The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state

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The transcription factor FNR globally regulates gene expression in response to oxygen deprivation in *Escherichia coli*. To understand how oxygen deprivation activates FNR, a constitutively active FNR* mutant protein, DA154, was studied to determine how this mutant bypassed the normal regulation pathway. When purified from aerobically grown cells, the DA154 protein had a larger apparent native molecular mass and an increased affinity for a consensus FNR target site as compared with wild-type FNR prepared under identical conditions. These results suggested that FNR* DA154 may bypass the normal regulation pathway by converting FNR from an inactive monomer to an active dimer under aerobic conditions. To determine whether wild-type FNR is active as a dimer under anaerobic conditions, FNR mutants were isolated that inhibit the activity of wild-type FNR by forming mixed dimers (i.e., dominant-negative mutants). These dominant-negative FNR mutants were shown to have substitutions in the putative DNA-binding domain and to be defective in binding to a consensus FNR DNA target site in vitro. One representative dominant-negative mutant, EK209, was also shown to be unable to form mixed oligomers in vivo under aerobic conditions, suggesting that FNR may be monomeric in the inactive state. Taken together, these data have led us to propose that under anaerobic conditions FNR is a dimer that is active for DNA binding, and under aerobic conditions, FNR is inactivated by conversion to a monomer.

The mechanism by which cells sense and adapt to changes in oxygen in their environment is poorly understood. In the bacterium *Escherichia coli*, like many other organisms, changes in oxygen concentration have profound effects on cellular physiology. Under aerobic conditions, oxygen is used as a terminal electron acceptor to generate energy by aerobic respiration reactions similar to those used in mitochondria. However, when oxygen is not available, anaerobic respiration can be used to generate energy if terminal electron acceptors, such as nitrate and fumarate, are present. The use of these novel pathways requires synthesis of distinct respiratory enzymes that specifically recognize these alternate electron acceptors.

The *E. coli* transcription factor FNR plays a central role in allowing *E. coli* to adapt to changes in oxygen availability (Spiro and Guest 1990). In the absence of oxygen, FNR not only activates synthesis of anaerobic respiratory enzymes but also represses synthesis of some aerobic respiratory enzymes. Because FNR levels do not differ between aerobically and anaerobically grown cells (Trageser et al. 1990), it appears that oxygen deprivation

modulates FNR-dependent transcription by regulating the activity of FNR. Our long-term goal is to understand the mechanism of this regulation.

FNR is similar to the well-studied *E. coli* transcription factor CAP, providing a framework for studying how FNR activity is regulated (Shaw et al. 1983). DNA-binding and transcription activation by CAP is regulated allosterically through the binding of cyclic AMP (cAMP) (Zubay et al. 1970). Although FNR contains a region similar to the cAMP-binding domain of CAP, the specific residues involved in cAMP binding are not conserved (McKay and Steitz 1981; Shaw et al. 1983). However, the location of the conserved residues in this region suggests that an overall β-roll structure may form in FNR as is the case in CAP (Cherfils et al. 1989). Thus, this region of FNR may also play a role in the allosteric regulation of the protein. The most striking similarity between CAP and FNR is in the DNA-binding domain. Substitution of just 3 amino acids in the putative FNR DNA-binding domain with those at the corresponding positions in the CAP DNA-binding domain is sufficient to convert the DNA-binding specificity of FNR to that of CAP-dependent promoters (Spiro and Guest 1987). Because of this, FNR is predicted to bind to DNA using a helix–turn–

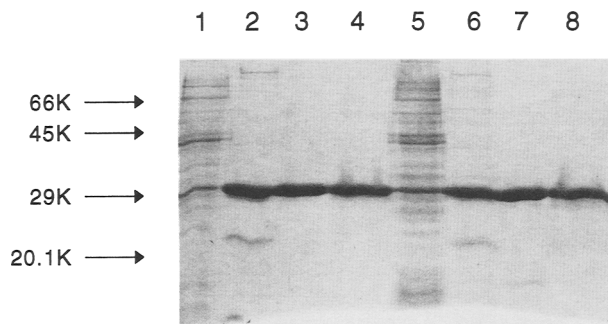[3]Corresponding author.

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Figure 1. The purity of FNR* DA154 (lanes *1–4*) and wild-type FNR protein (lanes *5–8*) at different stages in the purification was assessed by SDS-PAGE. One microgram of protein was loaded in each lane. Lanes *1* and *5* contain the crude cell extract loaded on the Bio-Rex 70 column; lanes *2* and *6* contain the Bio-Rex 70 material loaded on the Q Sepharose column; lanes *3* and *7* contain the Q Sepharose material loaded on the Sephacryl S-200 column; and lanes *4* and *8* contain the major peak of FNR from the Sephacryl S-200 column. On the left side are indicated the position of molecular mass markers, and their size is indicated in kilodaltons (K).

helix motif as has been shown for CAP (Schultz et al. 1991).

The sequence of the consensus FNR target site is also very similar to the sequence of the CAP target site, differing at only one critical symmetrical base pair (Bell et al. 1989). As expected, in vitro DNA-footprinting experiments have shown that purified FNR binds specifically to this consensus FNR target site (Green et al. 1991). Furthermore, the fact that a symmetric 22-bp region was protected indicated that FNR was bound as a dimer. Thus, it was expected that the active DNA-binding species of FNR would be dimeric, similar to CAP (McKay and Steitz 1981). However, wild-type FNR purified from either aerobic or anaerobic cells was found to be monomeric (Trageser et al. 1990; Green et al. 1991). Thus, these footprinting experiments do not distinguish whether the DNA-binding activity of this protein is the result of two monomers recognizing each half of the FNR target site independently or whether this activity is the result of a small, undetectable population of dimers. In addition, neither DNA-binding nor transcription activation by purified FNR is dependent on oxygen deprivation in vitro as it is in vivo (Sharrocks et al. 1991). This biochemical approach has not yet resolved the oligomeric state of the active form of FNR or the mechanism by which oxygen deprivation activates this protein.

To determine how the activity of FNR is regulated in response to oxygen deprivation, Kiley and Reznikoff (1991) isolated mutants that have FNR activity under aerobic conditions (FNR* mutants). One class of FNR* mutants has substitutions in the region of the protein analogous to the CAP dimerization site (Cherfils et al. 1989). In this study one such FNR* mutant, DA154, was analyzed biochemically to understand how a single amino acid substitution in the putative dimerization domain of FNR allowed function of this protein in the pres-

ence of oxygen. In addition, inactive FNR mutants were isolated and analyzed to further dissect the oligomeric state of the active form of FNR and the mechanism of regulation by oxygen.

Results

FNR* *mutant protein DA154 has a larger apparent molecular mass than wild-type FNR*

To determine the biochemical basis for the difference between the in vivo activity of wild-type FNR and the FNR* mutant DA154 under aerobic conditions, we purified both proteins from strains that contain each gene under the control of an inducible T7 RNA polymerase promoter. SDS-PAGE analysis following each purification step is shown in Figure 1. Both wild-type and DA154 proteins fractionated similarly through both cation (Bio-Rex 70) and anion (Q Sepharose) exchange columns, exhibiting only minor differences in the salt concentration at which they eluted (data not shown). The FNR protein was >95% pure after elution through the Q Sepharose column as judged by densitometry scaning of the proteins on a SDS–polyacrylamide gel. No difference was observed between DA154 and wild-type FNR subunit molecular weight (~30,000) as determined by SDS-PAGE (Fig. 1, lanes 4 and 8, respectively).

In contrast, a difference in the native molecular mass of these two proteins was observed when the highly purified fractions from the Q Sepharose column were analyzed at the same protein concentration by S-200 gel-exclusion chromatography (Fig. 2). At 80 μM wild-type FNR, the apparent molecular weight was ~33,000 (Fig.
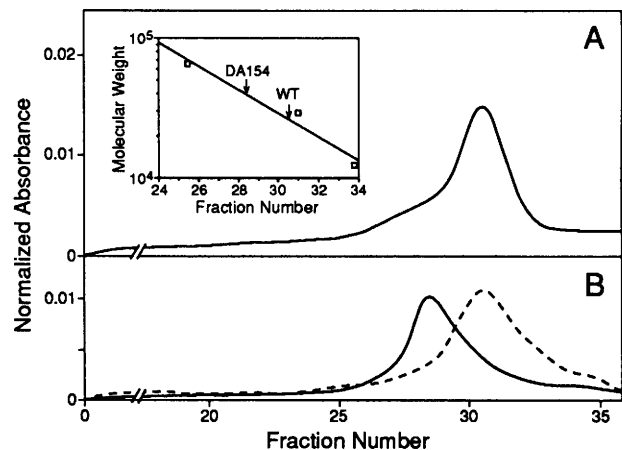

Figure 2. Gel-filtration analysis of wild-type and DA154 protein. The absorbance of the eluant at 280 nm from the Sephacryl S-200 column has been normalized and is plotted versus the fraction number. Shown are the elution profiles of wild-type FNR protein loaded at 80 μM concentration (*A*) and FNR* DA154 loaded at 80 μM (solid line) and 8.0 μM (broken line) concentration (*B*). (*Inset*) A calibration curve using BSA, carbonic anhydrase, and cytochrome *c* as standards. The elution peak of FNR* DA154 and wild-type FNR proteins, when loaded at 80 μM concentration, is indicated on the graph by arrows.

2A). This value is consistent with previous observations that wild-type FNR has a molecular mass of a monomer (Green et al. 1991). In contrast, at the same concentration of protein, DA154 eluted reproducibly at a larger apparent molecular weight of 45,000 (Fig. 2B). Such a difference could be attributable to a conformational change of the monomeric protein that is induced by the DA154 substitution, or it may reflect a change in the oligomeric state of the majority of the DA154 protein.

To test which of these possibilities was correct, the DA154 preparation was diluted 10-fold and analyzed by S-200 gel-exclusion chromatography (Fig. 2B). Its apparent molecular mass shifted to that of the monomeric form. This result suggests that the larger apparent molecular mass of the DA154 mutant is not attributable to a conformational change in a DA154 monomer, as the apparent molecular mass of a monomeric species should not be concentration dependent. Therefore, an alteration in oligomeric state of the DA154 protein is the likely cause of the increased apparent molecular mass. Because the DA154 substitution is located in a region of FNR that is analogous to the CAP site of dimerization, the simplest interpretation of these experiments is that the DA154 substitution appears to change the dimerization constant of the FNR protein to favor the formation of dimers.

FNR* mutant DA154 has increased DNA-binding activity relative to wild-type FNR

If the increased activity of DA154 under aerobic conditions in vivo is attributable to the presence of a higher concentration of dimers, then the purified DA154 protein might also be more active than the monomeric wild-type protein in vitro. To test this possibility, the DNA-binding activity of purified DA154 and wild-type FNR protein was compared in a gel-retardation assay using a consensus FNR site as the DNA target (Fig. 3). The DA154 and wild-type proteins produced the same size protein–DNA complex, indicating that the same protein species is bound to the DNA. However, at the highest
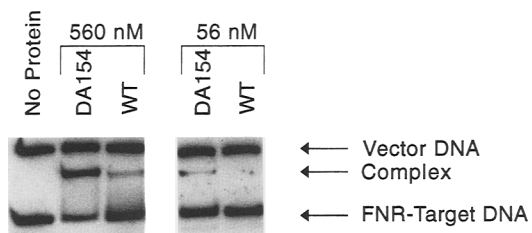

Figure 3. Comparison of the DNA-binding activity of FNR* DA154 and wild-type FNR proteins. The source of the protein for this gel-retardation analysis was from the peak FNR fraction of the Q Sepharose column. The migration of the 2.7-kb plasmid DNA (Vector DNA), the 48-bp consensus FNR target site (FNR–Target DNA), and the FNR/DNA complex (Complex) are indicated. The concentration and protein assayed are indicated in each lane.

concentration of protein tested, DA154 bound a greater percentage of the target DNA than wild-type FNR. Moreover, at lower concentrations of protein, 56 nM, DNA-binding activity was only detected for DA154. Thus, it appears that the FNR* DA154 protein has increased DNA-binding activity relative to wild-type FNR in vitro under aerobic conditions. Because this increased DNA-binding activity is correlated with an increased oligomeric state of DA154, this suggests that the dimer form of FNR may be the active DNA-binding species.

Dominant-negative FNR mutants inhibit the activity of wild-type FNR

Work with other oligomeric DNA-binding proteins has shown that DNA-binding defective mutants are dominant to the wild-type protein as a result of to the formation of inactive mixed oligomers (Miwa and Sadler 1977; Isackson and Bertrand 1985; Kelley and Yanofsky 1985). If a dimer of FNR represents the DNA-binding competent form of this protein, then it should be possible to isolate dominant-negative (DN) mutants that inactivate the wild-type FNR by subunit poisoning. Four independent FNR mutants that had a DN phenotype were isolated and characterized.

To demonstrate directly that the mutants have a DN phenotype, the FNR phenotype of the DN mutants in both a Δfnr and fnr+ background was compared. The FNR phenotype was determined by the level of transcription activation of a Φ(narG–lacZ) fusion as assayed by the amount of β-galactosidase activity produced under anaerobic conditions in vivo. As expected on the basis of the initial plate phenotype of these mutants in a Δfnr strain, all of the DN mutants showed a complete loss of FNR function, exhibiting as little β-galactosidase activity under anaerobic conditions as the parental Δfnr strain (Fig. 4A). To quantitate the dominance phenotype of the mutants, we assayed the levels of β-galactosidase in strains containing a chromosomal fnr+ gene and a plasmid-encoded DN fnr− gene under anaerobic conditions. Depending on the particular mutant allele, the DN mutants reduced the level of β-galactosidase activity 5- to 14-fold (Fig. 4B). The phenotype of these mutants is not specific to the narG promoter, as these mutants were also defective in activating an FNR-dependent, mutant lac promoter (Zhang and Ebright 1990) (Fig. 4C), and inhibited wild-type FNR activation of this promoter in vivo (data not shown).

Sequence changes in the DN FNR mutants

DNA sequence analysis showed that each DN mutant contained a single amino acid substitution in the putative DNA-binding domain of FNR (Fig. 5). The Glu → Lys substitution at position 209 (EK209) was isolated five times independently. The other three substitutions, a Ser → Arg and a Ser → Asn change at position 212 and an Arg → Cys at position 213, were each isolated only once. All of the FNR DN substitutions affect amino acids predicted to be involved in site-specific DNA bind-
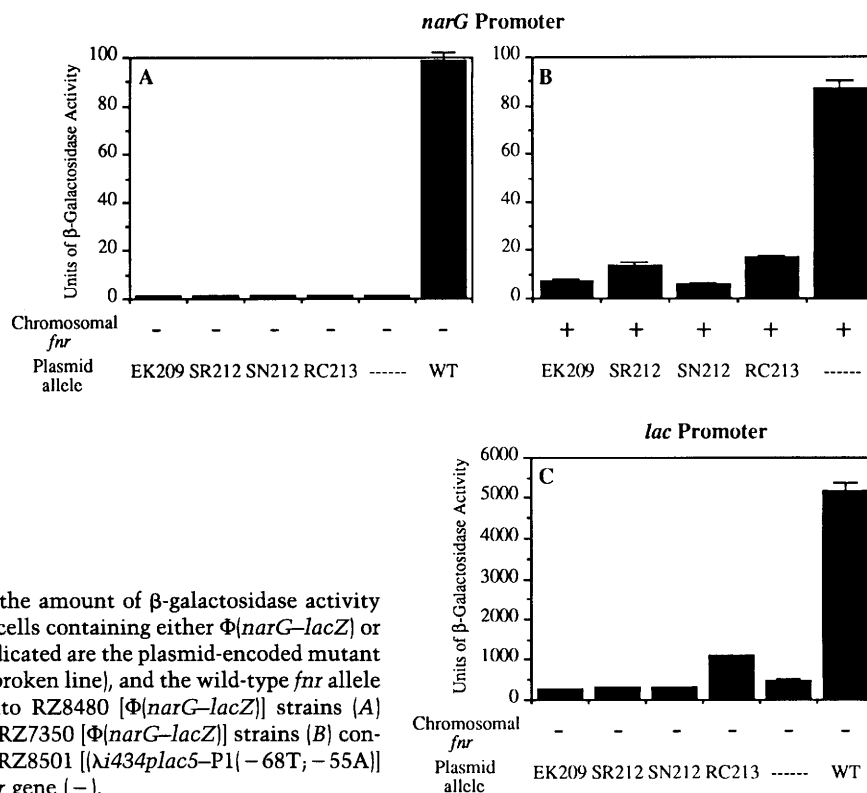
Figure 4. FNR activity was measured by the amount of β-galactosidase activity (Miller units) produced anaerobically from cells containing either Φ(narG–lacZ) or a FNR-dependent, mutant lac promoter. Indicated are the plasmid-encoded mutant fnr alleles, the control vector, pACYC184 (broken line), and the wild-type fnr allele (WT). These plasmids were introduced into RZ8480 [Φ(narG–lacZ)] strains (A) deleted for the chromosomal fnr gene (−), RZ7350 [Φ(narG–lacZ)] strains (B) containing a chromosomal fnr⁺ gene (+), and RZ8501 [(λi434plac5–P1(−68T; −55A)] strains (C) deleted for the chromosomal fnr gene (−).

ing (Schultz et al. 1991; Spiro and Guest 1990). Therefore, these DN substitutions are likely to disrupt base-specific contacts between FNR and its DNA target site (see Discussion and Fig. 8, below).
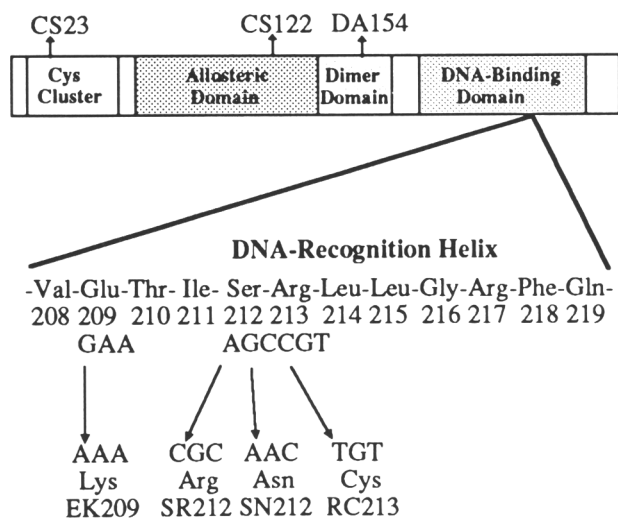

Figure 5. Sequence changes in DN FNR mutants. The base substitutions were determined by DNA sequencing as described in Materials and methods and are indicated by arrows. The assignment of the domains in FNR is based on the alignment of FNR to CAP (Shaw et al. 1983). The locations of other FNR substitutions used in this study are indicated above the schematic of the FNR protein.

DN FNR mutants are defective in binding DNA in vitro

Considering the location of the mutant substitutions, the most likely explanation for their dominance phenotype is that they are defective in DNA binding and poison the activity of wild-type FNR by forming mixed oligomers of wild-type and mutant subunits. Alternatively, the mutant proteins could bind DNA but fail to activate transcription. In this case, the DN mutants would exhibit a dominant phenotype because they occlude the DNA site and prevent wild-type FNR binding.

To distinguish between these possibilities, we tested whether the DN mutant proteins bind specifically to an FNR target site. Because the levels of wild-type FNR produced from a multicopy plasmid are not sufficient to demonstrate DNA binding in crude cell extracts using a gel-retardation assay, we took advantage of the fact that the DA154 protein shows site-specific DNA binding under these conditions and placed the DN mutations in the context of the DA154 protein. As expected, binding of the FNR target site is dependent on the extracts containing DA154 (Fig. 6, lane 3), as no binding was observed with extracts from strains carrying only the plasmid vector (Fig. 6, lane 2). However, introduction of any of the four DN substitutions into DA154 abolished formation of this specific complex (Fig. 6, lane 4–7). This lack of DNA binding was not attributable to reduced levels of these mutant proteins in these extracts, as immunoblot analysis with anti-FNR serum indicated that the abundance of these mutant proteins was equal to or greater
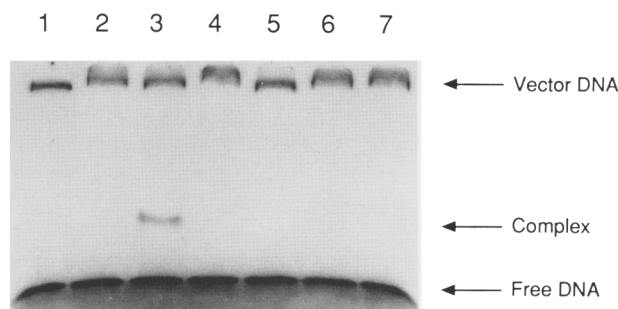
Figure 6. The DNA-binding activities of FNR* mutant DA154 and the DN derivatives of DA154 were determined by gel-retardation assay. The source of cell extracts in the assay (final concentration, 440 ng/ml) was from strains containing either the vector pGem-2 (lane 2) or plasmids carrying either FNR* DA154 (lane 3) or the derivatives of FNR* DA154 which also contain the DN substitutions (lane 4, DA154–EK209; lane 5, DA154–SR212; lane 6, DA154–SN212; lane 7, DA154–RC213). Lane 1 contains no cell extract. The migration of the 2.7-kb plasmid DNA (Vector DNA), the 48-bp consensus FNR target site (Free DNA), and the FNR–DNA complex (Complex) are indicated.

than that of DA154 (data not shown). Lack of DNA binding does not appear to be attributable to a disruption of FNR conformation in the DN derivatives of DA154, as these derivatives of DA154 retained their DN phenotype in vivo (data not shown). Taken together, these results indicate that the DN mutant proteins have a specific defect in binding to an FNR target site. Therefore, we conclude that the dominance phenotype of the mutants is likely to result from the formation of inactive mixed oligomers between mutant and wild-type FNR subunits rather than from the occlusion of the target site.

The dominance of DNA-binding domain mutant EK209 is abolished by aerobic conditions

Because our studies suggest that the DA154 substitution activates FNR aerobically by stabilizing dimerization, we then considered the possibility that wild-type FNR is inactive under aerobic conditions because it is monomeric. To determine the oligomeric state of the inactive form of FNR, we sought to determine whether DN FNR mutants would form mixed oligomers under aerobic conditions. The oligomeric state of the DN mutants under aerobic conditions should represent that of wild-type FNR, as the mutants appear to be only specifically defective in DNA binding and should be regulated by oxygen deprivation as with wild-type FNR. It is not possible to test directly whether the DN mutants inhibit wild-type FNR under aerobic conditions, because wild-type FNR is inactive under these conditions. However, as the FNR* mutant DA154 is active in the presence of oxygen, we tested whether one of the DNA-binding defective mutants, EK209, could inhibit the activity of DA154 in vivo.

To determine whether EK209 and DA154 are capable of forming mixed oligomers, we first tested whether EK209 could inhibit DA154 activity under anaerobic

conditions, as this is the condition where both FNR derivatives are expected to be oligomeric. To serve as a control for mixed oligomer formation, we also determined whether a mutant protein containing both the DA154 and EK209 substitutions was able to inhibit the activity of DA154. Strains were constructed in which the EK209 mutant, alone or in combination with the DA154 substitution, was introduced on a multicopy plasmid into a strain containing the FNR* DA154 allele on the chromosome and the Φ(narG–lacZ) fusion. The level of transcription activation by DA154 of the Φ(narG–lacZ) fusion decreased fourfold under anaerobic conditions in the presence of the EK209 mutant or the mutant containing both the DA154 and EK209 substitutions (Table 1), indicating that the EK209 and DA154 mutant proteins were able to form mixed oligomers. To determine whether the DA154 and EK209 mutant proteins were able to form mixed oligomers under aerobic conditions in vivo, these same strains were assayed under aerobic conditions. The mutant protein containing both the DA154 and EK209 substitutions inhibited the activity of DA154 15-fold (Table 1). In contrast, the EK209 mutant only inhibited activation by DA154 twofold under these conditions. A simple explanation for the inability of EK209 subunits to inhibit activity of DA154 subunits under aerobic conditions is that in the absence of the DA154 substitution, the EK209 subunits are largely monomeric.

The essential cysteines of FNR are required for the dominance phenotype of the DNA-binding domain mutant EK209

To further test the idea that wild-type FNR is monomeric in the inactive state, we tested the oligomeric state of FNR mutants that are inactive. Substitutions in individual cysteines of FNR are thought to lock the protein in an inactive conformation, because mutation of these cysteines prevents FNR activation under anaerobic conditions (Spiro and Guest 1988; Trageser and Unden 1989; Melville and Gunsalus 1990). These cysteine mutants are not dominant because they do not inhibit the

Table 1. The dominance phenotype of the DNA-binding domain mutant, EK209, is abolished by aerobic conditions

Location and source of *fnr* allele[a]		Units of β-galactosidase[b]	
chromosome	plasmid	aerobic	anaerobic
DA154	—	304 (±25)	929 (±39)
DA154	DA154–EK209	20 (±1)	235 (±10)
DA154	EK209	142 (±3)	212 (±10)

[a]The *fnr* allele encoded on the chromosome is carried by a λ–*fnr* derivative (Kiley and Reznikoff 1991). The plasmids encoding the *fnr* alleles are derivatives of pRZ7411. These strains are derivatives of PK465.
[b]The units of β-galactosidase are reported in Miller units. The strains were grown either aerobically or anaerobically in the presence of 20 mM KNO_3 as described in Materials and methods. The numbers in parentheses indicate the standard error.

activity of wild-type FNR under anaerobic conditions (Melville and Gunsalus 1990). This could be the result of the cysteine mutants being in a monomeric conformation, or it could be the result of FNR not requiring both subunits of a dimer to possess functional cysteines to be active. Therefore, to assay the oligomeric state of these cysteine mutants, the EK209 substitution was placed in the context of two different Cys → Ser mutants (CS23 and CS122), and the ability of these mutant subunits to form mixed oligomers with wild-type FNR in vivo under anaerobic conditions was determined.

The CS alleles abolished the dominance phenotype of EK209, as each of the plasmid-encoded CS–EK209 derivatives inhibited the activity of a chromosomal *fnr*⁺ gene only twofold under anaerobic conditions (Fig. 7B). The twofold inhibition by the CS–EK209 subunits was similar to that observed by the CS subunits not containing the EK209 substitution. In contrast, the EK209 subunit inhibited wild-type FNR activity 12-fold under these conditions. A simple explanation for the inability of the CS–EK209 derivatives to inhibit wild-type FNR is that these cysteine substitutions lock FNR in a conformation that does not allow oligomerization under anaerobic conditions. This suggests that the inactive form of wild-type FNR may be monomeric. These data, along with the evidence that the DNA-binding domain mutants lack a dominant phenotype under aerobic conditions, suggest that at least one step in FNR activation involves a change in oligomeric state.

Discussion

The DN FNR mutants are defective in DNA binding

The isolation of DN FNR mutants has provided additional support for the predicted DNA contacts made by this protein. DN mutants of several other oligomeric DNA-binding proteins commonly map to amino acids involved in target site recognition (Miwa and Sadler 1977; Isackson and Bertrand 1985; Kelley and Yanofsky 1985); therefore, we expected a major class of these mutants to disrupt the protein–DNA interactions of FNR. All of the substitutions mapped to the 3 amino acids in the putative DNA-binding domain of FNR predicted to make base-specific contacts with the FNR target site based on the similarity of FNR to CAP and genetic experiments altering the DNA-binding specificity of FNR (Fig. 8). In CAP, Glu-181 contacts the third position, C, in the DNA half-site, and Arg-185 contacts both the fourth position, T, and the third position, G, on the other strand (Schultz et al. 1991). This Glu (E209) and Arg (R213), as well as the bases that they contact, are conserved in both the FNR protein and its DNA half-site. Two of the three amino acids mutated in the DN mutants map to these positions (EK209 and RC213). Spiro and Guest (1990) have also isolated different substitutions at Glu-209 that prevent FNR function, but this is the first report of a substitution at Arg-213. The third amino acid altered in the DN FNR mutants, Ser-212, is not conserved in CAP, but other genetic experiments (Spiro and Guest 1987) suggest that Ser-212 of FNR participates in discrimination between the CAP and FNR sites by contacting the first position T : A base pair in the FNR half-site.

A defect in DNA binding by these mutants was shown by the inability of the DN mutants to bind to an FNR consensus–DNA site in a gel-retardation assay. This suggests that alterations in any of the indicated amino acids decrease DNA binding by disrupting a contact between FNR and its DNA target site. However, further in vitro experiments will be needed to determine the magnitude of their effect on DNA binding.

Is the activity of FNR regulated by a change in oligomeric state?

In addition to gaining insights about the protein–DNA contacts of FNR, analysis of the changes in the dominance phenotype of these DN mutants in response to oxygen has yielded new information about how the activity of FNR may be regulated. The data presented in this paper have led us to propose a model in which one aspect of the regulation of FNR activity by oxygen deprivation involves a change in the oligomeric state of FNR (Fig. 9). In the presence of oxygen, we propose that FNR

Figure 7. The effect of the cysteine substitutions on the dominance phenotype of the EK209 mutant was determined by measuring the ability of the EK209 derivatives to inhibit wild-type FNR activation of Φ(*narG–lacZ*) under anaerobic conditions. The level of Φ(*narG–lacZ*) expression is reported in Miller units of β-galactosidase activity. Indicated are the plasmid-encoded mutant *fnr* alleles, the control vector, pACYC184 (broken line), and the wild-type *fnr* allele (WT). These plasmids were introduced into RZ8480 strains (*A*) deleted for the chromosomal *fnr* gene (−), and RZ7350 strains (*B*) containing a chromosomal *fnr*⁺ gene (+).
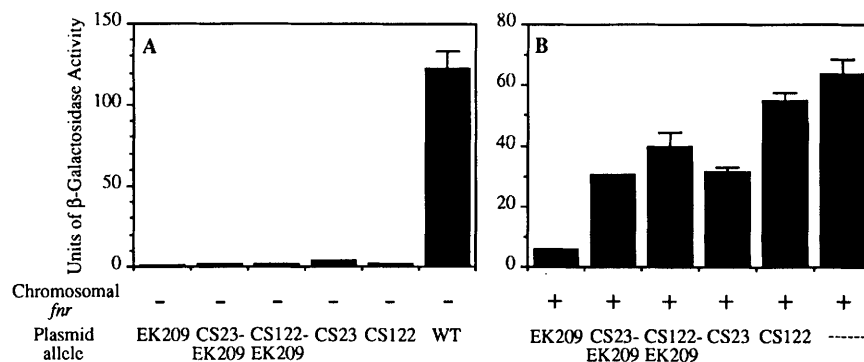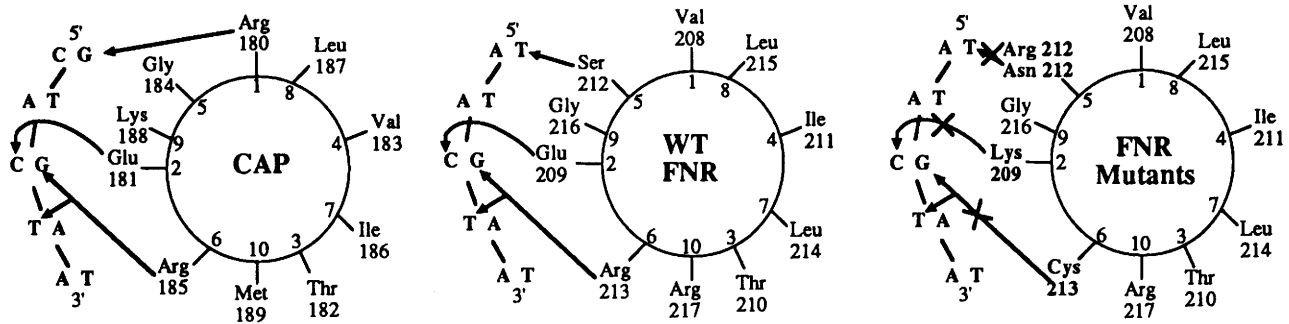
Figure 8. DNA recognition helices of CAP, FNR, and mutant FNR derivatives are shown, as well as their DNA target sites. The contacts shown between CAP and its target site are based on the interactions seen in the crystal structure of CAP bound to its DNA site (Schultz et al. 1991). On the basis of this recent crystal structure, we have modified the figure of Spiro and Guest (1990) to include the additional amino acid contact of Arg-213 of FNR with its DNA target site. The Xs are drawn to suggest a disrupted contact between a mutant FNR and its DNA target site.

is largely in a monomeric form and, thus, is unable to bind to the FNR DNA target site with high specificity and affinity. Under anaerobic conditions, a conformational change in FNR occurs, which then promotes dimerization, and it is the dimeric form of FNR that is able to bind to DNA target sites with high specificity and regulate transcription. The data that support this model are discussed.

The FNR* mutant DA154 has an altered monomer-to-dimer equilibrium

If FNR is regulated by a change in oligomeric state, then one mechanism by which a mutant can bypass the normal regulation pathway and be active in the presence of oxygen is to alter the monomer-to-dimer equilibrium to favor the formation of dimers. The analysis of the FNR* mutant DA154 suggested that this may be the mechanism by which this mutant bypasses the normal regulation pathway. This analysis showed that DA154 had a larger apparent molecular weight of 45,000, as compared with wild-type FNR of 33,000. The simplest interpretation of the increased molecular mass is that DA154 is dimeric, because it was possible to shift the molecular mass of DA154 to that of the monomer form by dilution. The less than twofold difference in the molecular mass of the monomer and dimer forms of DA154 may be attributable to underestimating the molecular mass of the dimer because it was dissociating during chromatography, as evidenced by the trailing edge of the protein elu-

Figure 9. Model of FNR regulation in response to oxygen deprivation. Each circle represents a monomeric subunit of FNR. A description of the model is provided in the text.

tion profile of DA154 (see Fig. 2). Also, the apparent molecular mass of the dimer need not be twice that of the monomer form if the subunits are more compact in the dimer than in the monomer. Thus, at the concentration of protein assayed, our data are consistent with the oligomeric state of a dimer of DA154, although our experiments do not rule out that a higher order oligomer could form at increased protein concentrations. However, we believe that a dimer is likely to be the relevant in vivo oligomeric species because the cellular concentration of FNR is in the micromolar range (Unden and Guest 1985), and this is similar to the concentrations of FNR used in this in vitro analysis.

Because the DA154 substitution maps to the region analogous to the CAP dimerization domain, the difference in the apparent molecular mass of DA154 and wild-type FNR could be attributable to the DA154 substitution directly altering the dimerization constant of FNR. The DA154 protein also showed greater specific DNA-binding activity than wild-type FNR. A change in the dimerization constant of DA154 would account for the increased binding activity of DA154 for the FNR target site relative to wild-type FNR because for proteins that bind to DNA as dimers, the apparent DNA-binding constant is a function of both the DNA-binding constant and the dimerization constant (Chadwick et al. 1971). Therefore, if a substitution favored dimerization, this would be manifest in an increased binding affinity of FNR for its target DNA. In the isolation of FNR* mutants that were active in the presence of oxygen (Kiley and Reznikoff 1991), four mutants were isolated that contained different substitutions in the putative dimerization domain of FNR. If the activity of FNR is regulated by a change in oligomeric state, then a major class of mutants that cause FNR to be active in the presence of oxygen should alter the dimerization constant of FNR to favor dimer formation. Future experiments will test whether other dimerization domain substitutions will have similar effects on the FNR protein.

Although the putative dimerization domain of FNR is defined on the basis of the alignment of the amino acid sequence of FNR with that of the transcription factor
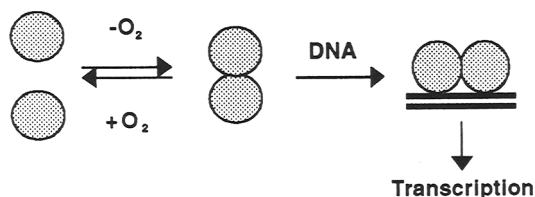
CAP (Cherfils et al. 1989), the dimerization properties of FNR and CAP appear to be different. Whereas the oligomeric state of FNR may change in response to its allosteric effector, CAP exists as a dimer in the presence and absence of its allosteric effector, cAMP (Anderson et al. 1971; Takahashi et al. 1980). It is not surprising that the dimerization properties of CAP and FNR should be different because CAP and FNR share very little amino acid sequence similarity in the dimerization region (Cherfils et al. 1989).

The active form of FNR is dimeric

On the basis of our current state of knowledge, our simplest model is that the active form of FNR is a dimer. The dominance phenotype of the DNA-binding defective mutants of FNR is consistent with this idea. Given our data, the simplest explanation for the dominance phenotype of these DNA-binding defective mutants is that the mutant subunits oligomerize with and inhibit the activity of the wild-type protein. It is likely that these mixed oligomers have a decreased affinity for the DNA target site. This is analogous to having a FNR target site with one half-site mutated, and FNR has been shown to have a decreased binding affinity for such mutated sites (Bell et al. 1989). DNA-binding domain mutants of other oligomeric transcription factors, such as LacR, TrpR, and TetR (Miwa and Sadler 1977; Isackson and Bertrand 1985; Kelley and Yanofsky 1985), also inhibit the activity of the wild-type protein by forming mixed oligomeric proteins. The fact that similar mutants of FNR inhibit the activity of the wild-type protein suggests that the functional form of FNR is oligomeric. Furthermore, the increased DNA-binding activity of the dimeric FNR* DA154 protein relative to monomeric wild-type FNR also suggests that the active DNA-binding species is a dimer. These two pieces of data taken together strongly suggest that the active form of FNR found in vivo is a dimer.

The inactive form of FNR is monomeric

The inability of the DNA-binding defective mutant EK209 to form mixed dimers with the FNR* mutant DA154 under aerobic conditions in vivo has provided genetic evidence to suggest that the oligomeric state of the inactive form of FNR is monomeric. We believe that the apparent monomeric state of the EK209 mutant protein under aerobic conditions represents that of wild-type FNR, because the EK209 substitution should only abolish DNA binding and not alter its regulation by oxygen. In addition, the CS23 and CS122 substitutions, which prevent FNR from being activated under anaerobic conditions (Spiro and Guest 1988; Trageser and Unden 1989; Melville and Gunsalus 1990), abolished the dominance phenotype of a subunit that also contained the EK209 substitution. Because the CS mutants are believed to be locked in the inactive state, this result also suggests that the inactive form of FNR is monomeric. If

the inactive form of FNR is monomeric, this explains why wild-type FNR from aerobic cells purifies as a monomer and further suggests that FNR purifies as a monomer from anaerobic cells because some factor is lost that is necessary to stabilize the dimer form.

Wild-type FNR exhibits specific DNA binding

The idea that FNR functions as a dimer is not inconsistent with the fact that purified FNR behaves as a monomer and has DNA-binding activity. DNA-binding activity was only observed at protein concentrations greater than was necessary for FNR* DA154 to bind to DNA. This requirement for increased protein concentration may define the limits necessary to detect a small population of active wild-type dimers capable of DNA binding. The ability to form active FNR at increased protein concentrations is also supported by the increased activity of FNR in vivo under aerobic conditions when fnr is placed on a multicopy plasmid (Shaw and Guest 1982). Increasing the concentration of FNR present in the cell may increase the amount of dimers present under aerobic conditions, and this could result in cells containing active FNR under conditions when it is normally inactive. Evidence that wild-type FNR protein may form dimers is supported by the protein elution profile of wild-type FNR on the gel-exclusion column. This showed a small leading edge that overlaps the elution peak obtained with the dimeric FNR* DA154 protein, suggesting that a small fraction of the wild-type protein at that concentration is dimeric (Fig. 2A). In addition, two other pieces of data also support that a dimer of wild-type FNR is the species bound to DNA. First, Green et al. (1991) have shown by DNA footprinting that FNR protects both halves of the palindromic target, which suggests that a dimer of FNR is bound to the DNA. Second, in our gel-retardation assay, the wild-type FNR–DNA complex migrated to the same position as the complex obtained with the largely dimeric DA154 protein. This also suggests that a dimer of wild-type FNR may be bound to the DNA.

Is the activity of FNR regulated by a change in oligomeric state?

Regulating the monomer-to-dimer equilibrium of an oligomeric protein would be a very effective way to control the DNA-binding activity of this protein. Precedence for regulating the activity of a protein by dimerization can be found in both prokaryotic and eukaryotic systems. For example, the requirement for a protein to dimerize to bind to a symmetrical DNA site with high affinity and specificity has been shown for the λ repressor protein (Pabo et al. 1979; Sauer et al. 1979; Weiss et al. 1987). Also, the RNA-binding activity of the BglG protein is regulated by dimerization of this protein in response to phosphorylation (Amster-Choder and Wright 1992). Dimerization is used to regulate the DNA-binding activity of eukaryotic transcription factors such as the

homeo domain protein, hepatocyte nuclear factor-1α, which requires a cofactor to dimerize and bind to DNA (Mendel et al. 1991). Also, the tyrosine kinase activity of eukaryotic growth factor receptors, such as the human growth hormone receptor (Cunningham et al. 1991) and the epidermal growth factor receptor (Greenfield et al. 1989), is regulated by dimerizing on binding of a specific hormone (Ullrich and Schlessinger 1990).

In summary, our genetic and biochemical analysis of mutant FNR proteins has led us to propose that one component in the mechanism of regulating FNR activity in response to oxygen deprivation is a change in its oligomeric state. Importantly, this model predicts that wild-type FNR purifies as a monomer from anaerobically grown cells (Green et al. 1991) because of the loss of some factor upon purification that is necessary to achieve or stabilize the dimeric form of FNR. The physiological factor that regulates FNR in response to oxygen deprivation is still unclear, but this investigation should help in the identification of this factor, as dimer formation may be used as an assay for FNR activation. For example, it is known that Fe^{2+} is required for FNR activity in vivo (Spiro et al. 1989), and recently, Green et al. (1991) have shown that iron appears to bind to FNR. Whether iron plays a role in regulating dimer formation of FNR in vivo is unclear, because iron is not sufficient to stimulate dimer formation of the wild-type protein in vitro (Green et al. 1991). However, this study has provided a model for the regulation of FNR activity that can be tested further biochemically.

Materials and methods

Growth conditions and strain constructions

For routine strain manipulations, cells were grown in LB medium at 37°C. When needed, chloramphenicol (Cm), spectinomycin (Sp), or streptomycin (Sm) were used at concentrations of 20 μg/ml; ampicillin (Ap) and tetracycline (Tc) were used at 50 and 10 μg/ml, respectively. Phage (P1 and λ) manipulations were performed essentially as described by Silhavy et al. (1984). Lambda lysogens were shown to contain a single copy of λ by the ter excision test (Mousset and Thomas 1969).

E. coli K12 strains and plasmids used in this study are described in Table 2. Chromosomal mutations were introduced into desired strains by P1 transduction. The Δ*fnr* allele is a deletion created by cloning an Sm^r, Sp^r Ω cassette (Prenkti and Krisch 1984) into the *BsmI–MluI* restriction sites of *fnr*. The Δ*crp-bs990* allele (Garges and Adhya 1988) was introduced by either selecting for the Sm-resistant phenotype of the closely linked *rpsL* and scoring for the CAP$^-$, small-colony phenotype on LB medium, or selecting for the Tc-resistant phenotype of the closely linked *zhe*-3085::Tn*10* (Singer et al. 1989). The *galE* allele (Malamy 1966) was transduced into a *nadA*57::Tn*10* (Singer et al. 1989) strain, selecting for the closely linked NadA$^+$ marker by growth on M63 minimal medium (Miller 1972) containing 20 mM glucose, and scoring NadA$^+$ transductants for sensitivity to 20 mM galactose.

Construction of FNR-overproducing strains

Plasmids were constructed that place either wild-type FNR or FNR* DA154 protein under the control of a T7 φ*10* promoter and the gene *10* ribosome-binding site (Dubendorff and Studier

Table 2. *Strains and plasmids used in this study*

Strain or plasmid	Relevant genotype	Reference
E. coli strains		
RZ4500	*lacZ*Δ145	Choe and Reznikoff (1991)
RZ7350	as RZ4500 but *narG*234::MudI1734	Kiley and Reznikoff (1991)
RZ8480	as RZ7350 but Δ*fnr*	this study
RZ8501	as RZ4500 but Δ*fnr*, Δ*crp–bs990*, *zhe*-3085::Tn*10*, (λ*i434plac5*–P1 (−68T; −55A) (Zhang and Ebright 1990)	this study
PK22	as BL21(DE53) but Δ*crp–bs990*, *rpsL*, Δ*fnr*, *zcj*-3061::Tn*10* (Singer et al. 1989)	this study
PK460	as RZ8480 but *galE*	this study
PK465	as RZ8480 but λ*fnr*–DA154	this study
BL21(DE53)	*hsdS*, *gal*, λDE53	Studier and Moffatt (1986)
Plasmids		
pET-11a	Ap^r, T7 φ*10* promoter and gene *10* translation start preceding *Nde*I site	Dubendorff and Studier (1991)
pPK14	Ap^r, *Hind*III–*Sac*I of pRZ7315, −521 to +817 of *fnr*–DA154 in pGem-2 (Promega)	this study
pPK810C	Ap^r, FNR–consensus site in *Hind*III site of pUC19 (Yanisch-Perron et al. 1985)	this study
pPK821	Ap^r, *Hind*III–*Bam*HI of *fnr*, −521 to +1115 of *fnr* with *Nde*I site at +1 in pUC118 (Vieira and Messing 1987)	this study
pPK822	same as pPK821 but *fnr*–DA154	this study
pPK823	Ap^r, *Nde*I–*Bam*HI of pPK821, +1 to +1115 of *fnr* in pET-11a	this study
pPK824	same as pPK823 but *fnr*–DA154	this study
pRZ7315	Ap^r, *Hind*III–*Hinc*II of *fnr*, −521 to +817 of *fnr* in *Hind*III and *Sma*I sites of pUC19	Kiley and Reznikoff (1991)
pRZ7411	Cm^r, *Hind*III–*Bam*HI of *fnr*, −521 to +1115 of *fnr* in pACYC184 (Chang and Cohen 1978)	this study

1991]. To clone *fnr* genes into the overexpression plasmid, site-directed mutagenesis (Kunkel 1989) was used to create a *NdeI* site that overlaps the *fnr* start codon. To do this, *fnr* genes were cloned as *Hind*III–*Bam*HI fragments (Kiley and Reznikoff 1991) into the same sites of the single-strand generating phagemid pUC118 (Vieira and Messing 1987) to generate pPK821 (wild-type FNR) and pPK822 (FNR* DA154). Single-stranded, uracil-containing DNA of this phagemid was obtained from the *dut ung* strain RZ1032 (Kunkel et al. 1987). Oligonucleotides were synthesized that change base −2 from a C to an A (base 1 is the A of the initiation ATG of *fnr*), creating an *NdeI* restriction site that overlaps the *fnr* start codon (CCTATG → CATATG). The oligonucleotides were annealed to the single-stranded template, and the complementary strand was synthesized using T4 DNA polymerase (Pharmacia). The entire *fnr* gene was sequenced from Ap^r clones to confirm the creation of the *NdeI* site and demonstrate the absence of any additional mutations. A *NdeI*–*Bam*HI fragment from each of the resulting plasmids was then cloned into the same sites of the expression vector pET-11a (Dubendorff and Studier 1991) to generate pPK823 (wild-type FNR) and pPK824 (FNR* DA154). In these plasmids, *fnr* transcription is under the control of a T7 promoter, and translation is controlled from the gene *10* ribosome-binding site. To make FNR synthesis isopropyl-thio-β-D-galactoside (IPTG) -inducible, these plasmids were introduced into strain PK22, a Δ*fnr*, Δ*crp* BL21 derivative, which has the gene for T7 RNA polymerase under the control of an IPTG-controlled *lac* promoter (Studier and Moffatt 1986).

Purification of FNR

FNR proteins were purified from PK22 strains carrying either pPK823 or pPK824. The cells were grown aerobically in 4 liters of LB medium plus 0.2% glucose and ampicillin at 37°C to an OD$_{600}$ of 0.3–0.5, and IPTG was added to a final concentration of 400 μM for 45 min to induce FNR synthesis. The cells were chilled on ice, and all subsequent steps in FNR purification were done at 4°C. The cells were harvested by centrifugation, concentrated 200-fold in buffer A [50 mM potassium phosphate (pH 6.8), 0.1 mM EDTA, 0.1 M KCl, 10% glycerol, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethanesulfonylfloride (PMSF)], and passed through a French press three times at 20,000 psi. The extracts were centrifuged in a SS-34 rotor at 7000 rpm for 30 min to remove cell debris and subsequently centrifuged at 45,000 rpm in a Beckman 70.1 Ti rotor for 1 hr to remove the membrane fraction. At each subsequent step, the protein concentration was estimated by a Bradford assay (Bradford 1976) using the Commassie Plus Protein Assay Reagent (Pierce), and purity was assessed on a 12% SDS–polyacrylamide gel (Bollag and Edelstein 1991) using a 30 : 0.8 acrylamide/bis-acrylamide ratio.

Cell extracts were passed over a Bio-Rex 70 (Bio-Rad) column (0.9 × 7.5 cm) at a flow rate of 10 ml/hr and eluted with a 70-ml linear gradient of 0.1–0.5 M KCl in buffer A. One-milliliter fractions were collected and assayed for DNA-binding activity by gel retardation. Fractions containing DNA-binding activity were pooled and dialyzed against buffer B [10 mM Tris (pH 7.9), 0.1 mM EDTA, 10% glycerol, 50 mM KCl, 1 mM DTT, and 0.1 mM PMSF]. After centrifuging in a SS-34 rotor at 10,000 rpm for 10 min to remove any precipitate, the dialyzed protein was then loaded onto a Q Sepharose (Pharmacia) column (0.9 × 7.5 cm) at 10 ml/hr and eluted with a 70-ml linear gradient of 0.05–0.5 M KCl in buffer B. Fractions of 1 ml were collected and assayed for DNA binding as described above.

To remove residual amounts of contaminating protein and assess the apparent molecular mass of FNR proteins, 400 μl of the Q Sepharose fractions containing FNR activity was adjusted to 0.2 M KCl and passed over a Sephacryl S-200 (Pharmacia) column (1.5 × 50 cm) equilibrated with buffer B at 0.2 M KCl at a flow rate of 2.5 ml/hr. The eluant from the Sephacryl S-200 column was monitored for absorbance at 280 nm using an LKB 2138 Uvicord S monitor coupled to an LKB 2210 Potentiometric Recorder (Pharmacia). Bovine serum albumin, carbonic anhydrase, and cytochrome *c* protein standards (Sigma) were used to calibrate the S-200 column. The molecular mass of the FNR proteins was determined by linear regression analysis. The purified protein was shown to react with anti-FNR antibody (kindly provided by G. Unden, Heinrich-Heine-Universitat, Düsseldorf, Germany) in an immunoblot analysis (data not shown) and to bind specifically to the FNR target site.

DNA-binding assay

The DNA-binding activity of FNR protein was assessed by a gel-retardation assay. A 48-bp fragment (5'-*AGCTT*GCAAC-GCAATAAATTTGATGTACATCAAATT*TT*AGGCACCCCA-3'), containing the 22-bp FNR–consensus site (bold) flanked by *lac* promoter sequences (underlined) and *Hind*III sites (italicized), was generated by annealing two synthetic complementary deoxyoligonucleotides. A portion of 5 mM of each oligonucleotide in 10 mM Tris, 0.1 mM EDTA, and 125 mM NaCl was heated together at 90°C for 10 min and then allowed to cool to room temperature. This annealed 48-bp fragment was then cloned into the *Hind*III site of pUC19 (Yanisch-Perron et al. 1985) to generate pPK810C, and this plasmid was shown to contain a single insert by sequencing. Cesium-banded (Sambrook et al. 1989) plasmid DNA of pPK810C was used as the source of FNR target DNA in this assay. This was cut with *Hind*III and end-labeled with 5 μCi of [α-^{32}P]dATP using AMV reverse transcriptase. The labeled DNA, consisting of both the 48-bp FNR consensus site and the 2.7-kb vector DNA, was then purified by extraction with phenol/chloroform and elution through a Sephadex G-25 spun column. The DNA-binding assay was performed by mixing protein with reaction buffer at 37°C for 10 min to achieve a final concentration of 5% glycerol, 50 mM EDTA, 30 μg/ml of calf thymus DNA, 1× KGB buffer (McClelland et al. 1988), and 58 pM labeled 48-bp fragment DNA. This mixture was loaded onto a prerun, 5% polyacrylamide gel (38 : 2 acrylamide/bis-acrylamide ratio) and electrophoresed in 1× TBE buffer (Sambrook et al. 1989) for 1.5 hr at 8.8 V/cm. Using these conditions, the DNA-binding activity has been shown to be specific for the FNR DNA site (Ziegelhoffer and Kiley, unpubl.).

Selection for DN FNR mutants

FNR mutants with a DN phenotype were identified in two steps. First, we selected for mutants that had an FNR$^-$ phenotype, and then we screened these candidates for dominance to wild-type FNR. The strategy used to isolate the FNR$^-$ mutants was to select for strains that would be unable to activate the FNR-dependent *narGHIJ* operon (Stewart 1982; Walker and DeMoss 1991) under anaerobic conditions. Mutants of this type were isolated by their inability to synthesize β-galactosidase from a Φ(*narG–lacZ*) fusion in a *galE* strain. The *galE* allele allowed us to select for FNR$^-$ strains because the Φ(*narG–lacZ*) strains lyse under anaerobic conditions in the presence of galactose (Malamy 1966), which is produced by β-galactosidase cleavage of the substrate phenylgalactoside.

To increase the frequency of isolating *fnr$^-$* mutations, mutagenesis was targeted to a plasmid containing *fnr*, pRZ7411. The plasmid pRZ7411 was constructed by cloning the *Hind*III–

BamHI *fnr*-containing fragment into the same sites of pA-CYC184 (Chang and Cohen 1978), disrupting the Tc^r gene. To generate independently mutagenized pools of *fnr*, pRZ7411 was transformed eight separate times into KD1067, which contains the *mutD5* mutator allele (Fowler et al. 1974). One colony from each transformation was grown for several generations in LB medium containing 10 μg/ml of thymidine to induce the mutator activity (Fowler et al. 1974), and the plasmid DNA was then isolated. The efficiency of mutagenesis (0.01%) was assessed as the percentage of the pRZ7411 plasmids giving rise to an FNR$^-$ phenotype. This was measured by transforming mutagenized pRZ7411 into RZ8480 [Φ(*narG–lacZ*), Δ*fnr*] and scoring aerobically incubated colonies for a Lac$^-$ phenotype on MacConkey's medium (Difco). Under aerobic conditions, FNR$^-$ colonies are white and FNR$^+$ colonies have red centers as a result of lactose utilization under the anaerobic condition that probably exists in the interior of a colony.

To select specifically for independent FNR$^-$ mutants, separately mutagenized pRZ7411 pools were electroporated (Dower et al. 1988) into PK460 [Δ*fnr*, Φ(*narG–lacZ*), *galE*], and ~10^5 transformed colony-forming units were plated anaerobically on M63 minimal medium (Miller 1972) containing 20 mM glucose, 0.05% phenyl-β-D-galactoside, and chloramphenicol. From each pool of mutagenized DNA, 100 FNR$^-$, Lac$^-$ colonies were pooled together, and the plasmid DNA was isolated. Each pool of plasmid DNA was transformed into a strain containing wild-type *fnr* on the chromosome, RZ7350 [Φ(*narG–lacZ*), *fnr*$^+$], to screen the mutants for a DN phenotype. The RZ7350 transformants were screened for a Lac$^-$ phenotype aerobically on Mac-Conkey's medium containing chloramphenicol (see above), because RZ7350 is normally Lac$^+$ under these conditions. Approximately 5% of the FNR$^-$ mutants showed a dominant phenotype as judged by this criteria. A single isolate bearing a Lac$^-$ phenotype in a *fnr*$^+$ strain was chosen from each independent pool of mutagenized plasmid DNA for further analysis.

Assay of β-galactosidase activity

β-Galactosidase activity was measured from derivatives either of RZ8480 [Δ*fnr*, Φ(*narG–lacZ*)], RZ7350 [*fnr*$^+$, Φ(*narG–lacZ*)], or RZ8501. To determine the levels of β-galactosidase produced under anaerobic conditions, cells were grown in 5 ml of M9 minimal medium (Miller 1972) containing 10 mM glucose, 0.16 μM ammonium molybdate, and a 1/100,000 dilution of trace elements solution (Sistrom 1960) in a N$_2$ atmosphere in butyl rubber-stoppered tubes at 37°C. To determine the levels of β-galactosidase produced aerobically, cells were grown in 4 ml of M9 minimal medium containing 10 mM glucose in test tubes (18 × 150 mm) at 37°C with shaking at 250 rpm. In all cases, cells were grown for more than eight generations under the growth condition to be tested to ensure steady-state β-galactosidase levels. Cell growth was terminated in mid-log phase (0.2–0.4 OD$_{600}$) by the addition of Sp to 400 μg/ml and immediate placement of the cultures on ice until assayed for β-galactosidase activity. Cells were treated with chloroform and 0.1% SDS, and β-galactosidase was assayed as described by Miller (1972).

DNA sequencing

fnr mutations were located by sequencing the FNR structural gene from mutants derivatives of plasmid pRZ7411. At least 60 bp upstream (which included the *fnr* promoter) to 50 bp downstream of *fnr* was sequenced using a *Taq* polymerase dideoxy sequencing kit (Promega) under conditions recommended by the manufacturer. DNA sequence was determined from at least one strand by using four sequence-specific oligonucleotides that

were synthesized at the University of Wisconsin Biotechnology Center.

Assay of DN mutants for DNA binding

The DN FNR mutants were assayed for DNA-binding activity after the mutations were introduced into the FNR* allele, DA154, because the DA154 protein showed greater DNA-binding activity in a gel-retardation assay. To combine the DA154 and DN mutations in the same gene, the DN mutations were introduced into pPK14. pPK14 was constructed by cloning the 1.4-kb *Hind*III–*Sac*I fragment, containing the entire *fnr* gene from −521 to +817, from a pRZ7315 derivative carrying the DA154 allele (Kiley and Reznikoff 1991) into the same sites of pGem-2 (Promega). The 139-bp *Eco*RV–*Mlu*I fragment from pPK14, which contains the DNA-binding domain of FNR, was replaced with that of the DN mutant derivatives of pRZ7411. All derivatives were verified by DNA sequencing.

To assay DNA binding in crude extracts, PK22 strains containing derivatives of pPK14 that have the DN mutations were grown aerobically in LB plus 0.2% glucose and ampicillin to an OD$_{600}$ of 0.5. The cells were resuspended in 10 mM Tris (pH 8.0), 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, and 0.1 mM PMSF, lysed two times in a French press at 20,000 psi, and centrifuged at 7000 rpm in a SS-34 rotor for 30 min. These crude extracts were used to assay DNA binding as described above.

Construction of the FNR CS23 and CS122 substitutions

Cys → Serine (CS) substitutions at amino acid positions 23 and 122 in FNR were created by site-directed mutagenesis as described above. Mutagenic oligonucleotides were synthesized that change base 166 (base 1 is the A of the initiation codon of *fnr*) from a T to an A, creating CS23, and base 364 from a C to a G, creating CS122. The CS23 and CS122 mutations were introduced subsequently into pRZ7411 by cloning the 1.1-kb *Hind*III–*Eco*RV fragment of the pPK821 derivatives containing the CS mutations into the same sites of pRZ7411. To combine the CS and EK209 mutations in the same gene, the same *Hind*III–*Eco*RV fragment was cloned into a pRZ7411 derivative containing the EK209 mutation. All plasmid constructs were verified by DNA sequencing.

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