Regulation of FNR Dimerization by Subunit Charge Repulsion*

Received for publication, August 31, 2006 Published, JBC Papers in Press, September 7, 2006, DOI 10.1074/jbc.M608331200

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Dimerization of the global anaerobic transcription factor FNR is essential for FNR activity. Under aerobic conditions FNR is an inactive monomeric species because it lacks the oxygen labile [4Fe-4S] cluster required for dimerization. In this study, we investigated the protein side chains that inhibit FNR dimerization under aerobic conditions. Substitution of Asp154 within the predicted dimerization helix with residues containing neutral or positively charged side chains increased FNR activity under aerobic conditions, whereas replacement of Asp154 with Glu inhibited FNR activity similar to WT-FNR. Similar results were obtained when making analogous substitutions of Glu150. In vitro analysis of representative FNR mutant proteins indicated that their increased activity under aerobic conditions resulted from an [4Fe-4S] independent mechanism of dimerization. In addition, simultaneous substitution of residues 150 and 154 with Lys restored inhibition of FNR activity under aerobic growth conditions. Collectively, these data indicate that charge repulsion by side chains at positions 150 and 154 is necessary to inhibit dimerization under aerobic conditions. They also suggest that a [4Fe-4S]-dependent conformational change overcomes charge repulsion between subunits under anaerobic conditions. Comparison of the trpsin sensitivity of [4Fe-4S]-FNR and apoFNR indicated that there are no major differences in protease sensitivity between these forms, whereas circular dichroism suggested that small changes in secondary structure occur between the cluster-containing FNR and apoFNR. Thus, the [4Fe-4S]-dependent conformational change necessary to overcome inter-subunit charge repulsion and create a subunit interface more favorable for dimerization must be small.

A major O2 sensor in Escherichia coli is the transcription factor FNR,2 which regulates transcription of genes under anaerobic growth conditions (1, 2). In the absence of O2, FNR is a ~60-kDa homodimer, with each subunit containing a [4Fe-4S] cluster (3–5). Dimerization is required for FNR activity, because only the dimeric form binds to specific sequences in the promoter region of target genes to activate or repress transcription (3). Under aerobic conditions, FNR target genes are not regulated because FNR is converted to a monomeric species, which is inactive for site-specific DNA binding and accordingly, transcriptional regulation (4, 5). The formation of the monomeric species results from the initial O2-dependent degradation of the [4Fe-4S] cluster to a [2Fe-2S] cluster (5, 6); the [2Fe-2S] cluster is then further degraded into clusterless apoFNR (7). Because the structure of FNR in any form has not been determined, a key question to address is how the [4Fe-4S] cluster alters the conformation of FNR to achieve regulated dimerization.

FNR belongs to the CRP/FNR superfamily of transcription factors, all of which are predicted to have similar tertiary structures but can be distinguished by their specificity for different effector ligands (8). The two best structurally characterized members of this family are the E. coli transcription factor CRP, which contains bound cAMP in the active form (9), and the Rhodospirillum rubrum transcription factor CooA, which contains a CO-bound heme cofactor when active (10). Unlike FNR, both CRP and CooA are dimeric in the absence of their effector molecules (10–12). The structure of CRP is characterized by a C-terminal helix turn helix DNA binding domain and an N-terminal effector binding domain, which contains an α-helix (known as the C-helix or dimerization helix) that promotes subunit-subunit interactions (Fig. 1) (9). Previous studies of FNR suggest that like the subunit interface of CRP, FNR promoters interact via a coiled-coil of a predicted α-helix and that the interface (positions a and d in a coiled-coil heptad repeat) contains mostly large hydrophobic residues (13). However, a notable exception within this interface is the negatively charged Asp residue at position 154. It is likely that this residue plays an inhibitory role in dimerization because a substitution to Ala promotes dimerization under aerobic conditions, yielding a transcriptionally active protein even when the [4Fe-4S] cluster is absent (14). Previous studies have shown that substitution to a Lys at Glu150, also results in an aerobically active transcription factor (15, 16), suggesting that both residues play a role in achieving regulated dimerization of FNR by O2. Because the location of the [4Fe-4S] cluster of FNR is predicted to be distant from the dimerization helix (Fig. 1), these data led to the hypothesis that ligation of the [4Fe-4S] cluster is coupled to a long range conformational change that overcomes the inhibitory effects of Asp154 and Glu150 to favor dimerization.

In this study we used site-directed mutagenesis followed by in vivo and in vitro studies to examine the connection between the charge of the residues at positions 154 and 150, the transcriptional activity, and extent of dimerization of the aerobic form of FNR. We also used circular dichroism spectroscopy and

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* This work was supported by Postdoctoral Training Grant GM19792 from the National Institutes of Health (to L. J. M.), an Illinois Wesleyan junior faculty leave grant (to L. J. M.), and National Institutes of Health Grant GM58444 (to P. J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: FNR, fumarate nitrate reduction; CD, circular dichroism; WT, wild type; CRP, cAMP receptor protein.
protease sensitivity assays to investigate the degree of the conformational differences between the [4Fe-4S]-FNR, [2Fe-2S]-FNR, and apoFNR.

**MATERIALS AND METHODS**

**Plasmid and Strain Construction**—Plasmids and strains used in this study are listed in Table 1. Amino acid substitutions of FNR were created via site-directed mutagenesis of pPK821, pPK822 (for mutant proteins also containing the Asp154Ala substitution), or pPK5111 (for mutant proteins also containing the D154K substitution) using oligonucleotide primers (Operon Technologies or Integrated DNA Technologies) as described previously (14). The mutations were recombined onto *λ*fr-C122S from the relevant pPK821, pPK822, or pPK5111 derivative. Because *λ*fr-C122S has a FNR° phenotype since the Fe-S ligand, Cys122, has been changed to Ser (15), it produces only white Lac° plaques when incubated with strain RZ8480 and overlaid in top agar with 100 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside. *λ*fr recombinants with the desired mutations were then identified as blue plaques (FNR° phenotype) as previously described (15). Individual *λ*fr recombinants were plaque purified, PCR amplified, and the resulting DNA fragments were sequenced at the University of Wisconsin sequencing facility to verify the incorporation of the desired mutation and the wild type (WT) codon for Cys122. At least 3 monolysogens of each *λ*fr derivative were isolated from RZ8480 as previously described (17). The *fr* coding sequences of some pPK821, pPK822, and pPK5111 derivatives were cloned into pET11a using the NdeI and BamHI restriction sites (13). The resulting plasmids were sequenced and used for either β-galactosidase assays or FNR protein overexpression.

**β-Galactosidase Assays**—The activity of FNR and its mutant derivatives was determined in *vivo* by assaying β-galactosidase activity expressed from a *lacZ* fusion to the FNR-dependent promoter *narG* as described previously (13). Strains were grown in M9 minimal media (18) with 0.2% glucose and 10 mM potassium nitrate. Aerobic cultures were grown to an optical density of 0.4 to 0.6.

**Dimerization of FNR**

![Figure 1. Proposed structure of a subunit of FNR based on the structure of its homolog, CRP (41).](Image)

The location of the DNA binding domain and the effector domain, which consists of the dimerization helix, the β-roll region, and the N-terminal Fe-S binding region, are shown. The 29 amino acids that are not present in CRP contain three of the four Cys (Cys20, Cys23, and Cys29) that ligate the [4Fe-4S] cluster. The fourth ligand (Cys122) is located within the β-roll region and is equivalent to position 96 in CRP. The likely location of the [4Fe-4S] cluster is predicted from the location of the cysteines. The probable locations of the two charged residues (Asp154 and Glu125) that are proposed to impede dimerization in the apo-form of FNR are shown.

**TABLE 1**

| Strain or plasmid | Relevant genotype | Ref. |
|-------------------|-------------------|------|
| **E. coli strains** |                   |      |
| RZ8480            | lacD145 Δ*λ*fr-Islp'/Sm'< narC::MudI1734 | 14   |
| PK22              | BL21(DE3) Δ*crp*-bo990 rpl Δ*fr-Islp'/Sm'< zc-3061::Tn10 | 14   |
| PK738             | RZ8480 but *λ*fr-D154A | 15   |
| PK739             | RZ8480 but *λ*fr   | 15   |
| PK912             | RZ8480 but *λ*fr-E150K | 15   |
| PK913             | RZ8480 but *λ*fr-D154G | 15   |
| PK914             | RZ8480 but *λ*fr-D154V | 15   |
| PK964             | RZ8480 but *λ*fr-E150A | This lab |
| PK963             | RZ8480 but *λ*fr-E150A/D150A | This lab |
| PK5128            | RZ8480 but *λ*fr-D154K | This study |
| PK5129            | RZ8480 but *λ*fr-E150K/D154K | This study |
| PK5135            | RZ8480 but *λ*fr-D154E | This study |
| PK5136            | RZ8480 but *λ*fr-K152E | This study |
| PK5138            | RZ8480 but *λ*fr-E150Q | This study |
| PK5139            | RZ8480 but *λ*fr-D154C | This study |
| PK5140            | RZ8480 but *λ*fr-D154N | This study |
| PK5141            | RZ8480 but *λ*fr-E150A/D154A | This study |
| LM008             | RZ8480 but *λ*fr-D154L | This study |
| **Plasmids**      |                   |      |
| pET-11a           | Ap<sup>+</sup>, T7 φ10 promoter and gene 10 translation start site preceding Ndel site | 42   |
| pPK821            | Ap<sup>+</sup>, HindIII-BamHI of *fur* 521 to +1155 of *fur* with Ndel site at +1 in pUC118 | 14   |
| pPK822            | Same as pPK821 except *fur*-D154A | 14   |
| pPK5111           | Same as pPK821 except *fur*-D154K | This study |
| pPK823            | Ap<sup>+</sup>, Ndel-BamHI of *fur*, +1 to +1115 of *fur* in pET11a | 14   |
| pPK824            | Same as pPK823 but *fur*-D154A | 14   |
| pPK1868           | Same as pPK823 but *fur*-L28H | 22   |
| pPK5112           | Same as pPK823 but *fur*-D154K | This study |
| pPK5123           | Same as pPK823 but *fur*-E150K/D154K | This study |
Dimerization of FNR

Protein Isolation—[4Fe-4S]-FNR (either WT or FNR-L28H) was isolated from cells grown in M9 minimal media supplemented with 0.2% glucose, 0.2% casamino acids, and ferric ammonium citrate (10 μg/ml) to an optical density at 600 nm of 0.4–0.6, induced with 400 μM isopropyl β-d-thiogalactopyranoside for 1 h, sparged overnight under argon at 4 °C, and purified anaerobically as described previously (19). The presence of the [4Fe-4S] cluster was verified by the characteristic visible absorbance spectrum and analysis of iron (20) and sulfur (21). The ratio of iron to sulfur was between 1 and 1.2 for all preparations and the amount of the FNR protein that contained the [4Fe-4S] cluster based on S²⁻ content was between 50 and 65%. The [2Fe-2S]-FNR for protease digestion studies was isolated by growing cells overproducing [4Fe-4S]-FNR as described above, aerating the cells to convert FNR to the [2Fe-2S] cluster form and then isolating [2Fe-2S]-FNR as previously described (7). The occupancy of [2Fe-2S]-FNR used in protease sensitivity studies was ~47%.

Apoprotein lacking any Fe-S cluster was isolated from PK22 derivatives containing WT fnr or relevant mutants grown to an optical density at 600 nm of 0.4 to 0.6 in either M9 minimal media with 0.2% glucose or Luria broth with 0.2% glucose. FNR synthesis was induced with 400 μM isopropyl β-d-thiogalactopyranoside for 1 h, the cells were harvested by centrifugation and resuspended in a potassium phosphate buffer (pH 6.8) with 100 mM KCl, 10% glycerol, and 1 mM dithiothreitol. The protein was isolated as described previously (14) except that a PolyCat A column (Nest Group) connected to a Beckman high performance liquid chromatography system was used in place of the Bio-Rex 70 and Q-Sepharose columns. No significant amount of iron or S²⁻ was found in the aerobically isolated protein.

Size Exclusion Chromatography—Size exclusion chromatography was performed using an Amersham Biosciences HR-12 Superose column (10 x 300 mm) connected to a Beckman high performance liquid chromatography system that was located in a Coy anaerobic chamber (atmosphere of 90% N₂, 5% CO₂, and 5% H₂) at 23 °C. Aliquots of FNR or its mutant derivatives (200 μl in 50 mM KPO₄ (pH 6.8), 400 mM KCl, and 1 mM dithiothreitol) were injected onto the column and the absorbance of the eluent was monitored from 200 to 600 nm using a photodiode array detector. The column was calibrated with cytochrome c, bovine serum albumin, and carbonic anhydrase. The chromatograms were analyzed using the Beckman System Gold Nouveau software.

Limited Proteolysis—Isolated [4Fe-4S]-FNR, [2Fe-2S]-FNR, [4Fe-4S]-FNR-L28H (22), or apoFNR were subjected to proteolysis by trypsin under anaerobic conditions in a Coy anaerobic chamber (atmosphere of 90% N₂, 5% CO₂, and 5% H₂) at 23 °C. FNR protein (20 μg) was incubated with trypsin (1 μg; 13 units) in 60 μl of 50 mM KPO₄ (pH 6.8), 10% glycerol, 0.4 M NaCl. The reaction was terminated by transferring to SDS loading buffer (62.5 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromphenol blue) and heating at 90 °C for 10 min. All samples were run on a 12% SDS-PAGE gel, stained with Coomassie Blue, and the amount of Coomassie-stained protein was quantified using ImageQuant. The trypsin cleavage sites were identified by mass spectrometry following precipitation of trypsin-treated apo- or [4Fe-4S]-FNR (90% cluster occupancy) protein (200 μl) with 10–15 μl of acetic acid, washing the protein pellet with 80% acetone, and solubilizing the pellet in formic acid. Mass spectra were acquired in the positive ion mode on an Applied Biosystems 32000 Q TRAP LC/MS/MS mass spectrometer equipped with an electrospray ionization source. Data acquisition, spectrum deconvolution, and analysis were performed using Analyst QS software.

CD Spectroscopy—Circular dichroism spectra were taken over a range of 200 to 300 nm at 1-nm increments and a 3-s acquisition time on an Aviv model 202SF CD spectropolarimeter. An anaerobic spectrum of [4Fe-4S]-FNR (5 μM FNR with 60% occupancy of the [4Fe-4S] cluster in 50 mM potassium phosphate (pH 6.8) and 200 mM KCl) was collected in a 1-mm sealed quartz cuvette. The sample was removed from the cuvette, exposed to air for a period of time sufficient to generate [2Fe-2S]-FNR (45 min), and another CD spectrum was recorded. A CD spectrum of apoFNR (5 μM) was also recorded under the same conditions except 1 mM tris-(2-carboxyethyl)phosphine hydrochloride (Molecular Probes), a thiol reducing agent that prevents aggregation of apoFNR was added to the solution. A fit of the CD line shape was carried out with the k2D algorithm (23).

RESULTS

Amino Acid Substitutions of Asp¹⁵⁴ Show That a Negative Charge Is Important for Proper O₂ Regulation of FNR—Previous studies showed that changing Asp¹⁵⁴ to Ala resulted in a FNR protein that was active (14–16) and dimerized under aerobic conditions in the absence of a [4Fe-4S] cluster (3, 14). Because, the molecular mechanism by which this substitution allowed dimerization was unknown, we replaced the Asp at position 154 with amino acid side chains of different properties to test which promote FNR activity and dimerization under aerobic conditions. FNR activity was assessed by measuring the level of β-galactosidase activity from strains containing lacZ under control of the FNR-dependent narG promoter (PnarG) and λfnr or its mutant derivatives integrated into the att site. When these strains were assayed for activity under aerobic conditions (Fig. 2A), only the FNR mutant with a Glu residue at 154 remained inactive like WT-FNR. In contrast, substitution of the negatively charged side chain with hydrophobic (Leu, Val, Ala, and Gly), polar (Cys and Asn), or basic (Lys) residues at this position yielded FNR proteins that were active under aerobic conditions. Surprisingly, the most active mutant protein under aerobic conditions contained the oppositely charged Lys in place of Asp (FNR-D154K). Western blot analysis showed that the FNR mutant proteins were present at levels similar to that of WT-FNR (data not shown), demonstrating that the change in activity is not due to a change in the protein concentration. Under anaerobic conditions, the mutants showed the same levels of β-galactosidase activity as WT-FNR (Fig. 2B), suggesting that all of the mutant proteins were still responsive to ligation of the [4Fe-4S] cluster.

To test if the increased activity of FNR-D154K under aerobic conditions was correlated with an increase in dimerization as was previously observed with FNR-D154A, size exclusion chromatography was performed (13) (Fig. 3). As expected from pre-
vious studies (3, 13, 14), apo-WT-FNR (10 μM, Fig. 3A) was eluted at 29 min, indicative of a monomeric (~30 kDa) protein, whereas, apoFNR-D154A (5 μM, Fig. 3B) and [4Fe-4S]-WT-FNR (data not shown) were eluted at ~27.2 min, indicative of a dimeric species (~60 kDa). FNR-D154K, lacking any Fe-S cluster, was eluted at the same position as FNR-D154A indicating that it is also dimeric under these solution conditions. Thus, these data suggest that the loss of the negative charge at position 154 is sufficient to increase dimerization of FNR under aerobic conditions.

A Negative Charge at Position 150 Is Also Important for Inhibiting the Aerobic Activity of FNR—The finding that only negatively charged residues at position 154 inhibited dimerization under aerobic conditions suggested that repulsion by another negatively charged residue within the dimerization helix of WT-FNR may prevent dimerization. To test this hypothesis, we assayed the activity of a library of Ala substitution mutants of the predicted FNR dimerization interface (Arg140 to Leu159) (13) under aerobic conditions. The only single Ala substitution along the dimerization helix that results in an active protein under aerobic conditions, in addition to FNR-D154A, is FNR-D154K (Table 2, Fig. 4). In addition, substituting Glu150 with a polar (Gln) residue also increased FNR activity in vivo under aerobic conditions (Fig. 4). As was found with position 154, the replacement of the negatively charged residue (Glu) with a positively charged residue (Lys) resulted in the highest activity of all the Glu150 substitutions (Fig. 4), although, the amount of β-galactosidase activity for all three mutants at position 150 was 2–3-fold less than similar replacements of position 154. Because Glu150 falls on the same face of the dimerization helix as Asp154, it seems likely that these two residues are in close proximity. We also tested whether placing a negative charge on the opposite face of the helix would affect FNR activity. The mutant containing a substitution of Lys152 to Glu had little activity under aerobic conditions like WT-FNR, suggesting that only the presence of negative charges at the predicted helical interface are important for regulating dimerization under aerobic conditions.

Similarly Charged Residues at Both Positions 150 and 154 Inhibit Aerobic Activity—To determine whether two positive charges at positions 150 and 154 would have an effect similar to the two negative charges found at these positions in WT-FNR, combinations of substitutions at both positions were made and their in vivo activity was analyzed (Fig. 5). The mutant containing the double substitution FNR-E150A/D154A, which removes both negative charges as well as some of the volume of the side chains, had less activity under aerobic growth conditions than the FNR-D154A mutant. This result suggests that in the absence of a negative charge at position 154, a Glu in position 150 is actually better at promoting dimerization than the Ala residue that has a side
chain with a smaller volume. However, replacement of Glu\textsuperscript{150} with Lys in combination with an Ala at position 154 retained the same \textit{in vivo} activity as the FNR-D154A mutant protein, indicating that a positively or negatively charged residue at position 150 is equally effective in promoting dimerization of FNR-D154A.

Finally, switching both positions 150 and 154 to positively charged Lys residues (FNR-E150K/D154K) resulted in much less FNR activity under aerobic conditions than proteins having a neutral residue at one position and a charged residue at the other. Because the presence of two positively charged residues at 150 and 154 is nearly as effective in inhibiting FNR activity as the having two negative charges at the same positions, this result suggests that charge repulsion between like charges of residues 150 and 154 is a key feature of maintaining FNR in an inactive state.

To test if the decreased activity of the FNR-E150K/D154K mutant under aerobic conditions was correlated with a decrease in dimerization, size exclusion chromatography of FNR-EK150/DK154 in the apoprotein state was performed (Fig. 3D). At the 5 \textmu M sample concentration, \textasciitilde80\% of the FNR-
E150K/D154K protein was eluted at ~29 min, indicating that a significant portion of the protein was monomeric. However, the small shoulder (20%) that is present at 27.7 min indicates that some protein is dimeric; as the concentration of the protein is increased, the shoulder becomes larger, increasing to 45% of the peak area at 20 μM, indicating that the monomer-dimer equilibrium of this mutant protein is indeed altered. The activity that remains in the FNR-E150K/D154K mutant may be a secondary effect of the length of the Lys side chain; the Glu and Asp side chains are shorter than the Lys side chain. Therefore, upon dimerization, the positive charges of Lys would not be positioned as close to one another as the negative charges from the Glu and Asp side chains in WT-FNR. Overall, these results support the hypothesis that charge repulsion between the two FNR subunits is important for disrupting dimerization.

**Alanine Substitutions of FNR-D154A Identify Additional Residues That May Contribute to Dimerization**—To investigate the contribution of residues within the predicted subunit interface to dimerization when charge repulsion is relieved, the activity of the FNR-D154A proteins with alanine substitutions from position Arg140 to Leu159 were assayed under aerobic conditions (Table 2). In [4Fe-4S]-WT-FNR this series of Ala substitutions showed that an Ala at every third or fourth hydrophobic residue caused a reduction of FNR activity, characteristic of the α and d positions of a coiled-coil subunit interface (13). The pattern of defects of the Ala substitutions of FNR-D154A that was observed in the lower part of the helix (residues 140–151) is similar to what was observed for [4Fe-4S]-WT-FNR, whereas the pattern in the upper part of the helix (residues 155–159) is different (Table 2). For example, substitution of Ala at Ile154, which disrupted dimerization completely in [4Fe-4S]-FNR, also decreased the activity of apo-FNR-D154A. In contrast to our findings with [4Fe-4S]-FNR (13), replacing Ile158 with Ala does not drastically decrease FNR-D154A activity, suggesting that Ile158 no longer plays a critical role in dimerization when the charge at Asp154 is removed.

Some Ala substitutions increased activity of FNR-D154A. Substitution of Gln142 to Ala increased FNR-D154A activity under both aerobic (Table 2) and anaerobic conditions (13). This substitution also increases the activity of [4Fe-4S]-WT-FNR by a similar amount (13). In contrast, substitutions of either Gly149 or Gly153, located in the middle of the predicted helix, increased the activity of FNR-D154A 2-fold under aerobic conditions (Table 2), even though these substitutions have little effect on [4Fe-4S]-WT-FNR activity or [4Fe-4S]-FRN-D154A activity (13). Therefore, the Gly residues appear to specifically affect activity of apo-D154A-FNR. The glycines could contribute to the structure of the helix that is required to keep the apoprotein inactive.

**Protease Susceptibility of [4Fe-4S]-FNR, [2Fe-2S]-FNR, and ApoFNR**—To assess the conformational differences between the cluster-containing and apoprotein forms of FNR, limited proteolysis experiments were performed (Fig. 6). FNR contains 30 possible trypsin proteolysis sites including residues close to the N terminus (Lys5, Arg6, Arg9, and Arg10), close to the C terminus (Lys227, Lys229, and Arg247), within the dimerization helix (Arg139, Arg145, and Lys157), and in the predicted hinge region between the effector domain and DNA binding domain (Lys163, Lys164, and Arg169) that could potentially report conformational differences in these regions. When [4Fe-4S]-FNR (58% occupied), [2Fe-2S]-FNR (47% occupied), or apo-FNR was incubated with trypsin under anaerobic conditions, a common well defined product of 29 kDa, which is slightly smaller than full-length FNR (apparent molecular mass of 30,000), was detectable by SDS-PAGE within 5 min of digestion. Similar results were obtained using a preparation of [4Fe-4S]-FNR, containing a higher occupancy (90%) of Fe-S cluster (data not shown) or an FNR mutant, FNR-L28H (62% occupied) (Fig. 6), which contains a [4Fe-4S] cluster that is resistant to destruction by O2 (22) or apo-D154A-FNR (data not shown). In addition, no further digestion was observed with incubation periods up to 20 min indicating that all forms of FNR are well folded, compact, and fairly resistant to proteolysis. Other proteases such as chymotrypsin were also tested and showed no detectable cleavage of any form of FNR (data not shown) even though there are numerous predicted sites for protease digestion by these enzymes.

Electrospray mass spectrometry of the trypsin digestion products for apo-FNR and [4Fe-4S]-FNR (90% occupied) indicated two major products for each species. Both apo- and [4Fe-4S]-FNR species were cleaved after either Arg9 or Arg10, whereas the C-terminal Arg247 of apo-FNR was more sensitive to proteolysis by trypsin. Thus, it appears that loss of the cluster does not result in large scale unfolding in the main body of the protein. Overall, these data indicate that all forms of FNR are well folded and not significantly different in tertiary structure.

**Circular Dichroism of [4Fe-4S]-FNR, [2Fe-2S]-FNR, and ApoFNR**—Circular dichroism spectroscopy was used as an independent method to examine conformational differences between the cluster and apo containing forms of FNR. The CD spectra of [4Fe-4S]-FNR and [2Fe-2S]-FNR are very similar, whereas the spectrum of the apoprotein has reduced ellipticity (Fig. 7). The k2D algorithm gave estimates of the α-helical and β-sheet that were very similar for the 4Fe- and 2Fe-FNR and slightly different for apo-FNR ([4Fe-4S]-FNR, 27% α-helix, 17% β-sheet; [2Fe-2S]-FNR, 28% α-helix, 18% β-sheet; apo-FNR, 32% α-helix, 16% β-sheet). Additionally, the CD spectrum of apo-FNR-D154A, which is dimeric under these conditions (14), is comparable in shape and magnitude (data not shown) to the monomeric apo-FNR. Taken together, these results suggest that any changes in the CD spectra most likely reflect a change of the N-terminal residues that are involved in ligation of both the [2Fe-2S] and [4Fe-4S] clusters.

**DISCUSSION**

Control of dimerization appears to be the principal means by which FNR activity is regulated by O2. Only forms of FNR that
Dimerization of FNR

A Critical Role for Charge Repulsion in Regulation of FNR Dimerization—Our previous studies of [4Fe-4S]-FNR dimerization led to the conclusion that dimerization proceeds via a coiled-coil interaction where residues Ile\(^{151}\) and Asp\(^{154}\) are in the dimerization interface (in the \(a\) and \(d\) positions of a heptad repeat of a coiled-coil (25)), along with other hydrophobic residues along the same face of helix (Met\(^{144}\), Met\(^{147}\), and Ile\(^{158}\)) (13) (Fig. 1). However, the finding that residue 150, which is not in an \(a\) or \(d\) interface position of the [4Fe-4S]-FNR dimerization helix, and residue 154 inhibit dimerization by charge repulsion indicates that the side chains of 150 and 154 are located at or near the interface in the monomeric species. These data also support and extend the critical role of Ile\(^{151}\) in promoting dimerization (13) by not only shielding Asp\(^{154}\) from its counterpart on the other subunit interface but also insulating Glu\(^{150}\). Accordingly, substitution of Ile\(^{151}\) has less of an effect on dimerization when FNR has Ala at position 154 because there is only the charge at 150 between subunits for Ile\(^{151}\) to shield. In addition, the data presented here suggest that flexibility of the predicted subunit interface may be a key feature to regulating dimerization via charge repulsion. First, even though position 154 is predicted to be at the interface, many different types of amino acid substitutions promoted dimerization of either the apo or the [4Fe-4S] form. The glycines at 149 and 153 may also be important because replacement of these residues with Ala further increases dimerization of only the form of FNR that lacks charge repulsion (FNR-D154A). This situation may be analogous to the reduction of dynamic fluctuations that are thought to occur upon cAMP binding in CRP (26, 27). Taken together, these data support a model of FNR inactivation in which the loss of the [4Fe-4S] changes the conformation of FNR, allowing two negative charges of residues 150 and 154 to be in close enough proximity that dimerization is inhibited by charge repulsion. Defining the structural basis of this mechanism is a challenge for the future.

Inspection of other Crp/FNR/CooA superfamily members indicates that the negative charges at positions 150 and 154 are conserved only in those orthologs that have a predicted Fe-S cluster binding region; a Blast alignment of FNR homologs with the same N-terminal Cys pattern (Cys-X_2-Cys-X_5-Cys) shows that most (45 out of 61) of those containing a N-terminal Fe-S cluster binding region have amino acids with negative charges at both of the positions analogous to 150 and 154 in FNR. Another 12 of these homologs have one negative charge at one of these positions. Furthermore, CRP/FNR/CooA family members that lack this Fe-S cluster region also do not have these negatively charged residues. For example, CRP has an Arg and a Thr at the analogous positions, whereas CooA has an Ala and Cys at the analogous positions. The presence of these conserved residues in the FNR family suggests that control of dimerization by the arrangement of negative charges may be a common means of regulation of homologs with Fe-S clusters.

Similarity of FNR Activation to Its Homologs—The change at the interface of FNR that leads to an alteration of the oligomeric state is an extreme form of the conformational changes that have been proposed to occur at the interface of subunits in the FNR homologs, CooA and CRP. Whereas these proteins are dimeric at low concentrations even in the absence of the effector molecule (cAMP and CO, respectively) (10–12), it has been suggested that effector binding leads to a tighter association between the subunits that is caused by realignment of the subunit interface (26, 28–32). Subunit realignment has also been used to explain the effects observed in amino acid substitution studies of the dimerization helix of both CooA and CRP (33, 34). Finally, a model for CooA activation has been proposed in which a “roll” of the dimerization helix (C-helix) and a repositioning of the adjacent heme occurs upon CO binding (35). This model may have relevance to the small rearrangement that we suggest alters the interface for dimerization in FNR. Thus, even though FNR changes oligomeric states in the process of activation, the conformational change at the dimerization helix may be similar to its homologs (33, 36, 37).

One reason to explain why FNR appears to be preferentially regulated by dimerization compared with its other superfamily members may be related to the mechanism of sensing of their respective small molecules. CRP and CooA sense the availability of cAMP and CO, respectively, by reversibly binding their relevant ligand. In contrast, sensing of O_2 by FNR destroys the Fe-S cluster requiring new synthesis of the cluster (38). We speculate that accumulation of fully occupied [4Fe-4S] dimeric FNR occurs more efficiently when FNR is in the monomeric state; rather than a mechanism where clusters assemble into apo-dimeric FNR, which would favor the formation of partially occupied, inactive, dimeric FNR.

Surprisingly, the conformational differences between the inactive and active forms of FNR observed in this study were small. Both limited proteolysis and circular dichroism data suggest that there are not large changes in structure when the [4Fe-
4S] cluster is degraded to the [2Fe-2S] form or is not present (apoFNR). In addition, the limited proteolysis results indicate that all forms of FNR are well folded because only the extreme N and C termini showed any sensitivity to trypsin, although there are many sites present in the protein. This finding is somewhat similar to what has been observed with CRP. Limited proteolysis indicated that CRP is also relatively resistant to proteolysis (39) except in the presence of cAMP, where a protease site between the effector and DNA binding domains (the “hinge region”) becomes accessible. For FNR, the only difference in protease sensitivity observed between apoFNR and the [4Fe-4S] form was the enhanced cleavage of the C-terminal region of apoFNR, not the N terminus, which contains 3 of the Fe-S cluster ligands, and not in the hinge region, as is seen in CRP (39). Nevertheless, the increased accessibility of the C terminus may explain how apoFNR is specifically targeted to proteolysis via ClpXP (40) because one of the ClpX binding sites maps to the last 2 amino acid residues of FNR. In addition, the overall well folded state of apoFNR may influence the rate of ClpX-dependent proteolysis and provide a rationale for balancing the slow but specific degradation of apoFNR with cluster reinsertion/destruction to readily accumulate active FNR when O2 suddenly becomes limiting.

In summary, it appears that several factors may contribute to FNR activity, but the presence of negative charges at positions 150 and 154 is critical for FNR to remain inactive under aerobic growth conditions in E. coli. We also postulate that the conformational changes, which occur in FNR in upon loss of the cluster, are relatively small. The relative contribution from conformational flexibility and shifts in the monomer-dimer equilibrium to FNR activity will need to be more fully explored to understand the complete pathway by which FNR dimerization is regulated.

Acknowledgments—We thank Dr. Helmut Beinert for the evaluation of FNR activity, but the presence of negative charges at positions 150 and 154 is critical for FNR to remain inactive under aerobic growth conditions in E. coli. We also postulate that the conformational changes, which occur in FNR upon loss of the cluster, are relatively small. The relative contribution from conformational flexibility and shifts in the monomer-dimer equilibrium to FNR activity will need to be more fully explored to understand the complete pathway by which FNR dimerization is regulated.

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