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Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding

(oxygen sensitivity/dimerization/3Fe cluster/transcriptional regulation)

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ABSTRACT **In the facultative anaerobe *Escherichia coli*, the transcription factor FNR (*f*umarate *n*itrate *r*eduction) regulates gene expression in response to oxygen deprivation. To investigate how the activity of FNR is regulated by oxygen availability, two mutant proteins, DA154 and LH28-DA154, which have enhanced *in vivo* activity in the presence of oxygen, were purified and compared. Unlike other previously examined FNR preparations, the absorption spectrum of LH28-DA154 had two maxima at 324 nm and 419 nm, typical of iron-sulfur (Fe-S)-containing proteins. Consistent with these data, metal analysis showed that only the LH28-DA154 protein contained a significant amount of iron and acid-labile sulfide, and, by low temperature EPR spectroscopy, a signal typical of a [3Fe-4S]$^+$ cluster was detected. The LH28-DA154 protein that contained the Fe-S cluster also contained a higher proportion of dimers and had a 3- to 4-fold higher apparent affinity for the target DNA than the DA154 protein. In agreement with this, we found that when the LH28-DA154 protein was treated with an iron chelator (α,α'-dipyridyl), it lost its characteristic absorption and the apparent affinity for DNA was reduced 6-fold. However, increased DNA binding and the characteristic absorption spectrum could be restored by *in vitro* reconstitution of the Fe-S center. DNA binding of the LH28-DA154 protein was also affected by the redox state of the Fe-S center, since protein exposed to oxygen bound 1/10th as much DNA as the protein reduced anaerobically with dithionite. The observation that DNA binding is enhanced when the Fe-S center is reduced indicates that the redox state of the Fe-S center affects the DNA-binding activity of this protein and suggests a possible mechanism for regulation of the wild-type protein.**

A fundamental problem in biology is to understand the biochemical and molecular events that allow cells to sense and adapt to changes in oxygen in the environment. To address this problem, we have been studying how the activity of the *Escherichia coli* transcription factor FNR (*f*umarate *n*itrate *r*eduction) is regulated by oxygen availability. In the absence of oxygen, FNR is converted to an active form that regulates the synthesis of many enzymes. A central question is to determine how FNR is converted to an active form.

One region of FNR proposed to be required for regulation by oxygen is located near the N terminus and is characterized by a cluster of four closely spaced cysteine residues (^{16}Cys-Xaa$_3$-Cys-Xaa$_2$-Cys-Xaa$_5$-Cys^{29}). Consistent with its supposed function as an oxygen regulatory site, this N-terminal region is located outside the regions of FNR that are similar to the DNA-binding and cAMP-binding domains of the well-studied, cAMP-dependent CAP protein (1). The FNR cysteine residues appear to be important components of the N-terminal region since mutational analysis has shown that three of the four cysteine residues (Cys-20, -23, -29) as well as one outside this region (Cys-122) are required for function (2).

Several studies (3–8) suggest that iron is also required for regulation of FNR activity by oxygen. For example, the transcription activating function of FNR can be manipulated *in vitro* (6, 7) and *in vivo* (6, 8) by Fe^{2+} levels. Moreover, the observation that Fe^{2+} is associated with FNR *in vitro* (3) and that the active, anaerobic form of FNR could be converted *in vivo* to an inactive form by varying the redox potential of the growth medium has led to the proposal that the bound iron is redox-sensitive and is required for a conformational change necessary to convert the FNR protein into an active form (6–10). Because iron is found at a stoichiometry of approximately 1 mol of Fe^{2+} per monomer of FNR, it has been proposed that Fe^{2+} is bound to FNR through four cysteines (possibly Cys-20, -23, -29, -122) as is found in iron-sulfur (Fe-S) centers of the rubredoxin type (11). However, spectroscopic analysis has not revealed any of the hallmarks typical for this class of proteins (3). Thus, the exact nature of the state of this iron has remained elusive.

The study of FNR mutant proteins that have activity *in vivo* in the presence of oxygen (referred to as FNR* mutants; ref. 12) has provided insight into understanding how FNR activity is regulated by oxygen availability (13–15). For example, a protein with an Asp → Ala substitution at position 154 (DA154) showed increased DNA binding and dimerization in the presence of oxygen, relative to wild-type protein. These results suggested that one function of FNR that is regulated by oxygen deprivation is its DNA-binding activity, which depends on the oligomeric state of the protein (13). Although alternative mechanisms have been proposed (6), these properties of FNR* mutants have led us to consider the hypothesis that one component of oxygen regulation of FNR is the conversion of inactive monomeric FNR into a dimeric form competent for DNA binding. As an approach to test this model, we surveyed the biochemical properties of several other FNR* mutant proteins (12). From this analysis we found that, unlike all other FNR preparations, a protein containing a Leu → His substitution at position 28 (LH28; ref. 12) and the DA154 change was brown in color, typical of iron-containing proteins. In this paper, we show that the LH28-DA154 protein contains a redox-sensitive, polynuclear Fe-S center.

MATERIALS AND METHODS

Protein Purification. FNR* proteins DA154 and LH28-DA154 were obtained from PK22 strains carrying these mutant genes under control of the inducible T7 promoter on the plasmid pPK824 (*fnrDA154*; ref. 13) or pPK2012 (*fnrLH28-*

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Abbreviations: DTT, dithiothreitol; FNR, *f*umarate *n*itrate *r*eduction.

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DA154). Plasmid pPK2012 was created by exchanging a restriction fragment containing both mutations (*LH28*, *DA154*) for the wild-type fragment from pPK823 (13) and verifying the construction by DNA sequencing. As described previously, the FNR protein was purified from cell lysates fractionated by cation- (Bio-Rex-70; Bio-Rad) and anion- (Resource Q; Pharmacia) exchange chromatography, except that EDTA and dithiothreitol (DTT) were omitted. To minimize protein precipitation, the pooled Bio-Rex-70 fractions were diluted with 10 mM Tris, pH 7.9/10% glycerol/0.1 mM phenylmethylsulfonyl fluoride to the same pH and conductivity of the loading buffer for the Resource Q chromatographic step, just prior to loading the column. Although purified aerobically, protein solutions were stored under an atmosphere of 90% N_2/5% CO_2/5% H_2 in sealed bottles to further minimize precipitation.

Protein concentrations were determined with the Coomassie Plus protein assay reagent (Pierce), using bovine serum albumin as a standard. The concentration of LH28-DA154 protein obtained by this method was 1.33 times higher than the value determined by amino acid analysis (data not shown). All protein concentrations determined colorimetrically were, therefore, corrected by dividing by 1.33.

Metal and Sulfide Analysis. Iron and sulfide were determined on samples analyzed in duplicate as described in refs. 16 and 17, respectively. To verify the formation of methylene blue from S^{2-} and *p*-phenylenediamine, the ratio of absorbances at 670, 710, and 750 nm was regularly measured (17).

Optical Spectroscopy. UV-visible spectra were obtained employing a Lambda 2 UV-visible spectrophotometer (Perkin–Elmer).

Low-Temperature EPR. EPR spectra were recorded at 9.11 GHz and 2 mW at 13 K with a modulation amplitude of 0.5 mT at 100 kHz.

Molecular Weight Analysis. Molecular sieve chromatography was performed using a Pharmacia FPLC system equipped with a Sepharose 12 HR column and eluting at a flow rate of 0.5 ml/min in 25 mM Tris·HCl, pH 7.9/10% glycerol/0.3 M KCl. Elution of the protein was detected at 280 nm.

DNA-Binding Assay. Site-specific DNA binding of mutant FNR proteins was measured using a gel-retardation assay as described (13), except that the DNA-binding reaction also included 1 mM dithionite (Fluka) and all manipulations were performed inside an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing approximately 90% N_2/5% H_2/5% CO_2. Following acrylamide gel electrophoresis to separate DNA–protein complexes from free DNA, the fraction of radioactively labeled DNA bound over a range of protein concentrations was quantified by use of a PhosphorImager (Molecular Dynamics). Since previous work has shown that FNR binding to DNA may also involve a monomer–dimer equilibrium, we report the differences in DNA binding as the amount of protein required to bind 50% of the target DNA (defined as $S_{0.5}$) rather than as a simple equilibrium constant, *K*. $S_{0.5}$ was determined after fitting the data to the following equation: $y = Y^*[P]^n/(K + [P]^n)$, where y = % DNA bound, Y = max % DNA bound, [P] = concentration of FNR (M_r 30,000). Using the least-squares sum method, the best fit of the experimental data was obtained when n (the number of protein units bound to the DNA) was set at 2.

Reconstitution of the Fe-S Center. All manipulations were performed in an anaerobic chamber (Coy Laboratory Products) as described above. To prepare deferrated (apo-Fe) protein, purified LH28-DA154 protein (0.04 μmol) was incubated with 0.8 μM iron chelator, α,α′-dipyridyl (Sigma), in the presence of 1 mM dithionite in a total volume of 0.2 ml at room temperature for 30 min. Apo-Fe protein was purified from the reaction mixture using an FPLC Sepharose 12 HR column equilibrated with 25 mM Tris·HCl buffer, pH 7.4/0.1 M KCl. Apo-Fe protein (4 nmol) was incubated with 0.3 nmol of NIFS protein (18) in the presence of 1 mM L-cysteine (Sigma), 2.5 mM DTT, and 2 mM ferrous ammonium sulfate in a total volume of 0.6 ml in 12.5 mM Tris·HCl, pH 7.4/50 mM KCl. Following addition of dithionite to a final concentration of 2 mM, reconstituted LH28-DA154 was purified from reactants as described above for the apo-Fe form. NIFS protein was kindly provided by Dennis R. Dean (Virginia Polytechnic Institute and State University, Blacksburg).

RESULTS

Spectral Properties of the LH28-DA154 and DA154 FNR* Proteins. In contrast to the colorless solution of the purified DA154 protein, the same concentration (approximately 1 mg/ml) of LH28-DA154 protein prepared under identical conditions was brown. UV-visible spectroscopy showed that the LH28-DA154 protein had absorption maxima at 324 and 419 nm (Fig. 1). Moreover, the ratio of the absorbances at 419 and 280 nm for LH28-DA154 was 0.16, typical of proteins with [4Fe-4S] clusters (19). In contrast, the DA154 protein had no detectable absorption peaks at these wavelengths (Fig. 1), suggesting the absence of an Fe-S center as has been previously observed for the wild-type protein (3).

Determination of the Iron and Sulfur Content of LH28-DA154 and DA154 Proteins. Five different preparations of DA154 and LH28-DA154 proteins were analyzed for Fe and S^{2-}. All preparations of the LH28-DA154 protein contained labile S^{2-}, indicating the presence of polynuclear Fe-S clusters. The values for Fe ranged from 0.4 to 1.7 mol, and those for S^{2-} ranged from 0.25 to 0.4 mol per mol of protein monomer. The DA154 protein contained <0.1 mol of Fe per mol of protein monomer and no detectable S^{2-} (<0.01 mol/mol). Thus, only the LH28-DA154 protein contained an Fe-S cluster.

We also observed that this Fe-S center was labile, since Fe and S^{2-} analysis carried out at intervals over a 24-hr period on LH28-DA154 protein stored at 0°C in the presence of oxygen showed that Fe and S^{2-} values decreased with time, although the S^{2-} values decreased more drastically. Simultaneously, turbidity was observed, indicative of protein precipitation, and protein determinations on the precipitates showed that substantial amounts of protein were lost during this period. Freezing the proteins led to even higher losses of Fe and S^{2-}. Unfortunately, concentrated protein samples were required for these analyses to avoid interference due to some of the nonprotein components of these solutions (e.g., high salt), which further contributed to precipitation. These observations could explain why the stoichiometries of Fe and S^{-2} to protein (as initially assayed) were lower than expected for a polynuclear Fe-S protein with at least one cluster per dimer (see *Discussion*). For example, for a polynuclear 4Fe-4S center, given our highest [S^{2-}] measurement, no more than 10% of the protein molecules could have contained intact Fe-S clusters.

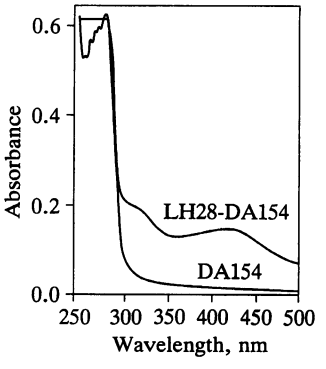

FIG. 1. Absorption spectra of FNR* proteins, DA154 (0.9 mg/ml) and LH28-DA154 (1.1 mg/ml).

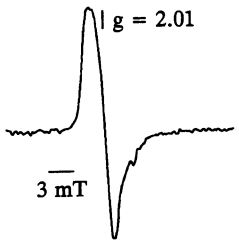

FIG. 2. EPR spectrum of LH28-DA154 protein at 9.11 GHz, 2 mW power, modulation of 0.5 mT at 100 KHz, and at 13 K. The signal is the average of nine scans.

Low-Temperature EPR Studies of LH28-DA154 Protein. As determination of the ratio of Fe and S^{2-} per monomer of mutant protein is not sufficient to distinguish whether a [2Fe-2S] or [4Fe-4S] cluster is present in the protein, low-temperature EPR spectroscopy was performed to further characterize the type of Fe-S center present. As shown in Fig. 2, a signal typical for a $[3Fe\text{-}4S]^+$ cluster was detected. According to a double integration of the signal, the concentration of the species represented in the signal was 1 μM. Since analysis for S^{2-} had shown 32 μM S^{2-}, the highest concentration of the $[3Fe\text{-}4S]^+$ cluster present could, therefore, have been 8 μM (0.25 $\times$ the $[S^{2-}]$). In our experience with similar proteins, the average recovery in the EPR signal of the concentration determined by this analysis is approximately 80% (20). Thus, given the fact that the protein precipitated and S^{2-} was lost over time, the recovery of 1 μM (15% of the [Fe-S] cluster estimated by $[S^{2-}]$) in the EPR signal of Fig. 2 is not unreasonable.

Spectral Changes of LH28-DA154 Protein upon Dithionite Reduction. Many proteins containing an Fe-S cluster participate in oxidation–reduction reactions. This led us to examine the redox sensitivity of this Fe-S center. In the presence of an excess of the reductant, dithionite, the absorbance of the LH28-DA154 protein at 419 nm decreased 60% (Fig. 3), a result typical for Fe-S proteins that undergo reversible oxidation–reduction. Subsequent exposure to air led to rapid reoxidation of the chromophore as determined by an increase in absorbance at 419 nm (data not shown). Thus, the cluster can be readily cycled through oxidation–reduction and might thus be suitable as a direct oxygen sensor.

DNA Binding of LH28-DA154 and DA154 Proteins. To investigate whether the presence of the Fe-S cluster in the purified LH28-DA154 protein affected its DNA-binding ability, specific DNA binding was analyzed by a gel retardation assay carried out under anaerobic conditions (Fig. 4*A*). Although the LH28-DA154 protein, containing the Fe-S center, and the DA154 protein, which lacked iron, formed a complex with the same apparent mobility, differences in the apparent binding affinities were observed. The amount of DA154 protein required to bind 50% of the DNA ($S_{0.5}$ = 29 nM) was found to be three to four times higher than that of the LH28-DA154 DNA complex ($S_{0.5}$ = 8 nM) (Fig. 4*B*). This suggested that the presence of the Fe-S center increased the apparent affinity of FNR for its DNA-binding site.

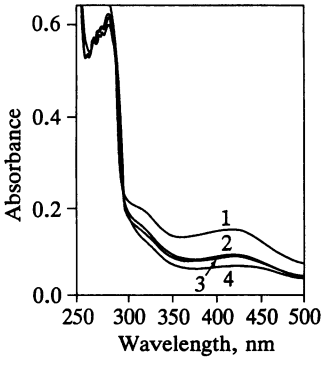

FIG. 3. Absorption spectra of dithionite-reduced LH28-DA154 FNR* protein (40 μM). Just prior to recording the absorption spectra, dithionite was added to the sample and control cuvettes at the following concentrations: 1, none; 2, 200 μM; 3, 280 μM; 4, 800 μM.

The Presence of the Fe-S Center Is Responsible for Enhanced DNA Binding. To determine if the changes in the DNA-binding properties of LH28-DA154 protein were imparted by the presence of the Fe-S center, we removed the iron by chelation with α,α'-dipyridyl, under anaerobic conditions in the presence of dithionite. Effective removal of the Fe-S cluster by this method was shown by the disappearance of the 419-nm absorbance (Fig. 5*A*). This resulted in a 6-fold decrease in the apparent binding affinity of the apo-Fe LH28-DA154 protein ($S_{0.5}$ = 50 nM) (Fig. 5*B*). In contrast, the amount of DNA bound by the DA154 protein under the same experimental conditions was not affected by treatment with an iron chelator (data not shown).

Reconstitution of the Fe-S Center in LH28-DA154 FNR with NIFS Protein from *A. vinelandii*. We investigated whether DNA binding of apo-Fe LH28-DA154 could be increased by reconstitution of the Fe-S center under anaerobic reducing conditions using ferrous ammonium sulfate and cysteine as donors of iron and sulfide, respectively. The NIFS protein from *A. vinelandii* was used to transfer the sulfur group from cysteine since this protein has been shown to form an enzyme-bound persulfide, which serves as the sulfide donor in nitrogenase Fe-S cluster formation (18). Reconstitution of the Fe-S cluster was observed by formation of a brown-colored protein solution and restoration of the characteristic absorption spectrum (Fig. 5*A*). As described above, the apo-Fe form of LH28-DA154 had one-sixth the apparent affinity for the DNA-target site ($S_{0.5}$ = 50 nM) as the same protein with an intact Fe-S center ($S_{0.5}$ = 8 nM), and DNA binding by the apo-Fe protein was increased 5-fold upon the reconstitution of the Fe-S cluster ($S_{0.5}$ = 11 nM) (Fig. 5*B*).

Redox-Dependent DNA Binding of LH28-DA154 Protein. The effect of oxygen on wild-type FNR activity *in vivo* can be

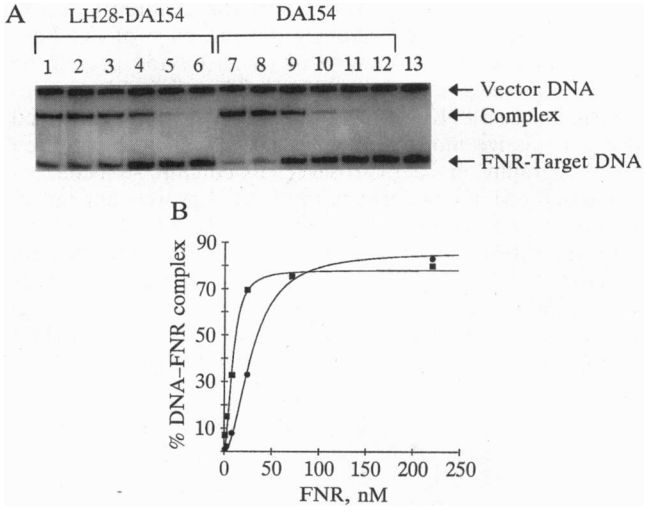

FIG. 4. DNA binding by DA154 and LH28-DA154 FNR* proteins. (*A*) Gel retardation assays were performed by incubating a ^{32}P-labeled 48-bp consensus DNA-target site (58 pM; ref. 13) with decreasing amounts of LH28-DA154 (lanes 1–6) or DA154 (lanes 7–12) protein. Protein concentrations were as follows: lanes 1 and 7, 220 nM; lanes 2 and 8, 71 nM; lanes 3 and 9, 24 nM; lanes 4 and 10, 8 nM; lanes 5 and 11, 2.7 nM; lanes 6 and 12, 0.9 nM; lane 13, no protein added. (*B*) Quantitation of DNA binding by LH28-DA154 (■) and DA154 (●) proteins. The DNA-binding curves represent the percentage of DNA in the FNR–DNA complex plotted as a function of protein concentration.

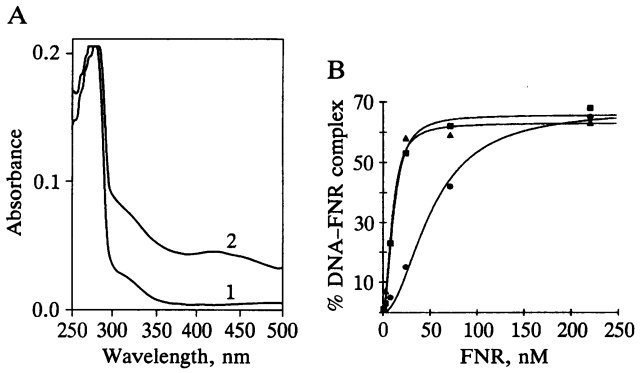

FIG. 5. Reconstitution of the Fe-S center in LH28-DA154 and effect on DNA binding. (*A*) Absorption spectra of LH28-DA154 protein after α,α'-dipyridyl treatment (apo-Fe LH28-DA154 protein) (1) and reconstitution of Fe-S center with *Azotobacter vinelandii* NIFS protein (2). (*B*) DNA-binding curves of LH28-DA154 FNR* protein: ■, untreated; ●, α,α'-dipyridyl treated; ▲, Fe-S center-containing protein after reconstitution. Protein was purified over Sepharose 12 hr before analysis.

mimicked by varying the redox potential of the cell growth medium (9). However, no effect of the redox state on *in vitro* DNA binding of purified wild-type protein has been observed previously. To investigate whether DNA binding of the LH28-DA154 protein, which contains the Fe-S center, would be affected by oxidation–reduction, the protein was treated with dithionite ($E_0' = -350$ mV) or exposed to air (oxygen/H_2O, E_0' = +816 mV) for 30 min with periodic mixing. After removal of dithionite, measurement of DNA binding (Fig. 6) showed that the reduced LH28-DA154 protein ($S_{0.5}$ = 8 nM) bound DNA 10-fold better than that of the air-oxidized form of the same protein ($S_{0.5}$ = 78 nM). An analogous experiment performed with DA154 showed no effect of these redox agents on DNA binding (data not shown).

The Presence of the Fe-S Center Increases Dimerization. It has been previously shown that the FNR* DA154 protein has increased DNA-binding activity relative to wild-type FNR apparently because of an increase in dimerization (13, 14). Therefore, we examined whether the presence of the Fe-S cluster increased DNA binding of LH28-DA154 protein by further stabilizing the active dimeric form of this mutant protein. Purified LH28-DA154 and DA154 proteins displayed different relative mobilities when analyzed by size-exclusion chromatography on a Sepharose-12 HR column. At a concentration of 8 μM, the majority of the DA154 protein appears in the monomer fraction with an apparent M_r of about 33,000 (data not shown; ref. 13). In contrast, at the same protein concentration (8 μM), LH28-DA154 was eluted at a larger apparent M_r of 66,000, consistent with the size of a dimer (Fig. 7). Upon a 10-fold dilution, some dissociation of the LH28-DA154 protein to monomers was observed (Fig. 7).

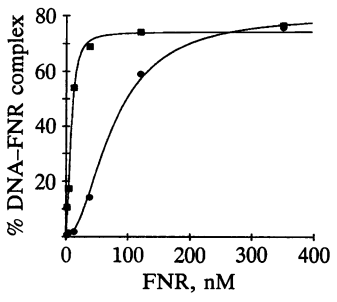

FIG. 6. Effect of oxidation–reduction on LH28-DA154 DNA binding. DNA-binding curves of LH28-DA154 protein reduced with dithionite (■) and oxidized by exposure to air for 30 min (●).

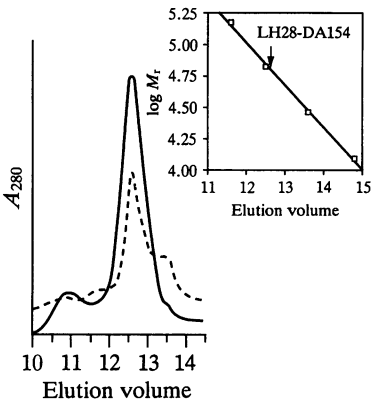

FIG. 7. Gel filtration analysis of LH28-DA154 FNR protein. Shown are the elution profiles of LH28-DA154 at protein concentrations of 8 μM (solid line) and 0.8 μM (dashed line). The absorbance values at 280 nm were normalized and plotted as a function of elution volume. (*Inset*) Calibration curve using alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* as standards. The elution peak for the dimer of the LH28-DA154 protein, when loaded at 8 μM, is indicated by an arrow.

DISCUSSION

Although the transcription factor FNR has been shown to be regulated by oxygen availability *in vivo*, we still do not have a complete understanding for the mechanistic basis for this regulation. *In vitro* and *in vivo* experiments designed to unravel the sensing mechanism have pointed to a role for bound iron (6–8). The work described here suggests that a [4Fe-4S] center may be the physiologically relevant form of Fe that is bound to FNR. Furthermore, the redox-sensitive DNA-binding properties of this protein have provided clues for understanding how FNR activity may be regulated by oxygen availability.

In this study, analysis of a FNR protein containing two different amino acid substitutions, LH28 and DA154, has demonstrated the presence of a redox-sensitive Fe-S center. Moreover, these results show that the redox state of this center is critical since the reduced form of this center was required for enhanced DNA binding of the LH28-DA154 protein. Interestingly, even though the FNR* substitutions were isolated based on their increased activity in the presence of oxygen, we found that the Fe-S center was sensitive to oxygen *in vitro*. Thus, these results suggest that DNA binding by this FNR* mutant can be regulated by the redox state. These results also contrast with previous studies of an Fe^{2+}-containing form of the wild-type protein that showed none of the characteristics of an Fe-S protein and where the presence of iron did not influence its DNA-binding characteristics (3). However, as discussed below, we consider it likely that the wild-type protein also contains such a center *in vivo* and that the iron found associated with the purified protein has arisen from the oxidative degradation of a very oxygen-sensitive Fe-S cluster.

Structure and Stability of the Fe-S Center. EPR analysis indicates that the aerobically purified LH28-DA154 protein contains a $[3Fe\text{-}4S]^+$ cluster. This is most likely the form that we also detected by visible spectroscopy (Fig. 1). In many cases, 3Fe clusters are artifacts of exposure of $[4Fe\text{-}4S]^{2+}$ clusters to oxygen (21). Since we have evidence for oxygen sensitivity of the Fe-S cluster present in the LH28-DA154 protein, it seems reasonable to assume that the cluster originally present was a [4Fe-4S] cluster. Although we attempted to detect an EPR signal of the reduced cluster ($[4Fe\text{-}4S]^+$), we were unable to achieve a concentration sufficient to obtain an unambiguous answer (data not shown), as was possible with the 3Fe cluster (Fig. 2). (The significant features of the 3Fe form have 1/20th the spread of those expected from the $[4Fe\text{-}4S]^{1+}$ form; an approximately 400-fold increase in con-

centration is therefore required for equally decisive identification of the latter.) Efforts to increase the concentration of the [4Fe-4S] center by anaerobic reconstitution from the 3Fe and apo forms also did not allow detection of the reduced cluster by EPR (data not shown). Since not all Fe-S clusters have four Cys ligands, we cannot unambiguously identify the cluster ligands. However, in general, clusters with only three Cys ligands are less stable. Thus, it seems possible to us that the His substitution in the LH28-DA154 protein becomes significant in this context. The His residue itself might be a cluster ligand or otherwise change accessibility to the cluster.

Despite our inability to detect the reduced [4Fe-4S] cluster, we emphasize the significance of having unambiguously found labile sulfide and an EPR signal of a 3Fe cluster, as together this provides an infallible sign of Fe-S clusters larger than [2Fe-2S]. We realize that we only found a fraction (10% Fe-S center per monomer, 20% per dimer) of what a reasonable stoichiometry would demand and what there may have been originally, but there is ample precedent that Fe-S clusters may be sufficiently fragile to lead to such a result (22, 23). If, as we are tempted to think, the [4Fe-4S] cluster is in fact an important component of the oxygen-sensing mechanism, we would expect that cluster to be very oxygen sensitive.

In view of our observations that the dimer of FNR is the active species (13, 14), we would expect a minimal stoichiometry to be, per dimer, one [4Fe-4S] cluster if we assume that the cluster is shared. Moreover, we expect that in addition to iron and sulfide, assembly of the Fe-S cluster might require additional factors or enzymes, as has been observed for nitrogenase from *Azotobacter* (18).

Role of Fe-S Center in FNR Activation. Fe-S proteins can have diverse functions in biology. Many are involved in electron transfer reactions as components of electron transfer systems (19). Some Fe-S clusters are constituents of enzyme active sites but are not involved in oxidation–reduction processes, such as aconitase (24). In other cases, the Fe-S center may have a strictly structural role, as in *E. coli* endonuclease III (25). Some proteins containing an Fe-S center are also regulatory proteins. In the case of SoxR, which controls the initial step of a redox-sensitive superoxide stress pathway, the Fe-S center appears to be involved in sensing of the redox state by oxidation–reduction (26). In the LH28-DA154 FNR protein, the Fe-S center appears to have two functions that may be interrelated: oxidation–reduction and stabilization of the protein in the active dimeric form.

Several lines of evidence suggest that the Fe-S center in LH28-DA154 protein is involved in stabilization of the active dimer conformation. Analysis of the Fe-S-containing LH28-DA154 protein by molecular sieve chromatography showed that a dimer form of FNR was present at concentrations much lower than that previously observed for DA154 protein (13). In addition, the effects of the Fe-S center and its redox state on the oligomeric state of the protein were also reflected in the shape of the DNA-binding curves. As has been previously observed with the DA154 protein (14), the sigmoidal character of the DNA-binding curves of the apo-Fe and oxidized form of the LH28-DA154 protein demonstrated cooperativity in the interaction of these proteins with DNA. The fact that FNR binds to DNA at concentrations where it is monomeric in solution suggests that the cooperativity in the binding curves reflects the presence of coupled equilibria. The FNR dimer–DNA complex could arise from either of two pathways: initial formation of dimers, which subsequently bind to DNA, or the binding of one monomer to DNA, which increases the binding of a second protein molecule. The DNA-binding data for the LH28-DA154 protein containing the reduced Fe-S center could be fitted to a hyperbolic function and thus was consistent with the data showing dimer formation even at low protein concentrations. These data are consistent with the model whereby specific DNA binding by FNR is dependent on the extent of dimerization, which is regulated by oxygen availability. If the stoichiometry of this protein is one [4Fe-4S] per dimer, this is most easily interpreted if the effect of the Fe-S center is to increase dimerization, which would then increase the pool of FNR molecules able to bind DNA.

The properties of an Fe-S center-containing mutant protein of FNR have provided important insight into the mechanism of FNR regulation by oxygen availability. Further work will be necessary to show how far these observations on mutant FNR proteins extend to the wild-type protein. A promising connecting link is the fact that iron has been consistently found in the wild-type protein (3) and there is no shortage of suitable ligands for the formation of Fe-S clusters. What had not been found previously is the sulfide, and here the properties of the LH28-DA154 mutant may just have given us enough of an edge to detect it as well as the EPR signal of a polynuclear cluster.

Note Added in Proof. Since the submission of the manuscript we have been able, by improved preparation and sampling procedures, to obtain a ratio of monomer:Fe:S^{2-} = 1:2.6:2.1 and to convert an EPR silent cluster to the 3Fe form (g = 2.01) by ferricyanide.

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