L28H has remarkable stability to O2, unlike the [4Fe-4S]2+ cluster in WT-FNR (13). Thus, it is possible that the aerobic activity (i.e., transcription activation) exhibited by FNR-L28H in vivo can be explained by the presence of the [4Fe-4S]2+ cluster under these conditions. To test this hypothesis, we studied with Mössbauer spectroscopy cells grown either under aerobic or anaerobic conditions where FNR-L28H was overexpressed for 60 min prior to cell harvesting. The Mössbauer spectra of both aerobic and anaerobic cells showed a central doublet that has parameters identical to those of the [4Fe-4S]2+ cluster of purified FNR-L28H (Fig. 6). Because our previous Mössbauer study of E. coli cells expressing WT-FNR and FNR-L28H (15) showed that a doublet with these parameters cannot arise from a cluster belonging to a protein other than FNR, we conclude that this doublet feature represents the contribution of the [4Fe-4S]2+ cluster in FNR-L28H. Quantitative evaluation of this doublet in cells expressing FNR-L28H shows that in either aerobic or anaerobically grown cultures, between 5 and 7% of the total iron in these samples is contained in the [4Fe-4S]2+ clusters of FNR. For illustration, the solid line in Fig. 6 shows a simulation of the spectrum of the isolated...
FNR-L28H scaled to represent 5% of the total absorption of this sample, superimposed on the data from the whole cells overexpressing this mutant protein. Because the total absorption in these spectra is associated with similar contents of iron, as shown by the iron analysis of sample aliquots (data not shown), we can conclude that the amount of 4Fe-FNR-L28H present in aerobic cells is similar to that of anaerobic cells using these growth conditions.

As an independent test for the presence of a [2Fe-2S]2+ cluster, FNR-L28H in aerobically grown cells, we tested whether FNR-L28H would retain a Fe-S cluster following purification from aerobically grown cells where all isolation steps were carried out anaerobically to avoid any potential loss of Fe-S cluster. FNR-L28H purified in this manner contained 2.14 mol of Fe and 2.16 mol of S2−/mol of FNR monomer (−50% [4Fe-4S]2+ cluster occupancy). These results along with the whole cell Mössbauer study demonstrate that FNR-L28H contains a [4Fe-4S]2+ cluster under aerobic growth conditions. Thus, the observation that FNR-L28H is able to activate transcription under aerobic growth conditions is explained most simply by the fact that this mutant protein contains a [4Fe-4S]2+ cluster, and it also supports the notion that FNR Fe-S cluster assembly can occur in vivo in the presence of O2.

**DISCUSSION**

Studies of FNR mutants that are altered in O2 regulation have been an integral part of establishing the role of a [4Fe-4S] cluster in regulating FNR activity (10, 21, 31). In this study, we have characterized the properties of the FNR− mutant, FNR-L28H, which activates transcription under aerobic growth conditions in contrast to WT-FNR (18). The properties of FNR-L28H described here have reaffirmed the key role of the [4Fe-4S]2+ cluster in forming an active FNR protein. Furthermore, our results show that the [4Fe-4S]2+ cluster of FNR-L28H can be assembled under aerobic growth conditions. As discussed below, this discovery has allowed us to extend our working model for how the activity of FNR is regulated by O2 in vivo. In particular, we discuss the possibility that for WT-FNR, O2-mediated turnover of the [4Fe-4S]2+ cluster of 4Fe-FNR keeps the protein in an inactive state under aerobic growth conditions.

**Stabilization of the [4Fe-4S]2+ Cluster of FNR-LH28—**FNR-L28H has a [4Fe-4S]2+ cluster that, by Mössbauer spectroscopy, is indistinguishable from WT-FNR under anaerobic conditions. However, in contrast to the rapid [4Fe-4S]2+ to [2Fe-2S]2+ cluster conversion that is seen with WT-FNR upon air exposure (−50% of 2Fe-FNR formed within 2 min (13)), our results show that substituting Leu-28 with His significantly alters the stability of the [4Fe-4S]2+ cluster of FNR-L28H. The increase in cluster stability of FNR-L28H appears to arise from His-28 influencing the process of [4Fe-4S]2+ to [2Fe-2S]2+ cluster conversion (Fig. 7) because cluster conversion occurred more slowly in air-exposed samples of 4Fe-FNR-L28H (−4% 2Fe-FNR formed within 60 min; data not shown). Although the simplest interpretation of these results is that His-28 acts directly to stabilize the [4Fe-4S]2+ cluster toward O2, our data cannot rule out the possibility that His-28 stabilizes an as yet unknown intermediate in the conversion of the [4Fe-4S]2+ to [2Fe-2S]2+ cluster, such as the [4Fe-4S]3+ or [3Fe-4S]1+ clusters observed with other proteins containing oxidizable [4Fe-4S]1+ clusters (32, 39); we have yet to look at very rapid events.

**Role of Histidine 28 in Stabilization of the Fe-S Cluster in FNR-L28H—**Our data indicate that His-28 does not functionally replace any of the cysteine residues as ligands to the Fe-S cluster. Rather, it is possible that the presence of the His-28 side chain in the immediate proximity of the [4Fe-4S] cluster alters the environment of the Fe-S cluster. The observation that amino acid side chains near the cluster ligands affect the stability of Fe-S clusters is not surprising given that model Fe-S clusters that are synthesized in the absence of protein ligands are degraded rapidly by exposure to air (33), whereas Fe-S clusters within proteins can be quite stable. Furthermore, substitution of aromatic amino acid residues that are in close proximity to the [4Fe-4S]2+ cluster of the high potential iron protein, HiPIP, have been shown to increase the accessibility of the cluster to O2 and promote the oxidative degradation of the [4Fe-4S]2+ cluster to a [3Fe-4S]1+ cluster (34). In agreement with these observations, recent work with model peptides has shown that hydrophobic amino acid residues neighboring the cluster ligands increase the stability of a [4Fe-4S] cluster (35). Because we are replacing a leucine with a histidine in our studies of FNR-L28H, this effect of hydrophobic residues is unlikely to explain the properties of our FNR mutant. Nevertheless, such studies highlight the importance of the protein environment in Fe-S cluster stability.

In considering how His-28 might alter cluster stability at the molecular level, the ferredoxin I structure from *Azotobacter vinelandii* (38) was used for molecular modeling because Leu-28 to Cys-29 of FNR would be equivalent to Leu-44 to Cys-45 of ferredoxin I (data not shown). Replacing Leu-44 with His in the ferredoxin I structure indicates that the imidazole ring of the His may be capable of forming a hydrogen bond with a cluster sulfide (estimated bond length 2.72 Å). Thus, if the structure of the region around the FNR [4Fe-4S] cluster is similar to ferredoxin I, it is possible that the His-28 side chain...
may provide a hydrogen bond to the cluster which would increase its stability.

The finding that the side chain composition near the [4Fe-4S]²⁺ cluster of E. coli FNR can dramatically alter cluster stability leads one to consider whether other FNR homologs show the same sensitivity to O₂ as E. coli FNR because there is significant variation of the amino acid residues around the Cys ligands (36). Furthermore, several other mutations (D22G (18), Q27R (37), D22S and S17a (20)) within the NH₂-terminal region also increase activity of E. coli FNR under aerobic growth conditions. Although none of these substitutions have yet been tested as to whether they increase the stability of the [4Fe-4S] cluster, their close proximity to the Cys ligands makes this an intriguing possibility.

**Similarity of FNR-L28H to FNR-L28H-D154A**—The increase in stability of the [4Fe-4S]²⁺ cluster by the His-28 substitution most likely explains why previous studies of a mutant protein containing both the L28H substitution, as well as an aspartate to alanine substitution at position 154 (FNR-L28H-D154A), exhibited low amounts of a Fe-S cluster when purified aerobically (31), although the major type of cluster that was present was not established by Mössbauer spectroscopy. In contrast, an FNR mutant protein containing the D154A substitution alone lacks any detectable Fe-S cluster when purified in the presence of O₂ (16), suggesting that the D154A substitution does not promote cluster stabilization. Whether the mutant FNR protein having both the L28H and D154A substitutions may have some properties that are different from those containing only the single amino acid changes remains to be determined.

**Increasing the Stability of the [4Fe-4S]²⁺ Cluster Generates an Air-tolerant FNR Protein with All of the Properties of Active WT-FNR**—Previously, we and others have demonstrated that the presence of a [4Fe-4S]²⁺ cluster in WT-FNR was correlated with an increase in dimerization (11) and site-specific DNA binding (11, 12). The failure of FNR-L28H dimers to dissociate upon air exposure supports the previous conclusion that the [4Fe-4S]²⁺ cluster was responsible for generating the dimeric form of FNR because exposure of WT-FNR to air rapidly disrupts the [4Fe-4S]²⁺ cluster concomitantly with its conversion to an inactive monomeric form (13). Furthermore, the data presented here indicate that the site-specific DNA binding properties of FNR-L28H under aerobic conditions can simply be attributed to the increased stability of the Fe-S cluster, suggesting that the [4Fe-4S]²⁺ cluster is the key component required for DNA binding activity of FNR (Fig. 7).

**FNR-L28H in Aerobically Grown Cells Contains a [4Fe-4S]²⁺ Cluster**—We also found that aerobically grown cells overexpressing FNR-L28H contain amounts of 4Fe-FNR similar to those grown under anaerobic conditions, indicating that FNR-L28H contains a [4Fe-4S]²⁺ cluster under aerobic growth conditions. This result explains why cysteine to serine acid replacements of two of the generally accepted cluster ligands abolished the activity of FNR-L28H in vivo (21). Thus, the demonstration that FNR-L28H contains a [4Fe-4S]²⁺ cluster under aerobic growth conditions led us to the important conclusion that Fe-S cluster assembly in FNR can occur under aerobic growth conditions.

**Implications for the Regulation of WT-FNR; O₂-mediated Turnover of 4Fe-FNR under Aerobic Growth Conditions**—Because the machinery for [4Fe-4S]²⁺ cluster formation of FNR is functional under aerobic conditions, the lack of WT-FNR activity under aerobic growth conditions cannot be simply explained by failure to assemble 4Fe-FNR. Rather, we propose that the [4Fe-4S]²⁺ cluster is assembled into WT-FNR under aerobic conditions but that 4Fe-FNR is inactivated rapidly because of the conversion of the [4Fe-4S]²⁺ cluster to a [2Fe-2S]²⁺ cluster which occurs when 4Fe-WT-FNR encounters O₂ (13) (Fig. 7). The fate of 2Fe-FNR formed in vivo under these conditions remains to be determined, but it is also likely that some 2Fe-FNR is converted to apo-FNR because this is the major form of FNR purified from aerobically grown cells (16, 17).

To test this model, we would have to determine the Fe-S cluster species present in WT-FNR from aerobically grown cells. However, the method for whole cell Mössbauer spectroscopy used in this study did not have the sensitivity to permit detection of WT-FNR even under anaerobic growth conditions (data not shown), although we know that 4Fe-WT-FNR can be purified from such anaerobic cells (11). Consistent with this notion, we previously found that when FNR induction is followed by sparging with argon overnight at 4 °C, the amount of 4Fe-FNR in whole cells overexpressing WT-FNR was approximately one-half to one-third less than that observed with FNR-L28H (15). Because we estimate that ~3% or more of the total Fe-S cluster remains to be present in FNR for detection by whole cell Mössbauer and ~5–7% of the total Fe-S was found in 4Fe-FNR from anaerobically grown cells containing FNR-L28H under the induction conditions used in this study, it appears that we are just below the limits to detect 4Fe-WT-FNR in anaerobically grown cells in this study. Experiments are in progress to improve our ability to detect iron-containing species of lower abundance to address these important questions. Nevertheless, it is clear from the results presented here as well as from those of a previous study (15) that the [4Fe-4S]²⁺ cluster of FNR-L28H accumulates to higher levels in anaerobic cells than that of WT-FNR. Whether the increase in stability of the [4Fe-4S]²⁺ cluster of FNR-L28H is sufficient to explain this observation remains to be determined.

In conclusion, we have shown that substitution of Leu with His at position 28 in FNR increases the stability of the [4Fe-4S]²⁺ cluster to O₂. As a consequence, the [4Fe-4S]²⁺ cluster of FNR-L28H has lost its regulatory function under aerobic growth conditions and therefore no longer acts as an effective O₂ sensor. The greater stability of the [4Fe-4S]²⁺ cluster also explains why the dimerization and DNA binding properties of FNR-L28H did not decrease rapidly following air exposure as observed with WT-FNR. These results reaffirm that FNR activity depends on the presence of a [4Fe-4S]²⁺ cluster and support the notion that stability of the [4Fe-4S]²⁺ cluster regulates the activity of FNR in response to O₂.

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