DNA Binding and Dimerization of the Fe-S-containing FNR Protein from Escherichia coli Are Regulated by Oxygen*

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The transcription factor FNR from Escherichia coli regulates transcription of genes in response to oxygen deprivation. To determine how the activity of FNR is regulated by oxygen, a form of FNR had to be isolated that had properties similar to those observed in vivo. This was accomplished by purification of an FNR fraction which exhibited enhanced DNA binding in the absence of oxygen. Iron and sulfide analyses of this FNR fraction indicated the presence of an Fe–S cluster. To determine the type of Fe–S cluster present, an oxygen-stable mutant protein LH28-DA154 was also analyzed since FNR LH28-DA154 purified anoxically contained almost 3-fold more iron and sulfide than the wild-type protein. Based on the sulfide analysis, the stoichiometry (3.3 mol of S2-/FNR monomer) was consistent with either one [4Fe–4S] or two [2Fe–2S] clusters per mutant FNR monomer. However, since FNR has only four Cys residues as potential cluster ligands and an EPR signal typical of a 3Fe–4S cluster was detected on oxidation, we conclude that there is one [4Fe–4S] cluster present per monomer of FNR LH28-DA154. We assume that the wild type also contains one [4Fe–4S] cluster per monomer and that the lower amounts of iron and sulfide observed per monomer were due to partial occupancy. Consistent with this, the Fe–S cluster in the wild-type protein was found to be extremely oxygen-labile. In addition, molecular-sieve chromatographic analysis showed that the majority of the anoxically purified protein was a dimer as compared to aerobically purified FNR which is a monomer. The loss of the Fe–S cluster by exposure to oxygen was associated with a conversion to the monomeric form and decreased DNA binding. Taken together, these observations suggest that oxygen regulates the activity of wild-type FNR through the lability of the Fe–S cluster to oxygen.

The ability to sense and adapt to changes in oxygen concentration is critical to the survival of many organisms. In Escherichia coli, the transcription factor FNR plays a central role in allowing the bacterium to adapt to changes in oxygen availability in its environment (1). When oxygen is limiting, FNR activates synthesis of many enzymes required to generate energy by anaerobic respiration and also represses synthesis of some enzymes involved in aerobic respiration. Since the levels of FNR do not significantly differ between aerobically and anaerobically grown cells, oxygen deprivation must modulate FNR-dependent transcription by regulating the activity of FNR (2).

Determining how oxygen regulates FNR activity should provide fundamental information regarding the strategies available to cells to sense changes in oxygen levels.

The isolation and analysis of FNR mutant proteins that are active in the presence of oxygen (FNRi) has provided important insights as to how wild-type FNR activity might be regulated by oxygen (3–5). For example, the in vitro analysis of one FNRi mutant protein, FNR-D1A54 which has an Asp to Ala substitution at position 154, led to the initial proposal that oxygen regulates the dimeric state of FNR. When purified in the presence of oxygen, FNR-D1A54 protein showed increased dimerization (3) and DNA binding (3, 5) relative to the monomeric wild-type protein (3, 6). This suggested that wild-type FNR becomes active under anaerobic conditions because of an increase in the formation of FNR dimers that presumably have maximal DNA binding affinity. In addition, the analysis of another FNRi mutant protein, FNR-LH28-DA154 which contains a Leu to His substitution at position 28 in addition to the DA154 substitution, identified a cofactor, an Fe–S cluster. This Fe–S cluster was associated with aerobically purified FNR-LH28-DA154 protein, and the association of this cluster enhanced DNA binding by the mutant protein (4).

Taken together, the results from the analysis of the FNRi mutant proteins led us to consider that, under anaerobic conditions, wild-type FNR contains an Fe–S cluster that increases dimerization and DNA binding. Consistent with this proposal, wild-type FNR requires Fe2+ for activity in vivo (7, 8), and FNR possesses four cysteines that are essential for FNR function and that could serve as potential ligands for an Fe cofactor (9–11). However, previously purified wild-type FNR did not contain an Fe–S cluster but contained Fe3+ at a stoichiometry of up to 1 mol of Fe3+ per monomer of FNR (6). This Fe3+ appeared to be loosely associated since its presence depended on providing Fe2+ in the purification buffer. Although Fe2+ bound to FNR in this way had little effect on DNA binding and FNR was still monomeric (6), some functional significance was attributed to the presence of this loosely bound Fe2+, because this form of FNR was reported to increase open complex formation at an FNR-dependent promoter (12, 13). To reconcile the apparent differences in the type of Fe cofactor associated with wild-type FNR and the FNRi mutant protein, we have...
considered that wild-type FNR may contain an oxygen-labile Fe–S cluster which is required for increased DNA binding and dimerization in the absence of oxygen, and that the Fe2+ found associated with purified wild-type FNR may be a remnant of a labile cluster.

A potential reason for the different properties of purified FNR* proteins and wild-type protein is that, until recently, a reliable functional assay was lacking to monitor purification of FNR. Thus, previously isolated FNR may have lost a component(s) during purification, such as an Fe–S cluster, necessary for maximum FNR activity. Therefore, we developed an assay to monitor DNA binding by FNR in cell extracts in the presence and absence of oxygen, because analysis of the FNR* mutants suggested that DNA binding by wild-type FNR should be regulated by oxygen. Using this assay, we purified and characterized a form of FNR which exhibited increased DNA binding under anoxic conditions. This preparation of FNR was also found to have an increased ability to dimerize and contained a Fe–S cluster.

MATERIALS AND METHODS

Anoxic Sample Handling—To minimize any exposure to oxygen, samples were manipulated either under a stream of copper-scrubbed argon gas or in an anaerobic chamber (Coy) which had an atmosphere of approximately 5% CO2, 5% H2, and 90% N2. Buffers were sparged with argon gas and then allowed to stand in an anaerobic chamber for at least 16 h. All sample-handling devices and test tubes were rinsed with anoxic buffers containing the reductant sodium dithionite. When it was necessary to work with protein samples lacking dithionite, this reductant was removed from all sample-handling materials by rinsing with anoxic buffer lacking dithionite.

DNA Binding Assay—The extent of DNA binding by FNR was assessed by a gel-retardation assay essentially as described by Lazazzera et al. (3). Briefly, protein was mixed in a 10-ml volume to achieve a final concentration of 0.1 g/ml bovine serum albumin (Pierce), 30 μg/ml calf thymus DNA, and 5% glycerol (Fisher, enzyme grade). When the assays were performed anoxically, sodium dithionite (Fluka, MicroSelect grade) (570 mmoles in 0.1 ml Tris-cl (pH 7.9)) was added to a final concentration of 1.7 mM. Following incubation at 37°C for 10 min, the reaction mixture was loaded onto a 10% polyacrylamide gel (38:2 acrylamide/bis-acrylamide ratio) in Tris borate-EDTA buffer (14), which had been electrophoresed for 30 min prior to loading, and was then run at 150 V for an hour. The amount of DNA bound by FNR was quantitated using a PhosphorImager from Molecular Dynamics.

Optical Spectroscopy—To remove dithionite from the protein for recording of spectra, the protein solution was diluted in buffer [10 mM KPO4 (pH 6.8), 10% glycerol] to achieve a final concentration of 0.1 mM KCl. This diluted protein was loaded on a one ml Bio-Rex 70 column which had been equilibrated with the same buffer. The protein was eluted from the column with one ml of buffer containing 0.8 M KCl. Spectra were recorded using a Lambda 2 spectrophotometer (Perkin-Elmer); anaerobiosis was maintained by capping cuvettes which were filled in an anaerobic chamber.

Iron and Sulfide Analysis—Iron and sulfide were determined, as described in Kennedy et al. (18) and Beinert (19) respectively, on three independent samples concentrated as described above for optical spectroscopy and analyzed in duplicate. To verify the formation of methylene blue from 5-′- and p-phenylenediamine, the ratio of absorbancies at 670, 710, and 750 nm was regularly measured when the color of methylene blue was not obvious to the eye (OD670 < 0.160 at a 1-cm path length) (19).

RESULTS

DNA Binding of FNR Is Enhanced under Anoxic Conditions—To develop an in vitro assay for detecting oxygen regulation of FNR activity, we chose to monitor changes in DNA binding by FNR, since previous analysis of FNR* mutant proteins (3–5) suggested that DNA binding of wild-type FNR should be increased under anoxic conditions. FNR was assayed for DNA binding by a gel-retardation assay. Using an extract prepared from anaerobically grown cells in which fnr is under T7 promoter control, a protein-DNA complex, which was dependent on FNR being present in the extracts (data not shown), was observed (Fig. 1A, lane 6). The size of this complex was identical to that observed with purified FNR from aerobically grown cells (data not shown), indicating that no additional proteins were present in the complex, and the electrophoretic mobility of the complex was the same whether the complex was formed in the presence or absence of oxygen (Fig. 1A, lanes 6 and 12). In addition, DNA binding by FNR appeared to be specific for the FNR-target site because the assay mixture also contained excess nonspecific, calf thymus DNA.

The amount of DNA binding by FNR that was present in the cell extracts, was also regulated by oxygen (Fig. 1). Under anaerobic conditions, FNR bound approximately 90% of the DNA containing the peak of DNA binding by FNR was pooled.
at the highest concentrations of protein assayed (Fig. 1A, lanes 2–6, and Fig. 1B). However, assaying these same extracts in the presence of oxygen decreased the amount of DNA bound to 20% even at the highest concentrations of protein assayed (Fig. 1A, lanes 7–12), which is similar to the amount of DNA bound by FNR purified from aerobic cells (5).

FNR Purified under Anoxic Conditions Exhibits Oxygen-regulated DNA Binding—To determine what property increased DNA binding by FNR in the absence of oxygen, we partially purified a form of FNR that demonstrated oxygen-regulated DNA binding. Extracts from anaerobically grown cells were fractionated by cation-exchange chromatography under anoxic conditions. A peak of DNA binding by FNR which was sensitive to oxygen was eluted at approximately 0.36 M KCl (Fig. 2). In contrast, the majority of FNR protein was eluted at approximately 0.27 M KCl (Fig. 2). This is also the concentration of KCl at which the majority of FNR is eluted under aerobic conditions (data not shown). The column fractions which exhibited oxygen-regulated DNA binding were pooled and were shown to contain approximately 90% FNR as judged by SDS-PAGE analysis (Fig. 3, lane 2). This FNR preparation exhibited properties similar to FNR present in cell extracts in that 90% of the DNA was bound at the highest concentrations of protein assayed under anoxic conditions, and only 20% of the DNA was bound under aerobic conditions (Fig. 4).

Despite the recovery of a form of FNR which behaved similar to that found in the cell extracts, only a fraction of the oxygen-regulated DNA binding activity that was originally present in cell extracts was recovered in this purification step (Table I). This was determined by two criteria. First, the 5-fold increase in the purity of the FNR protein following cation-exchange chromatography (Fig. 3) does not correspond to the 1.5-fold decrease in the amount of FNR protein required for half-maximal DNA binding. Second, only approximately 30% of the oxygen regulated DNA binding activity present in cell extracts was recovered in the purified fraction. This loss of FNR which possessed oxygen-regulated DNA binding activity suggests that this activity is labile. In addition, it was difficult to further purify this form of FNR, since such attempts resulted in a substantial loss of oxygen-regulated DNA binding.

The Apparent Molecular Weight of FNR Changes on Exposure to Oxygen—Previous in vivo analysis of FNR dominant-negative mutants indicated that FNR was converted from a monomer to an oligomer in response to oxygen deprivation (3). However, all previous preparations of wild-type FNR yielded only monomeric protein (3, 6). Therefore, to test in vitro if the oligomeric state of FNR was increased in the absence of oxygen as was indicated by our in vivo experiments, we examined the size of the anoxically purified FNR protein by gel-exclusion chromatography (Fig. 5). When the cation-exchange purified...
fraction of FNR, which showed oxygen-regulated DNA binding, was analyzed under anoxic conditions, the majority of the protein was eluted in a broad peak with a molecular weight which varied from 56,000 to 45,000, depending on the protein preparation analyzed. A dimer of FNR would have a molecular weight of 60,000, and therefore, the molecular weights ob-

erved most likely represent dimers of FNR which are dissociating during the analysis. When this FNR preparation was briefly exposed to air (less than 1 min) prior to loading the protein on the gel-exclusion column, the distribution of monomers and dimers was reversed. Only a portion of the FNR protein remained dimeric, and the majority was eluted with a molecular weight of an FNR monomer, 30,000. It seems likely that further dissociation of FNR would occur during longer exposures of FNR to oxygen, but precipitation of the protein under these conditions prevented such an analysis. These data are consistent with our previous in vivo analysis of FNR dominant-negative mutants that suggested that the form of wild-type FNR active in DNA binding is a dimer and that the oligomeric state of FNR is regulated by oxygen. Thus, this purified form of FNR has the same characteristics that wild-type FNR was observed to have in vivo.

Spectral Properties of Anoxically Purified FNR—The anoxically purified FNR protein possessed a yellow color which suggested that a cofactor(s) was associated with this protein that could be responsible for the increased dimerization and DNA binding properties. To determine the nature of the compound(s) that might be imparting this color to FNR, ultraviolet/visible spectra of the protein were recorded under anoxic conditions (Fig. 6). In addition to the protein absorption maximum at 280 nm, a shoulder at 320 nm and a broad peak with an absorption maximum around 420 nm were observed. This is similar to that observed for [4Fe–4S]–containing proteins (20) and the aerobically purified FNR* mutant protein, FNR-LH28-DA154, which contains an Fe–S cluster (4).

An Iron–Sulfur Cluster Is Associated with FNR—To determine whether the spectral properties imparted on the FNR protein were due to an Fe–S cluster, iron and sulfide analyses were performed. These showed the presence of iron which ranged from 0.44 to 1.9 mol of iron/mol of FNR monomer and sulfide which ranged from 0.36 to 1.2 mol of inorganic sulfide/mol of FNR monomer, indicating that an Fe–S cluster was associated with FNR. To determine the type of Fe–S cluster that was associated with FNR, low temperature EPR spectroscopy was performed. In some preparations, only a small signal typical of [3Fe–4S]1+ clusters (g = 2.01) was observed, which accounted for only a small fraction of the Fe–S cluster concentration expected from sulfide determination (data not shown). This signal increased severalfold upon addition of ferriyanide (data not shown). This suggests that an EPR silent [4Fe–4S]2+ may have been associated with the FNR protein as [3Fe–4S] clusters are known to arise only from [4Fe–4S] clusters but not from [2Fe–2S] centers (21). We have not yet been able to detect
Oxygen Regulation of FNR

Two [4Fe-4S] Clusters Are Associated with a Dimer of Anoxically Purified FNR* Mutant Protein, FNR-LH28-DA154 — Unlike the wild-type protein, when the FNR* mutant protein, FNR-LH28-DA154, was purified in the presence of oxygen, a small fraction of this protein contained an Fe-S cluster, suggesting that the amino acid substitutions in this protein increase the stability of this cluster against oxygen. It seemed possible that the Fe-S cluster associated with this FNR* mutant protein would be further stabilized by the absence of oxygen. To determine if this was true, we purified FNR-LH28-DA154 protein under anoxic conditions. FNR-LH28-DA154 protein was resolved on the cation-exchange column under anoxic conditions into the same fractions as seen under aerobic conditions (data not shown). The anoxically purified protein differed from that purified aerobically in that the protein was more visibly colored (data not shown), suggesting that a greater percentage of the anoxically purified FNR-LH28-DA154 protein contained an Fe-S cluster.

The purification of a form of FNR which has oxygen-regulated activity has shown that an Fe-S cluster is associated with wild-type FNR and that this Fe-S cluster appears to be required for the increased DNA binding and dimerization of this protein in the absence of oxygen. This study also extends the previous analysis of an FNR* mutant protein, FNR-LH28-DA154, which was more suitable for determining the stoichiometry of the Fe-S cluster relative to the FNR-LH28-DA154 protein, iron and sulfide analyses were performed. Iron and sulfide were found associated at maximally 4.0 mol of iron and 3.3 mol of inorganic sulfide/mol of FNR-LH28-DA154 monomer. Based on the concentration of inorganic sulfide, the stoichiometry was calculated to be 1.65 mol of [4Fe-4S] cluster/mol of FNR dimer which indicates that there are two [4Fe-4S] clusters per dimer of FNR-LH28-DA154 protein.

**DISCUSSION**

The aim of this study has been to gain additional insight into the mechanism of the regulation of FNR activity by oxygen. The development of an in vitro functional assay, which mimics how regulation of FNR occurs in vivo, provides an important tool to dissect what factors are involved in mediating oxygen regulation. The purification of a form of FNR which has oxygen-regulated activity has shown that an Fe-S cluster is associated with wild-type FNR and that this Fe-S cluster appears to be required for the increased DNA binding and dimerization of this protein in the absence of oxygen. This study also extends the previous analysis of an FNR* mutant protein, FNR-LH28-DA154, which was more suitable for determining the stoichiometry of the Fe-S cluster relative to the FNR-LH28-DA154 protein.
proteins appear to contain a [4Fe–4S] cluster, the percentage of protein requires a three-dimensional structure of FNR. Units. To confirm the location of the Fe–S cluster in the FNR, it seems most likely that one monomer would provide all the dimerizations should occur along the C-helix (Fig. 8). Therefore, it is likely that the cluster would only be associated with FNR under anaerobic conditions. The apparent requirement of the Fe–S cluster for increased DNA binding and dimerization of FNR, inactive refers to the form of FNR that is predominant under aerobic conditions and shows decreased DNA binding activity. Active refers to the form that shows enhanced DNA binding activity and is present predominantly under anaerobic conditions.

![Model for the association of the Fe-S cluster with FNR and regulation of FNR activity by oxygen.](image)

**A**. A, a hypothetical model of an FNR monomer. This was constructed by attaching the N-terminal extension of FNR (depicted by a thin line) to the existing ribbon diagram of the CAP structure (22); the letters and numbers are the CAP nomenclature for α-helices and β-sheets, respectively. Indicated in gray is the dimerization, C-helix. The N terminus of FNR in the presence of the Fe–S cluster (solid line) and in the absence of the Fe–S cluster (dashed line) is indicated, as well as the structure of a [4Fe-4S] cluster. B, a model for regulation of FNR activity by the stability of a [4Fe-4S] cluster to oxygen. Inactive refers to the form of FNR that is predominantly under aerobic conditions and shows decreased DNA binding activity. Active refers to the form that shows enhanced DNA binding activity and is present predominantly under anaerobic conditions.

1 E. C. Ziegelhoffer and P. J. Kiley, unpublished data.

**B**. Model for the association of the Fe–S cluster with FNR and regulation of FNR activity by oxygen. A, a hypothetical model of an FNR monomer. This was constructed by attaching the N-terminal extension of FNR (depicted by a thin line) to the existing ribbon diagram of the CAP structure (22); the letters and numbers are the CAP nomenclature for α-helices and β-sheets, respectively. Indicated in gray is the dimerization, C-helix. The N terminus of FNR in the presence of the Fe–S cluster (solid line) and in the absence of the Fe–S cluster (dashed line) is indicated, as well as the structure of a [4Fe–4S] cluster. B, a model for regulation of FNR activity by the stability of a [4Fe–4S] cluster to oxygen. Inactive refers to the form of FNR that is predominant under aerobic conditions and shows decreased DNA binding activity. Active refers to the form that shows enhanced DNA binding activity and is present predominantly under anaerobic conditions.

The instability of the Fe–S cluster to oxygen regulates the activity of FNR—The Fe–S cluster of FNR appears to be oxygen labile since absorption in the visible region is lost after exposure to the Fe–S-containing form of FNR to oxygen. Thus, a simple model to explain how the Fe–S cluster can be mediating oxygen regulation of FNR activity in vivo is that degradation of the Fe–S cluster by oxygen regulates the activity of FNR (see Fig. 8). The oxygen lability of the Fe–S cluster strongly suggests that the cluster would only be associated with FNR under anaerobic conditions. The apparent requirement of the Fe–S cluster for increased DNA binding and dimerization of FNR, suggests that degradation of the Fe–S cluster upon exposure to oxygen is the cause for the loss of these properties in the presence of oxygen. The Fe–S-containing form of FNR appears to be also capable of transcription activation, although the presence of RNases in the axonically purified protein has, so far, precluded a quantitative analysis. Therefore, the Fe–S cluster of FNR appears to function as a direct oxygen sensor. The Fe–S clusters of hydrolyase proteins and glutamine phosphoribosylpyrophosphate amidotransferase have been shown to be degraded by oxygen both in vitro and in vivo (29, 32). A mechanism for this degradation of the Fe–S clusters in the hydrolyase proteins involves the attack by superoxide (29). Consistent with this, the clusters of some of these proteins have been shown to be protected from degradation by the presence of superoxide dismutase both in vitro and in vivo (29, 32, 33). It must be pointed out, however, that the Fe–S clusters in hydrolyases have only three Cys ligands and, therefore, one accessible cluster iron to which substrate can be bound. Although it is not certain, it is nevertheless likely that the Fe–S cluster in FNR has four Cys ligands so that a different mechanism of degradation may have to be invoked. Whether superoxide is involved in the degradation of the Fe–S cluster of FNR is not known, and it will be interesting to determine if superoxide dismutase protects the Fe–S cluster of FNR from degra-

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1 E. C. Ziegelhoffer and P. J. Kiley, unpublished data.
vation by oxygen. If FNR is inactivated by superoxide, then the Fe-S cluster of FNR must be exquisitely sensitive to superoxide because FNR activity is lost in vivo under normal atmospheric conditions, whereas the hydroxyase proteins are inactivated in vivo only when the concentration of oxygen is increased.

How Does Association of the Fe–S Cluster Increase FNR Activity?—Since the Fe–S cluster associated with FNR is unstable in the presence of oxygen, we favor the major role of the cluster to be in stabilizing an active conformation of FNR under anaerobic conditions. This conformation could be achieved by a simple reorganization of the N-terminal domain of FNR in the presence of the Fe–S cluster. Non-redox roles for Fe-S clusters have been observed with a number of enzymes, and for at least one protein, glutamine phosphoribosylpyrophosphate amidotransferase, the role of the Fe–S cluster appears to be solely to stabilize a conformation resistant to degradation in vivo (34). As Fe-S clusters can occur in different oxidation states (24), it is still possible that oxidation or reduction of the Fe–S cluster could further modulate the activity of FNR.

When the observed in vitro properties of the wild-type and FNR* mutant proteins are considered, the association of an Fe–S cluster appears to stabilize a conformation that increases the dimerization constant. This increase in the number of FNR dimers, which presumably have maximal DNA binding affinity, would result in an increase in transcriptional regulation under anaerobic conditions in vivo. Analysis of FNR* mutants has indicated that amino acid 154 is a critical determinant in dimer formation (3, 25). This amino acid is located at the putative dimerization interface (as shown in Fig. 8), and in wild-type FNR, this amino acid is a negatively charged aspartic acid. Changing this position to a neutral amino acid, alanine, also results in an increase in dimerization which suggests that under aerobic conditions, the aspartic acid is inhibitory to dimer formation. Therefore, a possible mechanism for the Fe–S cluster increasing the ability of FNR to dimerize is that a long range conformational change is induced by the presence of the Fe-S clusters that either moves the aspartic acid side chain away from the dimer interface or brings in close contact a positively charged group to neutralize Asp-154.

In summary, we have provided evidence that anoxically purified FNR contains a [4Fe-4S] cluster, with a stoichiometry of apparently two clusters per FNR dimer. This Fe-S-containing FNR is capable of oxygen-regulated DNA binding and dimerization. On this basis, we propose that the Fe-S cluster is required for FNR activity and the regulation of this activity by oxygen. The data presented in this study provide important information about how FNR may be functioning as an oxygen sensor. Recently, Fe centers have been found in other regulatory proteins. As far as involvement of Fe-S clusters in regulation is concerned, the iron-regulatory protein is an excellent example as to how Fe-S cluster formation or degradation can determine its function (26). In addition, the SoxR transcription factor has been shown to contain a [2Fe-2S] cluster (35–36). In this case, the protein is activated by oxidative stress in vivo, but the role of the Fe-S cluster in mediating regulation has yet to be elucidated. A different Fe center, a heme moiety, is utilized by the sensor component of the FixK two-component system of Rhizobium meliloti to regulate the kinase activity of FixL on FixJ in response to oxygen deprivation (28, 37). Thus, it appears that FNR, as do other proteins, utilizes the versatile coordination and redox chemistry of iron for regulatory functions.

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