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In vivo and in vitro mutants of FNR the anaerobic transcriptional regulator of *E. coli*

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FNR regulates the expression of target genes in response to anaerobiosis. It resembles the catabolite gene activator or cAMP-receptor protein (CRP) except for the presence of an N-terminal cysteine cluster, which may form a redox-sensing iron-binding site. Site-directed mutagenesis has shown that 3 of the 4 cysteine residues in the N-terminal cluster (Cys-20, -23 and -29, but not Cys-16) and the only other cysteine residue (Cys-122), are essential for the normal activation and repression of FNR-dependent promoters. Deletion of residues Pro-3–Arg-9 (inclusive) had no effect, but FNR was inactivated by a frameshift extending through the C-terminal DNA-binding domain. Four independent in vivo mutants contained identical Gly-96→Asp substitutions, which may inactivate FNR by distorting a sharp turn between β-strands in the predicted structure.

FNR; Transcriptional regulation; Anaerobic gene expression; Cysteine residue; *fnr* mutant; *Escherichia coli*

1. INTRODUCTION

The *fnr* gene of *Escherichia coli* encodes a transcriptional regulator (FNR), which activates the expression of a variety of anaerobic functions in response to anoxia [1–3]. FNR also regulates its own synthesis and represses the NADH dehydrogenase II gene, *ndh*, under anaerobic conditions [4,5]. It has been predicted that the FNR protein is structurally analogous to the cAMP receptor protein (CRP), which activates genes that are subject to catabolite repression (Fig. 1). Furthermore, both appear to regulate transcription by similar mechanisms because FNR derivatives which activate CRP-dependent genes in response to anoxia, and CRP derivatives which control FNR-regulated genes in response to cAMP, have been constructed by targeted mutagenesis in the corresponding DNA-recognition helices [3,7].

The mechanism by which FNR senses and responds to anoxia is not known, but attention has focussed on the cysteine-rich N-terminal extension as a potential iron-binding site and sensor (Fig. 1). This is because: (1) FNR is inactivated by deleting residues 3–30 and by a Cys-20→Ser substitution in FNR-197 [8], (2) chelating agents mimic oxygen in inhibiting the anaerobic activation or repression of FNR-regulated genes [5] and increasing the rate of FNR cysteine-residue carboxymethylation in permeabilized cells [9]; and (3) monomeric FNR (M_r 30,000) binds up to 1 mol of iron per mol, the iron and sulphydryl contents being inversely related [3].

Here the structure-function relationships of FNR have been investigated by defining the molecular lesions of several previously characterized *fnr* mutants and by examining the consequences of further site-directed substitutions and deletions.

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Abbreviations: CRP, cAMP receptor protein; PCR, polymerase chain reaction; SSB, single-stranded DNA binding protein

2. EXPERIMENTAL

2.1. *DNA manipulation, sequencing and materials*

Standard procedures were used for DNA isolation, DNA manipulation, and dideoxy-sequencing of M13 and plasmid templates [10], with commercial enzymes (BRL, NBL and USB) and [α-^{35}S]dATP (NEN). Mutagenic oligonucleotides and primers for sequencing the *fnr* gene were synthesized with an Applied Biosystems DNA Synthesizer (model 381A).

2.2. *Isolation of mutant* fnr *genes by PCR*

The *fnr* genes of 4 independent mutants (JRG861a,b,c and d, formerly: E9, *fnr-1*; 01, *fnr-8*; F3, *fnr-2*; and J2, *fnr-4*) [11], were isolated from genomic DNA by PCR [10] with two primers flanking the *fnr* gene: S166 (5′-TTTCAAAT^{C}GATAGACATAT-3′, coordinates 392-411 in *fnr* [1]), which incorporates an A→C mismatch at position 400 to create a *Cla*I site; and S167 (ACAACTGTCAACGCAGTTTG, 1387–1407 [1]), which contains a *Hin*cII site. The amplified 1-kb fragments were treated with *Cla*I and *Hin*cII, ligated into the *Acc*I and *Sma*I sites of pUC13, and 4 clones of each type were sequenced. The plasmid derivatives, designated pGS396, contained inserts with identical DNA sequences (Table I).

2.3. *Site-directed mutagenesis*

Oligonucleotides were used in the Kunkel single-primer method [12] to direct the following mutations: Cys-16→Ala, S161 (GGATAGCA^{GC}ACCGCCAGACTG, 553–575); Cys-23→Ala, S162 (GGCTGATGCTG^{GC}ATCCTGGCA, 577–598); Cys-122→Ala, S164 (GGATTTCA^{GC}TACCATCGAGG, 872–892), Cys-29→Gly/Ala,

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Table I

Plasmids expressing mutant FNR proteins and their abilities to complement an *fnr* deletion strain

Plasmid			Protein	Amino acid substitution or deletion	Growth phenotype Fnr$^+$/Fnr$^-$
pBR322	pBR328	pUC			
pGS24	pGS388	pGS336	FNR	Wild-type	+
		pGS396	FNR-396	Gly-96→Asp	−
pGS387	pGS395		FNR-387	Δ (Pro-3-Arg-9)	+
		pGS398	FNR-398	Δ (Phe-191-Ala-250)*	−
pGS382	pGS389		FNR-382	Cys-16→Ala	+
pGS197	pGS390		FNR-197	Cys-20→Ser	−
pGS383	pGS391		FNR-383	Cys-23→Gly	−
pGS384	pGS392		FNR-384	Gys-29→Gly	−
pGS385	pGS393		FNR-385	Cys-122→Ala	−
pGS386	pGS394		FNR-386	Glu-64→Gln	+

*The deleted segment in this construct is replaced by 19 unrelated amino acid residues, see section 2.3

S169 (TGTGAACGGGATG$^{C/GC}$AAGCTGGCTG, 594–618); Glu-64→Gln, S174 (CGATTTAAGTT^{G}ATCACCAG, 701–720); deletion of residues 3–9 inclusive, S165 (GACTGAATGCGGAC-CATGGGTC, 515–525 and 547–557). The template was an M13mp9 derivative containing an *fnr* gene with a unique *Nco*I site at the translation initiation site [4]. With S169, SSB (4 μg) was added to the template prior to oligonucleotide annealing. Mutant *fnr* genes were fully sequenced, rendered double-stranded by primer extension, and subcloned as 1.6 kb *Hin*dIII-*Bam*HI fragments into pBR322 and pBR328. All mutations were as directed except with S162 where Cys-23→Gly (GGC) rather than Ala(GCC) was consistently recovered. The wild-type and mutant (Cys-20→Ser) *fnr* genes were similarly transferred from pGS24 [1] and pGS197 [8] to pBR328 and pUC19. The altered FNR proteins and the plasmids from which they are expressed are listed in Table I.

The pUC18 derivative, pGS398, encoding FNR-398 in which 60 C-terminal residues are replaced by 19 residues (LIPSDYDSR-RYRQLSRLNH) from an altered reading-frame was constructed by 'filling in' the *Eco*RI site at position 1087 in an *fnr-hlyX* hybrid gene (Sharrocks and Guest, unpublished).

2.4. *Phenotypic tests for* fnr *mutations*

The pUC and pBR322 derivatives expressing altered *fnr* genes were transformed into JRG1728(Δ *lac* Δ *fnr*) [8] to test their ability to restore anaerobic growth on glycerol-fumarate medium [11], and for Western blotting to confirm that an FNR protein is produced [4].

The ability to regulate gene expression under aerobic and anaerobic conditions was quantified as described previously [8] by assaying β-galactosidase synthesis in transformants of two strains: JRG2087, which contains an FNR-dependent *melR-lacZ* fusion plasmid (FF/pRW2A, Tc^R [13]) in JRG1728 (Δ *lac* Δ *fnr*), and JRG1991, which is a λ*ndh-lacZ* (λG221) lysogen of RK5279 (Δ *lac fnr-250*) [5]. The former was host for the pUC and pBR322 derivatives (Ap^R selection), and the latter for the pBR328 derivatives (Cm^R selection).

3. RESULTS AND DISCUSSION

3.1. *Molecular characterization of the mutations in* fnr *mutants*

The defective *fnr* genes of 4 *fnr* mutants, obtained by nitrosoguanidine-treatment of cultures from independent single colonies of the parental strain [11], each contained a single G→A transition at coordinate 806 [1]. This generates a Gly-96→Asp substitution in the corresponding FNR-396 proteins, which are essentially inactive in the phenotypic tests (Table I; Fig. 2a). Gly-96 is located in a sharp turn between β-strands 5 and 6 in the proposed FNR structure (Fig. 1), and based on this small selection of mutants, it would appear to be a hot-spot for inactivating mutagenesis. In CRP the analogous turn is a type II reverse turn [14], which requires glycine in the i + 2 position and aspartate would inevitably distort the structure. The same distortion is predicted for the FNR molecule and the substitution could further interfere with the formation of the putative iron-binding site or sensor, and any allosteric changes associated with the activation of FNR.

3.2. *N- and C-terminal deletion mutants*

Purified FNR generally has an apparent M_r of 29,000, which is about 1000 less than the protein in intact bacteria, due to the removal of 9 N-terminal amino acid residues [15]. To determine whether the truncated

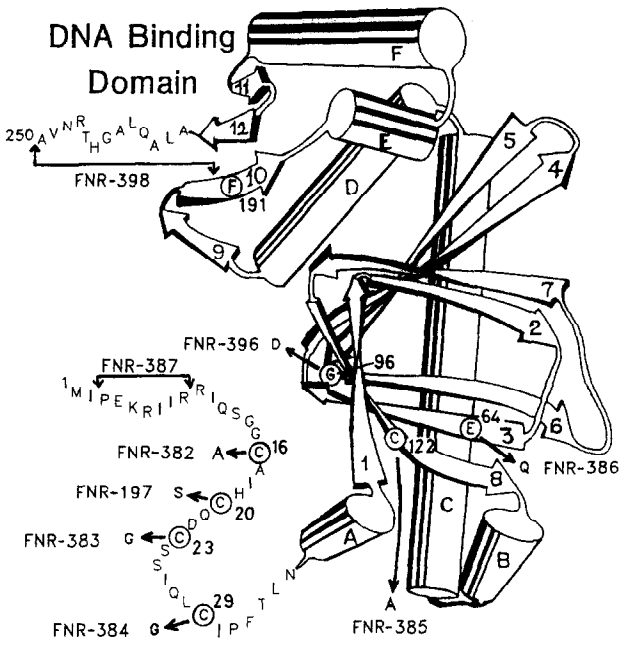

Fig. 1. Predicted 3-dimensional structure of FNR based on the CRP monomer [6], with putative α-helices (A–F), β-strands (1–12) and segments of unknown secondary structure at the N- and C-terminal ends. The amino acid substitutions and deletions in specific mutant FNR proteins are indicated.

protein retains its biological activity, plasmids which express a comparable protein (FNR-387) were constructed by site-directed deletion (Table I; Fig. 1). The results show that the deletion in the N-terminal region has no significant effect on the anaerobic activation or repression of FNR regulated promoters (Fig. 2b,c). This contrasts with the total inactivation that accompanies the more extensive Pro-3–Ile-30 deletion [8], and it suggests that proteolytic cleavage in the N-terminal region is not a physiologically significant processing event.

The mutant protein (FNR-398) created by a frameshift which replaces 60 C-terminal residues (including the helix-turn-helix motif of the DNA binding domain) with 19 unrelated residues (Table I; Fig. 1), was inactive (Fig. 2a). This suggests that unless the substituent residues interfere, the DNA binding domain performs an essential role in transcriptional activation.

3.3. *Cysteine-residue mutagenesis*

Site-directed substitution of each of the 5 cysteine residues (Table I; Fig. 1) indicated that all but Cys-16 are essential for both activating and repressing gene expression in response to anaerobiosis (Fig. 2b,c). The Cys-122→Ala substitution is particularly deleterious. An arbitrary missense mutation (Glu-64→Gln in FNR-386) was created with a mutagenic sequencing primer, and it had no effect on FNR activity (Fig. 2b,c).

The results are consistent with the cysteine sulphydryls having roles in iron-binding and/or disulphide bridge formation in the anaerobic activation of FNR. Significantly, the 4 essential cysteine residues are conserved in a **Cys-X_2-Cys-X_{5-7}-Cys-X_{87-92}-Cys** motif in two recent additions to the CRP-FNR family: HylX, a potential regulator of haemolysin synthesis in *Actinobacillus pleuropneumoniae* (J. MacInnes, personal communication; [3]); and FnrN, a regulator of microaerobic N_2-fixation in *Rhizobium leguminosarum* (U. Preifer, personal communication; [3]). There are also two pairs of essential cysteine residues in the central domain and adjacent interdomain linker of the redox-sensitive NifA regulator of *Bradyrhizobium japonicum* [17]. The CRP-FNR family of transcriptional regulators control the expression of a diverse range of genes and the presence of the cysteine cluster seems to provide a sensing-switching mechanism for mediating the response to environmental signals in several of its members.

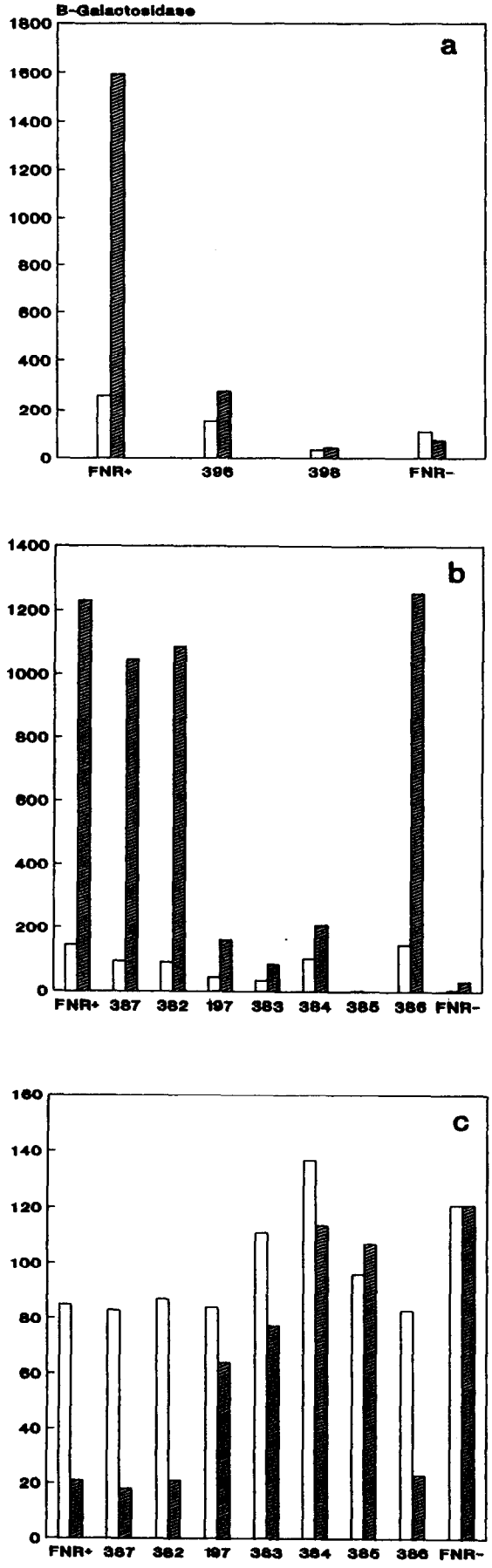

Fig. 2. Activation and repression of FNR-regulated *lacZ* fusions with altered FNR proteins listed in Table I. Aerobic (open) and anaerobic (shaded) expression of: FNR-activated *melR-lacZ* fusion with pUC plasmids (a) and pBR322 plasmids (b); and FNR-repressed *ndh-lacZ* fusion with pBR322 plasmids (c). FNR^+ and FNR^- denote controls with the wild-type *fnr* gene or no insert in the corresponding vector. Average specific activities are quoted in Miller units [16] for duplicate assays of β-galactosidase in at least 3 independent cultures (standard deviations, <10%).

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