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Characterization of FNR* Mutant Proteins Indicates Two Distinct Mechanisms for Altering Oxygen Regulation of the *Escherichia coli* Transcription Factor FNR

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In order to gain insight into the mechanism by which the *Escherichia coli* transcription factor FNR is activated in response to anaerobiosis, we have analyzed FNR* mutant proteins which, unlike the wild-type protein, stimulate gene expression in the presence of oxygen in vivo. Cell extracts containing seven different FNR* mutant proteins were tested in vitro for the ability to bind to the FNR consensus DNA site in a gel retardation assay under aerobic conditions. At the concentration of protein tested, only extracts which contained FNR* mutant proteins with amino acid substitutions at position 154 showed significant DNA binding. The three position-154 FNR* mutant proteins could be further distinguished from the other mutant proteins by analysis of the in vivo phenotypes of FNR* proteins containing amino acid substitutions at either of two essential cysteine residues. In the presence of oxygen, FNR* mutant proteins with amino acid substitutions at position 154 were the least affected when either Cys-23 or Cys-122 was substituted for Ser. On the basis of these in vivo and in vitro analyses, FNR* mutant proteins appear to segregate into at least two classes. Thus, it appears that each class of FNR* substitutions alters the normal pathway of FNR activation in response to oxygen deprivation by a different mechanism.

FNR is a global regulatory protein of *Escherichia coli* which controls gene expression in response to oxygen deprivation (recently reviewed in reference 27). When oxygen is limiting, FNR functions to activate transcription of genes whose products participate in anaerobic energy-generating pathways. Under the same conditions, it also represses transcription of genes which are needed for aerobic respiration. These activities of FNR allow *E. coli* to maximize its energy-generating potential in response to the availability of oxygen.

FNR belongs to a family of transcriptional regulators which are related to the well-studied catabolite activator protein CAP (20 [for a review, see reference 22]). Like all members of this family, the region of FNR involved in site-specific DNA recognition is located in the C-terminal half of the protein (Fig. 1 [DNA-binding domain]). FNR also contains a region which is similar to the CAP cyclic AMP (cAMP)-binding domain (Fig. 1 [allosteric domain]), although cAMP is not an allosteric regulator of FNR (for a review, see reference 24). As with other oxygen-regulated members of the FNR family, *E. coli* FNR contains a conserved cysteine within the allosteric domain as well as several cysteines in the N terminus (Fig. 1 [Cys cluster]). In FNR, the cysteine residues at positions 20, 23, 29, and 122 have been shown by mutational studies to be required for FNR function in vivo (13, 18). Several lines of evidence suggest that these cysteine residues are essential, because they function as an iron-binding, redox-active site (4–7, 25, 26). Consistent with this view, our recent finding that an FNR* mutant protein, LH28-DA154, contains a $[3Fe\text{-}4S]^{1+}$ center (8) makes it likely that one role of these cysteine residues in the wild-type (WT) protein is to serve as ligands for the iron in this center. However, a polynuclear Fe-S center has not been described for WT FNR (7, 24).

Determining the mechanism by which oxygen availability regulates FNR-dependent transcription is an active area of investigation (22, 24, 27). In vitro studies of WT FNR indicated that DNA binding does not appear to be regulated by oxygen availability or iron content (7, 19, 24). However, two FNR mutant (FNR*) proteins have been analyzed (see below) which show enhanced DNA binding in vitro (8, 11, 30) relative to WT FNR protein, suggesting that regulation of DNA binding cannot be excluded. Furthermore, the enhanced DNA binding observed for one of these FNR* mutant proteins appears to be dependent on an Fe-S center (8). It is also possible that oxygen deprivation may control transcription activation by FNR subsequent to DNA binding because open complex formation at one FNR-dependent promoter appears to be increased in vivo under anaerobic conditions and in vitro with an iron-containing form of WT FNR (4).

In order to further our understanding of the mechanism by which FNR becomes activated in response to oxygen deprivation, our approach has been to study FNR* mutant proteins which we have previously shown to function in vivo in the presence of oxygen (9). Two of these FNR* mutant proteins, DG22 and LH28, have amino acid substitutions at positions adjacent to essential N-terminal cysteine residues (Fig. 1). The remaining five FNR* mutant proteins (HR93, EK150, DA154, DG154, and DV154) map to the analogous domain in CAP that is responsible for the binding of cAMP (allosteric domain [Fig. 1]). The fact that the position analogous to D154 is located in the dimerization helix within the CAP allosteric domain (12, 20) led us to consider that substitutions at this position might affect FNR dimerization (11). Indeed, purification and analysis of the DA154 FNR* mutant protein in the presence of oxygen showed that it contains a larger population of dimers than the largely monomeric WT FNR protein. In addition, the ability of FNR to form dimers correlates with an

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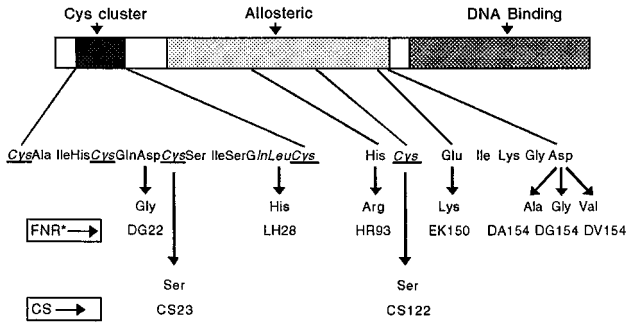

FIG. 1. A linear representation of the FNR protein, with the assignment of putative functional domains based on its homology to CAP (20). The locations of amino acid substitutions resulting in an FNR* phenotype as well as the conservative cysteine-to-serine substitutions at positions 23 and 122 that result in a decrease in FNR activity are shown. FNR mutant nomenclature follows the single-letter code for amino acids, in which the first letter indicates the amino acid found at the position indicated, with the second letter representing the amino acid substitution. To account for the presence of the N-terminal methionine in the *fnr* gene product, the numbering system for the designation of FNR* amino acid substitutions has been increased by one from that previously described (9) (i.e., DA153 is now designated DA154, etc.).

increase in the level of DNA binding in vitro (11). Furthermore, the observation that the FNR* mutant protein (LH28-DA154) which contains an Fe-S center shows enhanced DNA binding and increased dimerization relative to the DA154 protein suggests that the Fe-S center plays a role in dimerization (8). On the basis of these data, we proposed that transcription activation of FNR is regulated by the formation of FNR dimers capable of binding to DNA.

In this study, we have compared the DNA-binding properties of the DA154 protein to those of the six other FNR* mutant proteins with single amino acid substitutions (9). In addition, we have examined the effects of Cys-to-Ser (CS) amino acid substitutions on FNR* activity in vivo and in vitro as a way to further characterize these FNR* proteins. The results of these experiments show that FNR* mutant proteins can be divided into at least two classes. From this analysis, there appear to be at least two mechanisms by which FNR* substitutions alter the mechanism of FNR activation.

MATERIALS AND METHODS

Parental strains and growth conditions. *E. coli* K-12 strains RZ8480, RZ8501, and DH5α and the *E. coli* B strain PK22 were used in this study (Table 1). For routine strain manipulations, cells were grown in Luria-Bertani (LB) medium at 37°C. When needed, chloramphenicol and ampicillin were used at concentrations of 20 and 50 μg/ml, respectively, except for the growth of λ*fnr* monolysogens, for which ampicillin was used at a concentration of 30 μg/ml.

Molecular biology techniques. Standard molecular biology techniques were performed as described by Sambrook et al. (17). In construction of plasmids, DH5α (Table 1) was used as a host strain for transformation. All oligonucleotide primers used for either site-directed mutagenesis (10, 11) or DNA sequencing (9) were synthesized at the University of Wisconsin Biotechnology Center.

Construction of plasmids and strains containing *fnr* alleles for in vivo and in vitro assays. The pACYC184 (2) derivative, pRZ7380 (Table 1), containing WT *fnr* cloned into the *Hin*dIII site and its *fnr** mutant derivatives have been previously described (9). These plasmids were used to construct a set of strains for in vivo assays of FNR function by transformation of RZ8501, which carries λ*i434plac5*-P1 (−68T; −55A) as a monolysogen (Table 1). A symmetrical base pair change in the *lacP1* CAP site (G to T at −68 and C to A at −55) of this phage converts the CAP-binding site to an FNR-binding site, which results in FNR-dependent transcription activation of the *lac* promoter in vivo in these strains (29).

To construct a set of strains for use in in vitro DNA-binding assays, a set of plasmids derived from pGEM-2 (Promega) were made. The 1.4-kb *Hin*dIII-*Sac*I fragment containing *fnr* or *fnr** alleles was cloned from the respective pRZ7380 plasmids into the same sites of pGEM-2 to generate pPK12 (WT *fnr*) or its *fnr** derivatives (Table 1). To create *fnr**-containing plasmids with mutations directing serine substitutions of Cys-23 and Cys-122, the pUC118 (28) derivatives containing *fnr-CS23* (pPK1605) and *fnr-CS122* (pPK1608), were used (11) (Table 1) as starting plasmids for most constructions. Depending on the location of the *fnr** mutation, these plasmids were constructed in one of three ways. One group was constructed by cloning the 0.4-kb *Ava*I-*Mlu*I fragment of pPK1608 (*fnr-CS122*) into the same sites of the *fnr**-containing pGEM-2 derivatives. A second group was constructed by cloning the 0.35-kb *Bst*BI-*Mlu*I fragment of the *fnr**-containing derivatives of pPK12 into the same sites of pPK1605 or pPK1608. The remaining *fnr*-CS* mutant alleles (*fnr-LH28-CS23*, *fnr-LH28-CS122*, *fnr-HR93-CS23*, and *fnr-DG22-CS23*) were constructed by site-directed mutagenesis with the oligonucleotides previously described (11), except for the construction of *fnr-DG22-CS23*, in which an oligonucleotide which changed base 64 from an A to a G and base 66 from a T to an A (base 1 refers to the A of the initiation codon of *fnr*) was used. All constructs were verified by DNA sequencing as described previously (9). The pUC118 derivatives containing *fnr-CS23*, *fnr-CS122*, or *fnr*-CS* alleles were cloned onto pGEM-2 by using the 1.65-kb *Hin*dIII-*Bam*HI fragment and were subsequently introduced into PK22 (Table 1) by transformation for use in in vitro DNA-binding assays. In some instances, the *Hin*dIII-*Bam*HI fragments were cloned into pACYC184 (pRZ7411 [Table 1]), and the resulting plasmids were used in the phage constructions described below.

Construction of λ phage and strains containing *fnr* alleles for in vivo assays of *narG* expression. *fnr-CS23*, *fnr-CS122*, and all of the *fnr*-CS* mutant alleles were genetically recombined onto λ*fnr* from their respective pGEM-2 or pACYC184 counterparts. λ*fnr* is a λD69 (15) derivative containing *fnr* and *bla* (9) (Table 1). These plasmids were transformed into RZ8480 (Table 1), from which

TABLE 1. Parental strains, plasmids, and λ phage derivatives used in this study

Construct	Relevant genotype	Reference or source
E. coli strains		
RZ8480	Δ*fnr lacZΔ145 narG*::MudI1734	11
RZ8501	Δ*fnr lacZΔ145 Δcrp-bs990 zhe-3085*::Tn*10* λ*i434plac5*-P1 (−68T; −55A)	11
PK22	BL21 (DE53) Δ*crp-bs-990 rpsL Δfnr zcj-3061*::Tn*10*	11
DH5α	ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44* λ*-thi-1 gyrA96 relA1*	BRL[a]
Plasmids		
pRZ7380[b]	Cm^r; Ap^r*Hin*dIII-*Hin*dIII of λ*fnr* in pACYC184	9
pRZ7411[b]	Cm^r; *Hin*dIII-*Bam*HI of *fnr* −521 to +1115 of *fnr* in pACYC184	11
pPK12[b]	Ap^r; *Hin*dIII-*Sac*I of pRZ7411; −521 to +817 of *fnr* in pGEM-2	This study
pPK810C	Ap^r; FNR consensus DNA site in *Hin*dIII site in pUC19	11
pPK1605	Ap^r; *Hin*dIII-*Bam*HI of *fnr*; −521 to +1115 of *fnr-CS23* in pUC118	11
pPK1608	As pPK1605 except *fnr-CS122*	11
λ phage		
λ*fnr*[b]	λD69 containing *fnr* and *bla*	9

[a] BRL, Bethesda Research Laboratories.
[b] Derivatives containing various *fnr* alleles are described in Materials and Methods.

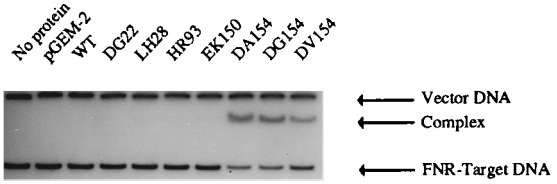

FIG. 2. DNA binding by FNR* mutant proteins was assessed by a gel retardation assay. The source of FNR protein was from cell extracts prepared from PK22 strains harboring the vector pGEM-2 or various *fnr**-containing derivatives. No protein indicates radiolabeled DNA in the absence of any cell extract. The migrations of the 2.7-kb plasmid vector fragment, the 48-bp consensus FNR target DNA, and the specific FNR-DNA complex are indicated. The binding reaction mixtures contained 84 μg of cell extract protein per ml (final concentration) and were run on a 10% polyacrylamide gel.

fnr is deleted. This strain also contains a chromosomally encoded FNR-dependent Φ(*narG-lacZ*) operon fusion from which β-galactosidase activity can be monitored when *fnr* is supplied from another source. The *fnr-CS23* and *fnr-CS122* mutations were recombined onto λ*fnr* (WT) by infecting RZ8480 containing pPK1605 (*fnr-CS23*) or pPK1608 (*fnr-CS122*) with this phage, and high-titer lysates were obtained (21). Recombinant phages having an FNR$^-$ phenotype (indicating the presence of *fnr-CS23* or *fnr-CS122*) were obtained by plating phages on RZ8480 and screening for white plaques on M9 minimal medium (14) containing 10 mM glucose and overlaid with top agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Bachem) at a final concentration of 400 μg/ml.

To identify λ*fnr* derivatives containing both *fnr-CS* and *fnr** mutations, a qualitative test of the effect of the Cys-to-Ser substitutions on the aerobic FNR* phenotype in which β-galactosidase was assayed from the chromosomal Φ(*narG-lacZ*) operon fusion was first performed with RZ8480 strains containing pACYC184 derivatives. λ*fnr* recombinants encoding FNR* mutant proteins which retained some in vivo activity in the presence of the Cys-to-Ser substitutions were obtained by infecting the appropriate *fnr*-CS23* or *fnr*-CS122* plasmid-containing strain with λ*fnr-CS23* or λ*fnr-CS122* and by screening high-titer lysates for blue plaques. Alternatively, recombinants were obtained by infecting either the *fnr*-CS23* or *fnr*-CS122* plasmid-containing strain with the appropriate λ*fnr** derivative (9) and by screening high-titer lysates for white plaques. *fnr-EK150-CS23* did not have a discernible phenotype upon recombination with either λ*fnr-CS23* or λ*fnr-EK150*. Consequently, this double mutation was cloned onto λD69 as described elsewhere (9). All recombinant phages were plaque purified, and high-titer lysates were obtained (21). In order to ascertain that the desired mutations were present, phage DNA was PCR amplified (3) with FNR-specific primers which annealed to bp 775 through 794 on the coding strand and bp −228 through −207 on the noncoding strand, and *fnr* DNA was sequenced. Monolysogens of each λ*fnr* derivative were obtained from RZ8480 and were verified by the *ter* excision test (16).

Assay for in vivo expression of the FNR-dependent *lac* and *narG* promoters. FNR mutant function was assayed in vivo by measuring the levels of β-galactosidase in strains containing *lacZ* either transcriptionally fused to the *narG* promoter (RZ8480) or under the control of an FNR-dependent *lac* promoter (RZ8501). Cells were grown in M9 minimal medium (14) as previously described (11), except that RZ8480 strains also contained 1.4 mM KNO_3. To terminate cell growth and any further protein synthesis, spectinomycin was added to a final concentration of 500 μg/ml for RZ8501 strains, chloramphenicol was added to a final concentration of 25 μg/ml for RZ8480 strains, and the cells were immediately placed on ice until they were assayed for activity. After treatment of cells with chloroform–0.1% sodium dodecyl sulfate, β-galactosidase was assayed as described by Miller (14). Assays were performed on at least two different occasions by using two or three independent isolates of each strain.

Preparation of cell extracts and assay of in vitro DNA binding. PK22 strains carrying pPK12 (WT *fnr*) and its mutant derivatives were grown aerobically in LB medium supplemented with 0.2% glucose and 50 μg of ampicillin per ml at 37°C to an optical density at 600 nm of approximately 0.5. PK22 is Δ*crp* Δ*fnr* and thus avoids any possible competition of the chromosomally encoded CAP or WT FNR protein for binding of the DNA target site in vitro with plasmid-directed mutant proteins. Cell extracts were prepared by lysis in a French pressure cell as previously described (11), except that the lysis buffer was 100 mM Tris (pH 7.9) and KCl was omitted. Protein concentrations were determined by the Bradford assay (1) by using the Commassie Plus protein assay reagent (Pierce) with bovine serum albumin as a standard.

DNA binding by FNR protein in crude cell extracts was assessed by a gel retardation assay as previously described (11). The assays were carried out under the binding conditions established for the purified FNR* mutant protein, DA154 (11). The FNR target DNA in this assay was generated first by cutting the plasmid pPK810C (Table 1) with *Hin*dIII, thereby liberating a 48-bp DNA fragment which contains the FNR consensus sequence, and then by radiolabeling with [α-^{32}P]dATP as previously described (11).

RESULTS

DNA-binding assays can distinguish position-154 FNR* mutants from the other FNR* mutant proteins. Since one previously observed characteristic of DA154 was that aerobically purified protein showed enhanced DNA binding relative to that of the WT FNR protein (11, 30), DNA binding was used to determine if other FNR* mutant proteins had a similar property in cell extracts. Cell extracts from a strain containing the pGEM-2 vector alone did not bind target DNA, indicating the absence of any nonspecific complexes (Fig. 2). At the highest concentration of cell extract protein that was possible in the DNA-binding assay mixture (~20 nM FNR as estimated by Western blot [immunoblot] analysis; data not shown), no specific binding to the 48-bp fragment containing an FNR consensus DNA site was observed for extracts containing WT FNR (Fig. 2). This was not surprising, since previous studies with purified FNR showed that >50 nM protein was necessary to reproducibly detect a specific complex under the same assay conditions (11). In contrast, cell extracts containing DA154 protein formed a complex with the 48-bp fragment, and the mobility of this complex was identical to the mobility of the complex observed with purified DA154 or WT protein (11; data not shown). Cell extracts containing FNR* mutant proteins with two other neutral amino acid substitutions at this position, DG154 or DV154, gave rise to a complex with the same size as that of DA154 protein (Fig. 2). In contrast, cell extracts from four other strains containing FNR* mutant proteins with amino acid substitutions LH28, DG22, HR93, or EK150 did not show any detectable complex formation in vitro at this protein concentration (Fig. 2). Thus, the concentration of FNR protein in these cell extracts serves as a convenient window to distinguish the position-154 FNR* mutant proteins from the other FNR* proteins. The lack of detectable DNA binding by WT FNR protein or particular FNR* derivatives observed in this assay was reproducible and was not due to significant differences in the amount of FNR protein present in these extracts, since visual inspection of Western blots did not indicate any major differences in the amount of FNR protein present (data not shown).

FNR* mutant proteins show increased expression of an FNR-dependent *lac* promoter in the presence of oxygen. Since the binding site used in the in vitro studies was derived from a promoter different than the one that was originally used to show the activity of these mutants in vivo (9), we assayed FNR-dependent expression in vivo from a promoter similar to the one used in the in vitro assay described in the legend to Fig. 2. The 48-bp DNA site used in the in vitro assay was derived from the *lacP1* element. Thus, we tested FNR* mutant protein DNA binding in vivo by assaying β-galactosidase activity from the FNR-dependent *lac* promoter (29) in RZ8501 (Δ*crp* Δ*fnr*), in which *fnr* alleles were present as pACYC184 derivatives (11).

In the presence of oxygen, all of the FNR* mutant proteins showed a reproducible increase in the level of expression from this *lac* promoter derivative, with β-galactosidase activities ranging from 1.2- to 3.0-fold over the activity seen with WT FNR (Fig. 3). Since transcription activation of this FNR-dependent promoter was observed with all of the FNR* mutants, this indicates that these FNR* proteins were able to bind to this site in vivo. Therefore, an inability to recognize this binding site cannot explain the failure to observe DNA binding in vitro for FNR* mutant proteins containing amino acid substitutions at positions other than amino acid 154. In fact, in contrast to the DNA-binding assays, the position-154 FNR* mutants showed the least amount of transcription activation

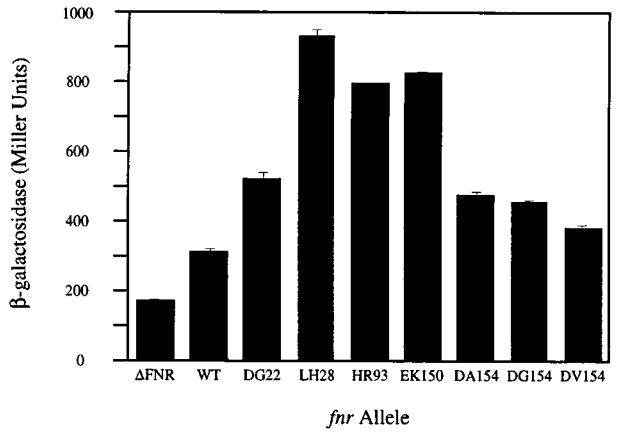

FIG. 3. Aerobic transcription activation was assessed in vivo by assaying for β-galactosidase produced from the FNR-dependent *lac* promoter in RZ8501-derived strains. *fnr* alleles were provided as pRZ7380 derivatives, with ΔFNR indicating the pACYC184 vector control. Cells were grown in minimal medium (14) containing 10 mM glucose under aerobic conditions.

from the *lac* promoter in vivo. These results show that FNR* mutants can be divided into two classes: those that showed detectable DNA binding, represented by the position-154 FNR* mutants, and those that lacked detectable DNA binding under the conditions used in vitro. This result may indicate that some FNR* proteins require a factor(s) for DNA binding which, although present under aerobic conditions in vivo, was not present at sufficient concentrations in our in vitro extracts to result in significant FNR* activity.

FNR* mutant proteins containing amino acid substitutions at position 154 are the least affected by substitution of two of the essential cysteine residues. In order to further distinguish the two classes of FNR* mutant proteins, we assayed the abilities of these proteins to activate transcription in vivo when either of two essential cysteine residues (Cys-23 or Cys-122) has been substituted for Ser. These particular cysteine residues were chosen since they are located within two different putative functional domains of FNR (Fig. 1).

Strains containing either WT FNR or the analogous mutant derivatives as monolysogens of RZ8480 (Table 1) were assayed for their abilities to activate the FNR-dependent *narG* promoter, which was transcriptionally fused to *lacZ*. From our previous work (9), the *narG* promoter shows a higher level of aerobic transcription activation by these FNR* mutant proteins than the *lac* promoter; thus, RZ8480 provides a more-sensitive in vivo assay to examine the effects of the CS substitutions on FNR* function. FNR* proteins with substitutions in the N-terminal Cys cluster (DG22 and LH28) showed a complete loss in FNR function when either Cys-23 or Cys-122 was substituted, since the β-galactosidase activity from strains containing any of these FNR*-CS mutant proteins was reduced to the activity observed from strains lacking functional FNR (ΔFNR, CS23, or CS122 [Fig. 4A]). Substitution of cysteine 122 had a similar deleterious effect on FNR* mutants HR93 and EK150, since placing the CS122 substitution in the context of either of these mutant proteins gave the same low amount of β-galactosidase activity as that observed with strains lacking functional FNR (Fig. 4A). However, unlike the effect of CS23 on DG22 and LH28, strains containing the HR93-CS23 and EK150-CS23 proteins retained some FNR function, since β-galactosidase activities were four- to eightfold greater than those observed from strains lacking functional FNR. However, this represents a large reduction in the magnitude of the FNR* phenotype, since strains containing HR93-CS23 and EK150-CS23 had only ~7.5 and 6.1% of the β-galactosidase activity observed from strains containing HR93 and EK150, respectively (Fig. 4A). Thus, it appears that in the context of DG22, LH28, HR93, or EK150, the CS substitutions, largely decrease the ability of these FNR* proteins to activate transcription in vivo in the presence of oxygen.

For FNR* proteins with amino acid substitutions at position 154, substitution of Cys for Ser at position 122 or 23 had a wider range of effects on the abilities of these proteins to activate transcription in vivo. The strain containing the CS122 substitution, in the context of the DA154 protein, had ~38% of the β-galactosidase activity observed from a strain containing DA154 alone (Fig. 4B). In the context of either DG154 or DV154, the CS122 substitution had a larger effect on FNR* activity than the effect of this substitution on DA154 protein,

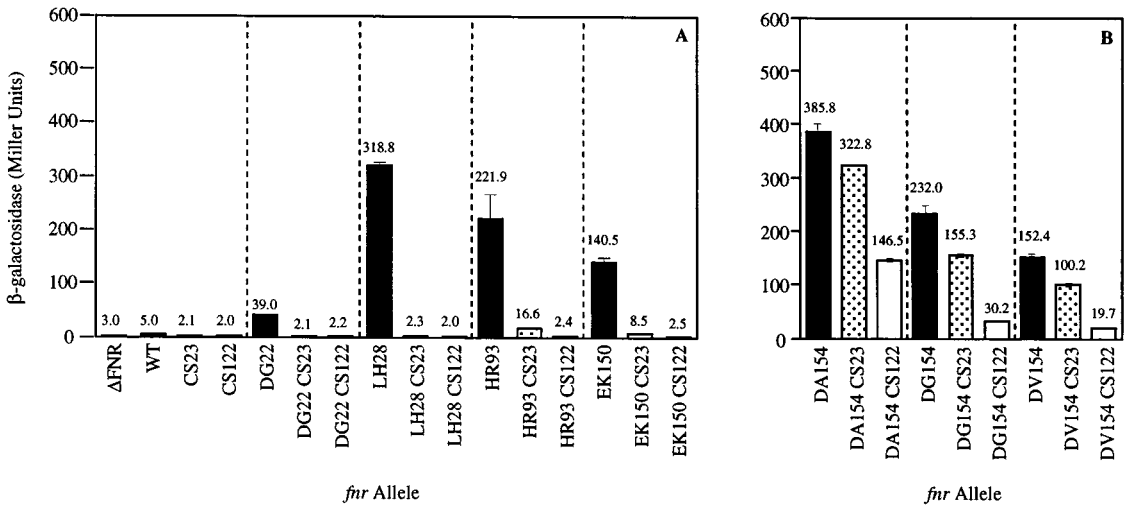

FIG. 4. Aerobic transcription activation by FNR* mutant proteins containing either the CS23 or CS122 substitution was measured by assaying for β-galactosidase produced from the FNR-dependent *narG* promoter fused to *lacZ*. (A) FNR* mutant proteins which are largely inactivated by the CS substitution; (B) FNR* mutant proteins which are least affected when either Cys-23 or Cys-122 is substituted for Ser (CS). *fnr* alleles were present as λ*fnr* derivatives integrated as monolysogens in RZ8480. Cells were grown aerobically in minimal medium (14) containing 10 mM glucose and 1.4 mM KNO_3.

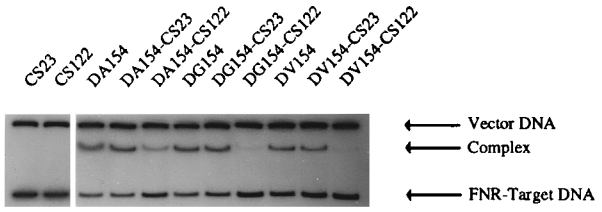

FIG. 5. The effect of the CS substitutions on the ability of the position-154 FNR* mutant proteins to bind to DNA was tested by a gel retardation assay. The source of FNR was from crude cell extracts prepared from PK22 strains harboring pGEM-2 derivatives containing *fnr* alleles as shown. The migrations of the 2.7-kb plasmid vector DNA, the 48-bp consensus FNR target DNA, and the specific FNR-DNA complex are indicated. The binding reaction mixtures contained 84 μg of crude cell protein per ml (final concentration) and were run on a 10% polyacrylamide gel.

since β-galactosidase activity was ~13% of that observed from strains containing DG154 or DV154 protein (Fig. 4B). Thus, in contrast to the FNR* mutant proteins in Fig. 4A, all of the position 154 FNR* mutant proteins containing the CS122 substitution retained some in vivo activity in the presence of oxygen. Substitution of Cys-23 for Ser had little effect on the position 154 FNR* mutant proteins since the amount of β-galactosidase activity observed from aerobically grown cells was ~66 to 84% of the activity observed from strains containing DA154, DG154, or DV154 protein alone (Fig. 4B). Thus, it appears that the ability of the position-154 FNR* mutant proteins to activate transcription under aerobic conditions in vivo is much less affected by the CS substitutions than the four FNR* mutant proteins in Fig. 4A.

To demonstrate that the amount of FNR activity observed was not due to differences in the levels of FNR protein, Western blots were performed on aliquots of cells used in the β-galactosidase assays. From visual inspection of the Western blots, we observed no major differences in the amount of FNR protein except for strains containing CS23 or CS122 proteins, which showed a small increase in FNR protein (data not shown). Thus, it does not appear that FNR protein levels can account for the large decreases in activity observed for some of the FNR*-CS mutant proteins.

Overall, these results indicate that the in vivo activities of the position-154 FNR* mutant proteins were much less affected by the CS substitutions. Coincidentally, the FNR* mutant proteins which showed no detectable DNA binding in vitro (i.e., DG22, LH28, HR93, and EK150) were the same FNR* mutant proteins whose in vivo activity was most affected by the CS substitutions (compare Fig. 2 and 4A). Thus, the effect of the CS substitutions on FNR*-dependent transcription activation in vivo further supports the idea that there are at least two classes of FNR* mutant proteins.

The Cys-to-Ser substitutions do not abolish in vitro DNA binding by the position-154 FNR* mutant proteins. The fact that the position-154 FNR* mutant proteins containing either a CS23 or CS122 substitution retained activity in vivo suggests that dimerization and DNA binding are occurring in vivo, albeit to different extents. To show that FNR*-dependent transcription activation in vivo could be correlated with DNA binding, we examined the ability of those FNR*-CS mutant proteins containing FNR* substitutions at position 154 to bind to DNA in vitro by the gel retardation assay. As expected from the behavior of WT FNR protein in cell extracts, cell extracts containing the CS23 or CS122 mutant proteins showed no detectable DNA binding to the DNA target site (Fig. 5). In contrast, DNA binding by the position-154 FNR* mutant proteins was not greatly affected by the presence of the CS23 substitution, since roughly the same amount of FNR target DNA was bound for the double mutant compared with that of the FNR* mutant alone (Fig. 5; compare DA154 with DA154-CS23, etc.). The CS122 substitution had a wider range of effects on DNA binding by the position-154 FNR* mutant proteins, which were similar to what was observed in vivo (compare Fig. 4B and 5). For the DA154 protein, substitution of Cys-122 resulted in a small decrease in DNA binding. In contrast, the CS122 substitution resulted in a larger decrease in DNA binding for the DG154 and DV154 proteins. However, these FNR*-CS mutant proteins still showed more DNA binding than CS122 alone. These results show that the CS substitutions do not abolish the ability of the position-154 FNR* mutant proteins to bind to DNA in vitro.

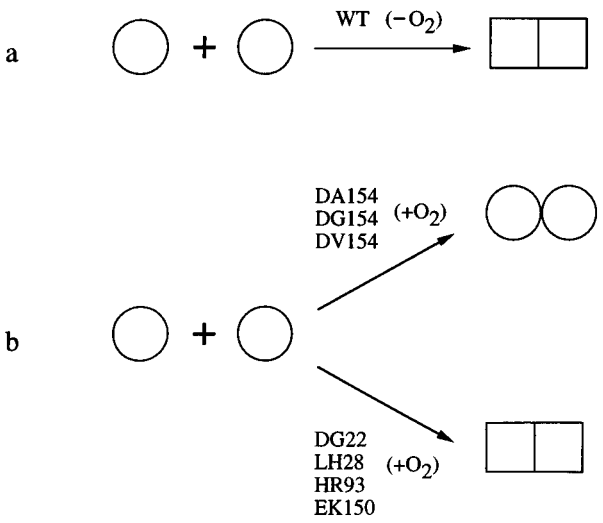

FIG. 6. A model for FNR activation in response to oxygen deprivation. (a) Pathway of activation of WT FNR, which consists of monomeric subunits (circles) undergoing a presumed conformational change upon oxygen deprivation to form dimers (squares); (b) two different classes of FNR* mutant proteins and how FNR* substitutions may affect this pathway. One class of FNR* substitutions, represented by DA154, DG154, and DV154, appears to cause FNR to dimerize directly in the presence of oxygen while the remaining FNR* substitutions have a distinct mechanism which is more similar to the activation of WT FNR and may result in altered interactions between FNR and an additional factor(s).

DISCUSSION

The characterization of FNR* mutant proteins that are altered in oxygen regulation has provided insight into the mechanism of FNR activation (8, 9, 11, 30). On the basis of the previous analysis of one such mutant protein, DA154, we proposed that DNA binding by FNR is regulated by an oxygen-dependent change in oligomeric state (11). From the characteristics of six additional FNR* mutants presented in this study, we suggest that FNR* amino acid substitutions can alter this pathway of FNR activation by at least two different mechanisms, on the basis of the observation that these mutant proteins appear to segregate into two classes. One class of FNR* substitutions (DA154, DG154, and DV154) appears to provide an alternate pathway of FNR activation by allowing the protein to dimerize directly in the presence of oxygen (Fig. 6). The remaining FNR* substitutions alter FNR in a manner which is distinct from that of the position-154 FNR* substitutions. As discussed below, these particular FNR* substitutions may result in altered interactions in vivo between the FNR protein and an additional factor(s) (Fig. 6).

FNR* mutant proteins with amino acid substitutions at position 154 are likely to alter dimerization. Analysis of FNR DNA binding revealed that, of the seven FNR* mutant proteins analyzed in this study, only the position-154 mutant proteins showed detectable DNA binding at the concentration tested in vitro. Previous in vitro analysis of one of these mutant proteins, DA154, showed that enhanced DNA binding is correlated with increased dimerization in the presence of oxygen (11). As shown in the present work, two other neutral substitutions at this position, glycine and valine, appear to alter the protein in a manner similar to that of DA154, since they also show increased DNA binding relative to WT FNR protein. Assuming that an increase in dimerization is responsible for the enhanced DNA binding for all three proteins, it appears that any of these three substitutions at position 154 can alter the dimerization constant of FNR, although this must be verified directly. This proposed increase in dimerization for all three mutant proteins is presumably responsible for their elevated aerobic activity in vivo, and, thus, these results support the idea that dimerization is a point of control of FNR activity.

Interestingly, all three amino acid substitutions which give rise to increased DNA binding in vitro involve the replacement of an acidic amino acid residue for a neutral residue. In CAP, the majority of the intersubunit interactions occur between the C α-helices of the homodimer (12). If FNR has a similarly oriented α-helix, then this would place Asp-154 of each monomer in close proximity in the FNR dimer. The fact that removal of a negative charge at position 154 enhances dimerization in the presence of oxygen suggests that in the WT protein, Asp-154 may interfere with dimerization, possibly via a steric repulsion generated upon close proximity of two negatively charged Asp residues. If this were true, then dimerization of WT FNR under anaerobic conditions would require removal of the negative charge at position 154 in either one or both subunits of the homodimer. For instance, an additional factor or another amino acid, which is positively charged under anaerobic conditions, may neutralize the negative charge of Asp-154 in one or both subunits, thereby contributing to dimerization.

The position-154 FNR* mutant proteins were also shown to be the least affected by substitution of Cys-23 or Cys-122, unlike WT FNR, which absolutely requires these residues for activity in vivo under anaerobic conditions (13, 18, 23). The fact that the position-154 FNR* mutant proteins can still function both in vivo and in vitro when Cys-23 or Cys-122 is substituted suggests that the position-154 FNR* substitutions are providing an alternate pathway for FNR activation. Since our data also suggest that increased dimerization is correlated with enhanced DNA binding (11), we propose that this alternate pathway consists of the ability to dimerize in the presence of oxygen by directly affecting the dimer contacts.

A logical extension of this idea would be that in the WT protein, Cys-23 and Cys-122 are required for dimerization under anaerobic conditions. Since previous results have shown that an FNR* mutant protein containing both the LH28 and DA154 substitutions contains a $[3Fe\text{-}4S]^{1+}$ center (8), then one expected role of these cysteine residues in WT FNR would be to serve as ligands for such an Fe-S center. Thus, any mechanism to explain the role of the cysteine residues in dimer formation must take into account the role of the Fe-S center. Furthermore, since neither cysteine 23 nor 122 is likely to be located at the dimerization interface which is analogous to the C-helix of CAP, then the effect of the Fe-S center on FNR dimerization must either be through an indirect conformational change and/or by stabilization of an additional dimerization interface.

Throughout this study, we have consistently noted that substitution of Cys-122 for Ser results in a more severe effect on the in vivo or in vitro activities of FNR* mutant proteins than substitution of Cys-23. This suggests that substitution of Cys-122 is not equivalent to that of Cys-23, perhaps because of an effect on the global conformation of FNR. The observation that the Cys-122 substitution has a larger effect on FNR function is consistent with previous biochemical studies of FNR mutant proteins with amino acid substitutions at these cysteines (6).

FNR* mutant proteins which lack detectable DNA binding in vitro may require an additional factor(s). All of the other FNR* mutant proteins (DG22, LH28, HR93, and EK150) showed properties similar to the WT FNR protein with respect to their lack of detectable in vitro DNA binding and the requirement for functional Cys-23 or Cys-122 in vivo. This was surprising since, unlike WT FNR, these FNR* proteins are active in the presence of oxygen in vivo. One interpretation of these results is that both WT FNR and the DNA-binding-deficient FNR* proteins require a common factor(s) which is necessary for increased dimerization and DNA binding in vitro. One possible candidate for this factor is an Fe-S center, since FNR* mutant protein LH28-DA154, which contains an Fe-S center, shows increased dimerization and DNA binding in vitro relative to protein lacking this center (8). If this interpretation is correct, then it also suggests that assembly of this Fe-S center is possible in aerobic cells since the DNA-binding-deficient FNR* proteins presumably require this center for their in vivo aerobic activity. Thus, one could explain the in vitro DNA-binding characteristics of WT and FNR* mutant proteins if the Fe-S center is labile to aerobic cell breakage, even though it is stabilized in vivo. As a result, DNA binding by these FNR* mutant proteins would be similar to that observed with aerobically purified WT FNR protein which lacks an Fe-S center. To determine whether this is true, these *fnr** alleles will have to be cloned onto an FNR expression vector in order to achieve FNR protein concentrations sufficient to monitor in vitro DNA binding in cell extracts and to purify their active forms.

The idea that the proposed factor-dependent FNR* mutant proteins may require an Fe-S center for their activity in vivo is supported by the effect of the CS substitutions on FNR* activity, since these residues are likely to be the ligands for an Fe-S center (8). However, it is possible that some of the FNR* mutant proteins requires a factor(s) in addition to an Fe-S center for their activity in vitro (such as a small molecule, RNA polymerase, etc.), which may explain why FNR* substitutions are located in two different regions of the FNR protein. Since any factors required for FNR* activity are presumably required for WT FNR activity under anaerobic conditions, the FNR* substitutions which are located at positions other than position 154 may alter the interactions with these factors to allow FNR to be active in the presence of oxygen. Biochemical analysis of the requirements for FNR* mutant activity in vitro should aid in the identification of any factors needed for FNR dimerization and DNA binding. In addition, determining how FNR* mutant proteins differ from WT FNR in their interactions with these factors may aid in the dissection of steps involved in converting WT FNR to an active form upon oxygen deprivation.

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