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Fnr Mutants That Activate Gene Expression in the Presence of Oxygen

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The regulatory protein Fnr is required for anaerobic expression of several anaerobic respiratory enzymes in *Escherichia coli*. To gain insight into how Fnr activity is regulated by oxygen, we have isolated Fnr mutants that increase expression of the nitrate reductase operon in the presence of oxygen (Fnr* mutants). Seven single-amino-acid substitutions thats mapped within two regions of Fnr have been characterized. Two mutants mapped adjacent to two Cys residues in the N-terminal Cys cluster. Five Fnr* substitutions mapped to a region of Fnr that is similar to the cyclic AMP-binding domain of the catabolite activator protein (CAP). Within this group, four mutants were clustered in a region analogous to the CAP C helix, which is important in CAP dimer subunit interactions. Taken together, these data implicate regions in Fnr that may be important either in sensing oxygen deprivation or in the conformational change proposed to be necessary for Fnr activation under anaerobic conditions.

For many organisms, the ability to sense and adapt to changes in oxygen tension in the environment is crucial to survival. Although oxygen plays a pivotal role in many biological systems, the molecular mechanism by which oxygen is sensed by cells is poorly understood. Fnr is a global regulatory protein that regulates gene expression in response to oxygen deprivation in *Escherichia coli* (15, 24). Fnr acts as both an activator and a repressor to coordinate gene expression for adaptation to anaerobic conditions. For example, Fnr positively regulates the anaerobic expression of several anaerobic respiratory enzymes (13, 14, 16) and negatively regulates the synthesis of at least one aerobic respiratory enzyme (31). Although Fnr has been shown to regulate transcription of several operons, little is known about how oxygen deprivation modulates this activity. Fnr levels are apparently not oxygen regulated (35), and the nature of the physiological signal induced by oxygen deprivation is unknown. Therefore, we are interested in determining how oxygen deprivation regulates Fnr activity.

The model for how Fnr activates transcription has been strongly influenced by the observation that Fnr shows homology to the catabolite activator protein (CAP), which regulates transcription of many carbon utilization operons in *E. coli* (26, 38). The most striking similarity between these two regulatory proteins is in the DNA-binding region. In fact, replacement of three amino acids in the Fnr DNA-binding domain with those found at equivalent positions in CAP is sufficient for Fnr to substitute for CAP in activation of the CAP-dependent *lac* operon (29). Recent experiments also suggest that the consensus sequence inferred for the Fnr DNA-binding site is very similar to that for CAP binding (3, 40). Fnr also contains a region that shows some similarity to the CAP cyclic AMP (cAMP)-binding domain. However, there is no evidence for a role for cAMP involvement in Fnr activation (35).

Although these similarities suggest that Fnr and CAP may share a common mode of transcriptional activation, Fnr contains a region at its N terminus that has no counterpart in CAP. A cluster of four Cys residues is located within the first 28 amino acids, and Fnr activity is lost when these amino acids are deleted (30). Unden and Guest (36) were the first to suggest that oxygen deprivation exerts its effect on Fnr activity in a reaction involving this Cys cluster. More recently, a role for the Cys cluster in metal ion binding has been proposed, since removal of metal ions from anaerobically growing cells inhibited Fnr activity (31, 34). According to this model, either metal binding or changes in the metal ion coordination state may be oxygen sensitive (31). Therefore, the oxygen-dependent control of Fnr activity may be effected by a metal ion-dependent reaction that induces a conformational change in Fnr similar to how cAMP regulates activity of CAP.

The best-characterized Fnr target operon is *narGHIJ*, which encodes the major nitrate reductase of *E. coli* (12, 16, 17, 32). Under anaerobic conditions, the energy-generating nitrate reductase reduces the electron acceptor nitrate to nitrite. The Fnr-responsive element has been localized to a region 55 bp upstream of the *nar* transcription start site by deletion analysis (17). In the presence of nitrate, a second regulatory protein, NarL, acts at a site approximately 145 bp upstream of the Fnr-binding site and stimulates *nar* expression an additional 10-fold in an Fnr-dependent fashion (17). How these two proteins function at the molecular level to activate *nar* transcription remains to be demonstrated. To begin to dissect the mechanism of Fnr-dependent regulation of gene expression, we have isolated and characterized *fnr* mutants that are able to express the *nar* operon in the presence of oxygen.

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MATERIALS AND METHODS

Strain constructions and growth conditions. For routine work, cells were grown in LB medium at 37°C. Phage (P1 and λ) manipulations were performed essentially as described by Silhavy et al. (27). RZ7350 (*narG234*::Mu dI1734) was constructed by P1 transduction of the Kan^r transposon

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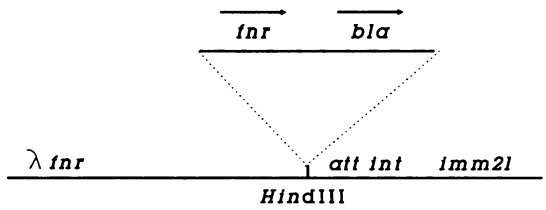

FIG. 1. λ D69 derivative showing the orientation of the *fnr*- and *bla*-containing *Hin*dIII fragment inserted into the unique *Hin*dIII site.

bacteriophage Mu dI1734 (5) from VJS882 (*narG234*::Mu dI1734; Valley Stewart, Cornell University) to MG1655 *lacZ*△*145* (RZ4500; Muhyeon Choe, this laboratory). Mu dI1734 contains a *trpA'-lacZ*$^+$ W209 gene fusion that places *lacZ* expression under control of the *narGHIJ* operon [Φ(*narG-lacZ*)] in strains VJS882 and RZ7350. Because of this insertion these strains are also deficient for the major nitrate reductase of *E. coli*. The Fnr$^-$ derivative of RZ7350, RZ7351, was constructed by transduction of *zcj*::Tn*10* (which is 95% linked to *fnr-501*) from EC2111 (*fnr-501 zcj*::Tn*10*; *fnr-501* is a null allele of *fnr*, obtained from J. Wild, University of Wisconsin) to RZ7350 and scoring the Tet^r transductants for a Lac$^-$ and anaerobic respiration-deficient phenotype. The anaerobic respiration-deficient phenotype was determined by the inability of the transductants to grow anaerobically on M63 minimal medium (19) containing 20 mM glycerol as a carbon source and 20 mM fumarate as an electron acceptor.

Plasmid and phage constructions. The *Hin*dIII-*Hin*cII fragment containing *fnr* was derived from phage λ18-259 (obtained from an *E. coli* MG1655 library; Donna Daniels and Fred Blattner, University of Wisconsin). This restriction fragment was cloned into the *Hin*dIII-*Sma*I sites of pUC19 (39) to generate pRZ7315. A *Hin*dIII site was placed downstream of *bla* (Ap^r gene) by partially digesting pRZ7315 with *Dra*I and inserting a *Sma*I fragment containing a Sp^r gene (which carries flanking *Hin*dIII restriction sites [23]) into the *Dra*I site located 44 bp downstream of *bla*. The *Hin*dIII fragment from this plasmid (pRZ7306) containing *fnr* and *bla* was isolated and inserted into the unique *Hin*dIII site of λD69 (20) to generate λ*fnr* (Fig. 1). The relevant genotype of this phage is λ *fnr*$^+$ *bla*$^+$ *att*$^+$ *int*$^+$ *imm*^{21}. Lysogenic integration of this phage at the *E. coli att* site can be directly selected by virtue of its ampicillin-resistant phenotype. λ *fnr* lysogens were isolated on LB medium containing 30 μg of ampicillin per ml. Lysogens were shown to contain a single copy of λ by the *ter* excision test (22).

Mutagenesis. λ *fnr* was grown on strain KD1067, which contains the *mutD5* mutator allele (8), in NZY medium (18). The mutator activity was induced after phage absorption by adding 10 μg of thymidine per ml. Phage lysates were collected 4 to 6 h later and usually contained 1 to 3% clear plaque mutants. The percentage of clear plaques was used as an indicator of the efficiency of mutagenesis, since the unmutagenized phage has a turbid-plaque phenotype.

Selection for *fnr* mutants. RZ7351 was grown to late exponential phase in LB medium. Cells were washed and resuspended in 10 mM $MgSO_4$. Approximately 2×10^8 cells were infected with control or mutagenized λ *fnr* at a multiplicity of infection of approximately 0.5. After 20 min for phage absorption, 1 ml of LB was added and cells were incubated for 1 h at 37°C to allow for *bla* expression. After being washed in M9 (19), cells were plated on M63 minimal medium (19) containing 10 mM lactose, 0.04% Casamino Acids (Difco Laboratories), 50 μg of ampicillin per ml, 80 μM KNO_3, 0.16 μM ammonium molybdate, and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml and incubated aerobically at 37°C. Lac$^+$ colonies appearing after 36 h were picked for further characterization.

Assay of β-galactosidase. For aerobic cultures, RZ7351 λ *fnr* and its derivatives were grown in M9 minimal medium (19) containing 20 mM glycerol as a carbon source. To minimize oxygen limitation of aerobic cultures, 4 ml of cells was shaken at 300 rpm in test tubes (18 by 150 mm) for more than eight generations without exceeding a culture optical density at 600 nm of 0.3. For anaerobic cultures, cells were grown to mid-log phase in M9 minimal medium (19) containing 10 mM glucose, 0.16 μM ammonium molybdate, and a 1/100,000 dilution of trace element solution (28) in an N_2 atmosphere in butyl rubber-stoppered tubes. To terminate cell growth and any further protein synthesis, chloramphenicol (30 μg/ml) was added and cells were immediately placed on ice until assayed for β-galactosidase. Cells were treated with chloroform–0.1% sodium dodecyl sulfate, and β-galactosidase was assayed as described by Miller (19).

DNA sequencing. Mutations were identified by sequencing either λ *fnr* phage DNA or *fnr*-containing plasmid DNA. Plasmid derivatives were obtained by cloning the *Hin*dIII restriction fragment from each phage containing *fnr* and *bla* into the *Hin*dIII site of pACYC184 (6). DNA sequence was determined from at least one strand by using four sequence-specific oligonucleotides. At least 60 bp upstream (which included the *fnr* promoter) to 50 bp downstream of *fnr* was sequenced by using a *Taq* polymerase dideoxy sequencing kit (Promega, Madison, Wis.) as recommended by the manufacturer. The four oligonucleotide primers used in sequencing were synthesized at the University of Wisconsin Biotechnology Center.

RESULTS

Selection for Fnr mutants that increase expression of the *nar* operon in the presence of oxygen. The *narGHIJ* operon, which requires Fnr for transcription, is induced by oxygen deprivation. We sought to isolate *fnr* mutants that would be able to express the *nar* operon in the presence of oxygen (hereafter referred to as Fnr* mutants). Since RZ7350 [Φ(*narG-LacZ*)] was phenotypically Lac$^-$ when grown aerobically, Fnr* mutants should be distinguished by a Lac$^+$ phenotype. The frequency of obtaining spontaneous Lac$^+$ mutants from RZ7350 (Fnr$^+$) was approximately 10^{-6}. Nine of these spontaneous mutants were mapped, and in each case the mutation was shown by P1 transduction to be linked to the *nar* operon (data not shown). Therefore, to increase the frequency of isolating *fnr* mutants with this phenotype, a λ phage carrying *fnr* (λ *fnr*) was mutagenized by propagating the phage on a *mutD* mutator strain (8). The mutagenized phage were introduced into RZ7351 (Fnr$^-$), and Fnr* mutants were selected by the ability to grow on lactose-containing media under aerobic conditions. Ap^r (also carried on λ *fnr*) lysogens bearing an Fnr* phenotype appeared 10 times more frequently from mutagenized λ *fnr* than from nonmutagenized phage. Ten Lac$^+$ Ap^r isolates were streak purified, and phage were obtained from these strains by spontaneous induction. Phage were collected from the supernatant of log-phase cultures, plaque purified, and then infected into RZ7351 to distinguish whether the Lac$^+$ phenotype was host or phage associated. For seven of the

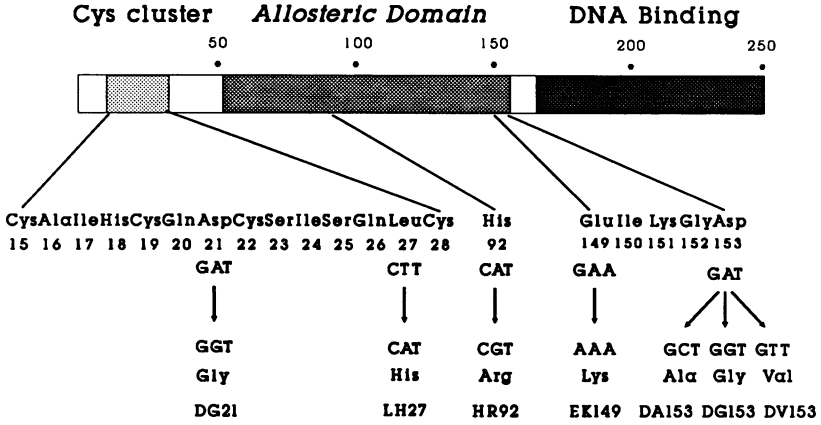

FIG. 2. Locations and designations of *fnr** mutations. Locations of the mutations were determined by DNA sequencing as described in Materials and Methods. The base substitutions are indicated by arrows. The correction of GC to CG, which replaces Ser-28 with Cys, is incorporated into the wild-type sequence. The assignment of the domains in Fnr is based on the alignment of Shaw et al. (26).

phage, the Lac$^+$ phenotype was phage associated; the characterization of these λ *fnr* mutants is described below.

Sequence changes in wild-type strains and Fnr* mutants. During sequencing, we discovered that the wild-type gene we had cloned from a λ library contained a Cys residue at position 28 instead of the Ser residue originally reported (25). This is of particular significance since it places a fourth Cys residue within six amino acids of the previously described Cys cluster that is proposed to function as a redox-sensitive site in Fnr. This Ser-to-Cys change at position 28 is in agreement with that recently reported by Trageser et al. (33). An additional change in the DNA sequence was found downstream of the *fnr* gene in *orfX* (25), where the G at position 1588 should be deleted. None of *orfX* is present on λ *fnr*.

The nomenclature used to designate the mutations consists of two single-letter amino acid abbreviations indicating the change, followed by the position (e.g., LH27 is a Leu-to-His change at position 27). The location of each mutation in *fnr* was determined by sequencing the entire gene. All seven mutants contained single amino acid substitutions that mapped to the N-terminal 60% of FNR (Fig. 2). No substitutions were identified in the carboxy-terminal DNA-binding domain. All of the mutations resulted in replacements to more basic amino acids. Two mutations mapped in the amino-terminal domain containing the Cys cluster. In mutant LH27, replacement of Leu-27 with positively charged His occurs adjacent to the newly identified Cys-28. The second mutation in this region, DG21, also changes the charge of the amino acid next to a Cys residue by removing one negative charge. One mutation, HR92, resulted in a substitution (His to Arg) in the region analogous to β-strand 5 of the CAP cAMP-binding domain. The last four mutations were clustered in a region similar to the CAP C helix. At position 149, Glu is replaced with Lys. At position 153, three different neutral substitutions (Ala, Gly, and Val) were obtained by second-position changes in the codon for the acidic amino acid Asp-153. The spectrum of mutations observed (four transitions and three transversions) showed a much higher frequency of transversions than is expected with use of this *mutD* allele (8).

Fnr* mutants increase expression of the *nar* operon in the presence of oxygen. Single-copy λ lysogens of RZ7351 bearing mutant or wild-type λ *fnr* were constructed to assess the effect of these mutations on Φ(*narG-lacZ*) expression. Glycerol was chosen as a carbon source for aerobic cultures, since we observed a small (two- to threefold) but reproducible repression for both wild-type and mutant strains when glucose was the carbon source (data not shown). Fnr* strains showed an increase in Φ(*narG-lacZ*) expression ranging from 1.7-fold (DG21) to 5.5-fold (LH27) relative to that of the wild-type *fnr* strain (Fig. 3A).

Nitrate amplifies the aerobic activity of Fnr* mutants. Previous studies have shown that nitrate stimulates *nar* expression through the regulatory protein NarL in an Fnr-dependent fashion (17, 32). It was not surprising then that the level of β-galactosidase was increased in all strains by the addition of 1.4 mM KNO_3 to the growth media (Fig. 3B). In fact, the nitrate stimulation was much greater for Fnr* mutants (23- to 48-fold) than for the Fnr$^+$ strain (7.7-fold). Therefore, all of the Fnr* mutants had significantly higher levels of Φ(*narG-lacZ*) expression (from 5.5- to 34.1-fold) than did the wild type in the presence of both oxygen and nitrate. Again, DG21 showed the lowest Φ(*narG-lacZ*) expression, while LH27 had the highest activity. The properties of these strains imply that NarL can activate *nar* transcription in the Fnr* mutants in the presence of oxygen.

LH27 shows reduced activity under anaerobic conditions. Wild-type Fnr normally shows maximal activity under anaerobic conditions. Therefore, Φ(*narG-lacZ*) expression was assayed for both wild-type and mutant strains grown anaerobically (Fig. 3C) to test the normal function of these mutants. Glucose was chosen as the carbon source for anaerobic growth, since this allowed cells to generate energy by fermentation and thus would not require Fnr function for growth. Wild-type Fnr induced Φ(*narG-lacZ*) expression approximately 77-fold under anaerobic conditions (relative to aerobic conditions). LH27 showed the weakest anaerobic induction of Φ(*narG-lacZ*) expression (threefold) and the lowest β-galactosidase activity of all strains tested. This anaerobic phenotype of LH27 is in contrast to what was observed under aerobic conditions, in which *nar* expression was the highest. All of the substitutions at position 153 showed small (24 to 42%) decreases in levels of β-galactosidase relative to the wild-type level, whereas DG21, HR92, and EK149 showed activities similar to that of the wild type.

Nitrate restores Fnr* mutants to wild-type activity under anaerobic conditions. Nitrate stimulated expression of

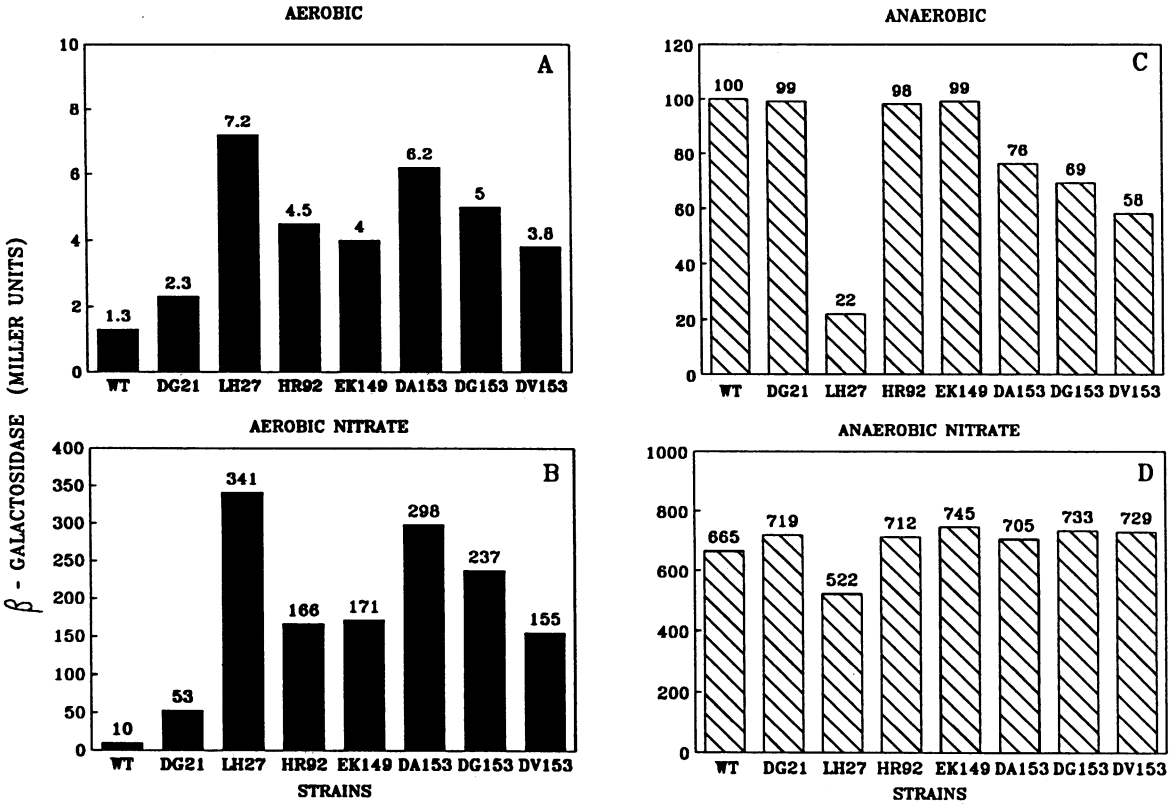

FIG. 3. Expression of *nar* as measured by β-galactosidase activities of wild-type (WT) and Fnr* mutant strains containing Φ(*narG-lacZ*), reported in Miller units. Cultures were grown aerobically in glycerol minimal medium (A and B) and anaerobically in glucose minimal medium (C and D). KNO_3 was added to 1.4 mM (B) and to 20 mM (D).

Φ(*narG-lacZ*) 6.7-fold in the Fnr^+ strain, similar to what was observed under aerobic conditions (7.7-fold stimulation). The nitrate induction ratio (7- to 12-fold) and the level of β-galactosidase for most of the Fnr* mutants were similar to values for the wild type (Fig. 3D). LH27 had just slightly lower levels of β-galactosidase (78%) and showed the largest induction by nitrate (24-fold) under anaerobic conditions. This observation indicates that although the anaerobic activity of LH27 is partially defective under anaerobic conditions, the presence of nitrate can overcome this defect.

DISCUSSION

This report describes Fnr structural gene mutations that allow activation of Fnr-dependent gene expression in the presence of oxygen. Since the physiological signal regulating oxygen control of gene expression is unknown, Fnr mutants that function in the presence of oxygen should be useful for studying how oxygen controls Fnr activity. A simple explanation for the phenotype of Fnr* mutants is that they no longer require the physiological signal induced by oxygen deprivation for activation of the wild-type protein. Thus, the mutant proteins may exist in a conformation that mimics active Fnr. Alternatively, the mutant proteins may have an altered threshold for sensing the physiological signal induced by oxygen deprivation.

Fnr* mutants that map to a region similar to the CAP cAMP-binding domain. It is difficult to determine the significance of the fact that Fnr contains a region similar to the cAMP-binding domain of CAP, since cAMP has no known role in regulating Fnr activity (35). However, the fact that five Fnr* mutants map to this region suggests that it provides an important function in Fnr activation. In addition to binding cAMP, the CAP cAMP-binding domain plays a critical role in the conformational change induced by cAMP which allows specific DNA binding of the CAP dimer (1, 10, 11, 38). Because the structure of CAP in the absence of cAMP is not known, the details of this conformational change have been largely inferred from analysis of mutants (e.g., CAP* mutants [1, 9–11, 37]). Therefore, it is possible that the similarity between Fnr and CAP in this domain reflects a conservation of residues required for transducing the conformational change rather than for binding an effector molecule.

None of our Fnr* mutants map to positions equivalent to those to which existing CAP* mutants map (Fig. 4). Since we have isolated each mutant only once, it is clear that we have yet to genetically saturate all of the sites that may allow for the Fnr* phenotype. Thus it may still be possible to identify Fnr* mutants that map at positions similar to those to which CAP* mutants map (the majority of the CAP* mutants map to the D helix within the DNA-binding domain [1, 9–11]). Yet despite the fact that the Fnr mutants do not map identically to CAP* alleles, the Fnr* substitutions are located in regions of CAP that are believed to play a role in the cAMP-induced conformational change. Therefore, we propose that the region of Fnr similar to the cAMP-binding domain of CAP be termed the allosteric domain to denote its involvement in the conformational change necessary for Fnr activation in response to the physiological signal of oxygen deprivation.

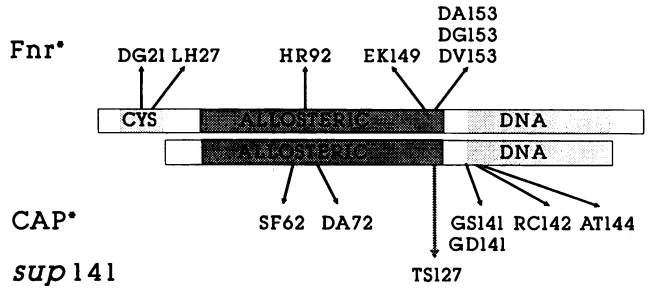

FIG. 4. Comparison of Fnr and CAP. Locations of Fnr* and CAP* (1, 9–11) mutants are indicated by arrows. A suppressor of CAP* GS141 that is no longer cAMP independent maps at positions 127. CAP127 is the position analogous to Fnrl53 (26).

Four Fnr* mutants (EK149, DA153, DG153, and DV153) map to a region similar to the CAP C helix. One cAMP-induced conformational alteration proposed to occur in CAP is subunit reorientation along the C helix in the CAP dimer (10). In particular, the position analogous to Fnr153 in CAP (T127) defines an important site for CAP activation by cAMP, since a Thr-to-Ser substitution at this position can suppress the cAMP independence of a CAP* mutant (10; Fig. 4). If the structure of Fnr is indeed similar to that of CAP (26, 38), then the location of the Fnr* mutants in the putative Fnr C helix is consistent with this being a site involved in the activation of the Fnr dimer.

In addition, all four of these Fnr* mutants result in acidic-to-neutral amino acid substitutions. This implies that disruption of a charge-charge interaction may be important in the conformational change that generates active Fnr. Moreover, the three Fnr* mutants at position 153 had different activities, which may be correlated with the flexibility or size of the substituted amino acid side chain. For example, valine has the largest side chain of the three substitutions obtained, and the DV153 protein has the least activity. Glycine has the smallest side chain, but it also has the greatest potential for destabilizing an α helix as a result of its flexibility. The DG153 protein exhibited activity intermediate between those of DV153 and DA153.

All three Fnr* substitutions isolated at residue 153 were second-position changes. In this regard, it will be interesting to see whether additional Fnr* mutants can be isolated by first-position changes in the D153 codon, since first position changes should lead to charged (His) or larger side chain (Tyr and Asn) substitutions which may or may not yield an Fnr* phenotype. Third-position changes at codon 153 would yield silent or conservative (Glu) amino acid replacements.

One Fnr* mutant (HR92) maps in a region analogous to CAP β-strand 5 in the cAMP-binding domain (Fig. 4). CAP β-strand 5 is also proposed to participate in CAP activation because of its close proximity to cAMP in the crystal structure (38), its interaction with helix E in the DNA-binding domain (38), and the location of a CAP* mutant in this β strand (1). It is possible that Fnr H92 is in a structure similar to β-strand 5 of CAP and serve an analogous function in Fnr.

Fnr* mutants that map to the Cys cluster. The fact that we obtained Fnr* mutants in the N-terminal Cys cluster is consistent with the function of this domain in regulating Fnr activity. It has previously been noted that clusters of Cys residues like those in Fnr are commonly found in cytochromes, iron-sulfur proteins, and metal-binding proteins (2, 7). In fact, other oxygen-sensitive transcriptional regulators such as the *Rhizobium meliloti nifA* gene product also contain Cys residues which are required for their activity (7). More recently, Trageser and Unden (34) and Spiro et al. (31) have suggested that the Cys cluster mediates its effect through metal binding. It is known that the redox properties of metal-binding clusters can be strongly influenced by the surrounding environment (21). Therefore, it is conceivable that Fnr* mutants LH27 and DG21 are altered in their redox properties and have an altered threshold for the signal of anaerobiosis. Alternatively, the particular amino acid substitution in LH27 or DG21 could modify the structure of the protein to mimic the conformation of tne protein present in anaerobic wild-type cells. It will be interesting to determine whether similar substitutions next to other Cys residues of Fnr or the Cys residues of NifA change the oxygen sensitivity of gene expression.

Mutant LH27 showed the most oxygen-independent phenotype of all of the mutants. In fact, LH27 had the highest expression of Φ(*narG-lacZ*) in the presence of oxygen but also showed impaired function under anaerobic conditions. If the LH27 substitution has induced a conformational change in this protein that mimics the active form of the wild type, it appears that this substitution also renders this protein unresponsive to the oxygen deprivation signal and therefore reduces its anaerobic activity. However, addition of nitrate to the media restored *nar* expression in LH27 to near wild-type levels anaerobically. This finding shows that *nar* expression by the LH27 protein is greatly enhanced by the presence of nitrate under anaerobic conditions (presumably through NarL).

Fnr* activity does not require nitrate. The oxygen-independent phenotype of all of the Fnr* mutants at the *nar* promoter was amplified by the presence of nitrate in the media. However, the Fnr* mutants activate other Fnr-dependent operons in a nitrate-independent fashion (14a). This finding suggests that the nitrate effect is specific to the *nar* operon and is presumably acting through the regulatory protein NarL.

Bonnefoy et al. (4) previously isolated mutants with increased expression of *nar* in the presence of oxygen. In one mutant studied in detail, the phenotype could be explained by the insertion of an IS*5* sequence upstream of *fnr*, causing increased expression of Fnr. These authors also showed that expression of Fnr from a multicopy plasmid resulted in *nar* expression in the presence of oxygen, suggesting that some fraction of Fnr is always in the active form (4). In our study, there is no indication that increased protein levels are responsible for the Fnr* phenotype, since there were no sequence changes in the *fnr* control region in any of the mutants and no differences were observed in levels of *fnr**-specific mRNA for three mutants examined.

In both cases, the aerobic expression of *nar* was enhanced by the presence of nitrate. One possible interpretation of the results for our mutants and the data of Bonnefoy et al. (4) is that maximal transcription from the *nar* promoter under aerobic conditions requires the interaction of NarL with Fnr. NarL could enhance transcriptional activation of *narGHIJ* either by increasing Fnr binding at the *nar* promoter and/or by facilitating transcription initiation. Clearly, the development of an in vitro system to study the properties of NarL and Fnr will be necessary to understand transcriptional activation at this promoter. However, the effect of nitrate on the Fnr* phenotype at the *nar* promoter may eventually provide clues to how NarL and Fnr function.

Anaerobic activity of Fnr* mutants. All of the Fnr* mutants

had close to wild-type activity anaerobically in the presence of nitrate, even though some mutants were not as active as the wild-type protein anaerobically in the absence of nitrate. Therefore, although the nitrate stimulation of *nar* expression for the mutants was variable, the maximal level of anaerobic *nar* expression in the presence of nitrate was always the same and was similar to that of wild-type Fnr. Presumably, this result reflects maximum *nar* promoter occupancy by either wild-type Fnr or Fnr* mutants.

In summary, these experiments have shown that it is possible to isolate mutant Fnr proteins which activate transcription in the presence of oxygen. Future studies to identify additional Fnr* mutants and elucidate the molecular basis for their phenotype may add to our understanding of the mechanism of oxygen-regulated gene expression. Finally, we hope to use purified Fnr* proteins to develop an in vitro system and correlate the structures of these mutant proteins with their functions as transcriptional regulators.

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