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(REVIEW)

Gene Regulation and Transcriptional Regulation in Bacteria

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ABSTRACT

The catabolite gene activator protein (CAP) and the fumurate nitrate reductase (FNR) are two founder members of the growing CRP-FNR protein superfamily. The consensus FNR binding site (TTGAT-N_4-ATCAA) closely resembles that of CRP to the extent that both contain a common core motif (**N**TGA**N**-N_4-**N**TCA**N**). The transcription factor FNR plays a role in altering gene expression between aerobic and anaerobic conditions but CRP regulators control the response to glucose starvation. Protein DNA interactions occur between the regulatory protein and DNA at the corresponding promoter using the consensus either CRP or FNR binding sites. Successful transcriptional activation generally requires contact between a DNA-bound activator and RNA polymerase in order to generate on effective complex. CRP promoters are grouped into two classes depending on the location of DNA binding site. Class I promoters contains transcription activator binding sites centered near position -61.5, -71, -82 or -92. Class II promoters the regulator proteins bind to a site centered at or near -41.5 so three possible contacts, α_{CTD}-AR1, α_{NTD}-AR2 and δ^{70}-AR3, occur between regulator and RNA polymerase. FNR and FLP act as a class II activator.

Key Words: *Transcription regulation, FNR, CAP, FLP.*

1. INTRODUCTION

The transcription of bacterial operons is regulated by the product of regulatory genes, which are often proteins called repressors and activators. These regulatory proteins bind close to the promoters of operons and regulate transcription from the promoter. Repressor and activator proteins work in opposite ways. Repressors bind to a site called the operator and turn off the promoter so preventing transcription of the operon genes. Activators bind the activator sites and turn on the promoter as a result providing transcription of the operon genes. An operon regulated by a repressor is negatively controlled in contrast to an operon regulated by an activator, which is positively regulated. Activator and repressor proteins usually bind to different regions of the DNA. Activators usually bind upstream of the -35 sequence of the promoter where they can make contact with RNA polymerase bound to the promoter. Repressors often bind to the promoter region itself, or very close, and block access by RNA polymerase to the promoter.

2. CRP-FNR SUPERFAMILY

The cAMP receptor protein (CRP) also known as a catabolite gene activator protein (CAP) and the fumurate nitrate reductase (FNR) are two founder members of the growing CRP-FNR protein superfamily. The CRP-FNR family has 369 members and different members control various aspects in response to different environmental stimuli [1]. The superfamily has been divided into three main groups as FNR, CRP and NtcA, based on structural relationships and functional specificities [2, 3, 4].

The transcription factor FNR plays a major role in altering gene expression between aerobic and anaerobic conditions to induce many enzymes required to generate

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energy by anaerobic respiration [5]. The major FNR group differs from CRP in being a more diverse group containing at least four distinct subgroups designated as FNR, FnrN, FixK and DNR/FLP. Within the major FNR group, the FNR and FnrN subgroups show all the criteria formerly assigned to FNR proteins [3] and have a greater degree of sequence identity and DNA binding specificity corresponding to that of FNR rather than CRP. The FixK subgroup contains a distinct but related cluster of regulators from the nitrogen-fixing bacteria. However, members of the FixK subgroup are not one-component sensor regulators and their regulatory activity is controlled at the transcriptional level by an independent sensor regulatory system that modulates their expression. The DNR/FLP subgroup is a distinct group of regulators having sensory functions as well as binding to a potential FNR binding site.

CRP regulators are the second main group and represent a fairly homogeneous group of proteins that control the response to glucose starvation. The sequence identity in this family of proteins is high, ranging from approximately 20 to 70% [6]. The consensus FNR site (TTGAT-N_4-ATCAA) closely resembles that of CRP to the extent that both contain a common core motif (**N**TGA**N**-N_4-**N**TCA**N**). Indeed a single base substitution in each FNR half site (from TTGAT to GTGAT and ATCAA to ATCAC) is sufficient to change an FNR binding site to a CRP target [7, 8].

The distinct NtcA group centres primarily on regulators of cyanobacterial nitrogen and sulfur metabolism. The *ntcA* gene encodes a 222 amino acid protein and homologous member of CRP [9]. The structural consensus cyanobacterial NtcA binding site contains the sequence signature GTA-N_4-TAC that is located 20-23 nucleotides upstream from the –10 box [10]. CRP residues Glu^{72} and Arg^{82} are essential but only the Arg residue is conserved in NtcA so it seems likely that ultimately both the NtcA and CRP groups will need subdividing [4].

3. CATABOLITE ACTIVATOR PROTEIN

The cyclic AMP receptor protein (CRP) is a transcription factor found in *Escherichia coli*. In the past, CRP has been known by various names such as CAP or CGA (catabolite gene receptor protein). CRP modulates the transcription activity of many genes in response to glucose levels. High levels of glucose reduce the levels of cyclic AMP (cAMP) within the cell. Conversely, glucose starvation leads to an increase in cAMP levels allowing a molecule of cAMP to bind to CRP.

CRP has a molecular mass of 45kDa and is a dimer of two identical subunits, each consisting of 209 amino acids [11]. The N-terminal domain (residues 1-139) is responsible for dimerization of CRP and the dimer form of CRP interacts with each CRP subunit interacting with one half of the DNA site. The C-terminal domain (residues 140-209) is responsible for interaction with DNA, mediating interaction with DNA through a helix-turn-helix DNA binding motif. CRP specifically recognizes sequences conforming to related consensus TGTGA-N_6-TCACA sequences [12] whereas FNR recognizes sites conforming to a related consensus TTGAT-N_4-ATCA [13]. These specificities have been correlated with the presence of distinct RE--R (CRP) and -E--SR (FNR) motifs in the binding face of the respective DNA recognition helices [6]. The CRP DNA complex is 2-fold symmetric: one CRP subunit interacts with one half of the DNA site, and the other CRP subunit interacts with the other half of the DNA site. CRP bends DNA sharply to an angle of ~80^o [14] but bending by CRP does not appear to play a critical role in transcriptional activation at simple CRP dependent promoters [15]. The orientation of CRP-induced DNA bend is such that the DNA wraps towards and around the sides of CRP.

CRP-dependent promoters are grouped into two classes depending on the location of the DNA binding site for CRP [16]. Class I promoters contain a transcription activator binding site that is located upstream of RNA polymerase at sites centred near position -61.5, -71, -82 or -92. At a class I promoter, the activating region 1 (AR1) is adjacent to the downstream subunit of the CRP dimer and interacts with the C-terminal domain of the α-subunit (α CTD) of RNA polymerase [15]. At class II promoters the regulator binds to a site centred at or near -41.5. Thus RNA polymerase surrounds the regulator and three possible contacts, α_{CTD}-AR1, α_{NTD}-AR2 and δ^{70}-AR3, occur between regulator and RNA polymerase [4].

4. FNR PROTEINS AND OXYGEN SENSING BY FNR IN *E. coli*

Facultative anaerobic bacteria adopt different metabolic modes in response to the availability of oxygen. The transcription factor FNR plays a central role in allowing the bacteria to adapt to changes in oxygen availability in its environment. While FNR is present in the cell under both aerobic and anaerobic growth conditions, it functions as an active transcription factor only when cells are grown anaerobically and controls the transcription of >100 genes whose products facilitate adaptation of *E. coli* to growth under O2 limiting conditions [17]. A hierarchy of metabolism exists in which aerobic respiration is preferred to anaerobic respiration, which in turn is preferred to fermentation. This order reflects the relative energetic benefit derived from each metabolic mode. When oxygen is limiting, FNR activates many enzymes required to generate energy by anaerobic respiration and also represses synthesis of some enzymes involved in aerobic respiration.

FNR is located in the cytoplasm [18] and anaerobically purified FNR contains one $[4Fe\text{-}4S]^{2+}$ cluster per subunit [19, 20]. $[4Fe\text{-}4S]^{2+}$ cluster is necessary for the function of FNR as a transcription factor. Specific ligation to the $[4Fe\text{-}4S]^{2+}$ cluster increases dimerization and site specific DNA binding under anaerobic conditions [19, 5]. Amino acid residues 140-159 of FNR (**R**QQM**M**RL**M**SGE**I**KG**D**QDM**I**L) have been proposed to function in subunit-subunit interactions because the position of these residues corresponds to the helix along which the closely related *E. coli*

transcription factor, the cAMP receptor protein (CRP) dimerizes via a coiled coil interaction [19].

Aerobic inactivation of FNR occurs when the iron-sulfur cluster is exposed to air (Figure 1). The [4Fe-4S] 2+ cluster of FNR is converted to a [2Fe-2S] 2+ cluster with a concomitant decrease in specific DNA binding due to dissociation of FNR dimers to monomers [19, 20]. The [4Fe-4S] 2+ to [2Fe-2S] 2+ cluster conversion also occurs in vivo since anaerobic cells, exposed to air for 15 min, showed ~50% conversion of [4Fe-4S] 2+ to the 2Fe form as judged by whole cell Mössbauer spectroscopy [17]. The [2Fe-2S] 2+ cluster is semi-stable and decays to apo-FNR upon prolonged exposure to oxygen [17, 5, 21]. Furthermore, the cysteinyl ligands of apo-FNR are sufficiently reactive to generate intramolecular disulfide bonds between Cys122 and one of the N-terminal cysteine residues with the production of an oxidized disulfide form of apo-FNR, designated [2SH, S-S] [22]. The apo-FNR reactivates with glutathione as a reducing agent [18] so that FNR activation/inactivation is a reversible process [17].

Met144, Met147, Ile151, and Ile158 form a hydrophobic interface that promotes FNR dimerization. Two charged residues, Arg140 and Asp154 are also important for FNR function. Replacing Arg140 with either the neutral Leu or negatively charged Glu residue results in an FNR mutant with little anaerobic activity. Also the Asp154 residue has an inhibitory effect on dimerization, replacing Asp154 with Ala repaired the defects caused by Ala substitutions of other residues located on the same helical face so Asp154 is also a key element in the inhibition of FNR dimerization by O2 availability [23].

Each FNR monomer can acquire a single [4Fe-4S] 2+ cluster and four potential iron ligands (Cys20, Cys23, Cys29, Cys122) are essential for normal FNR activity in vivo [24]. Three of the essential residues (Cys20, Cys23 and Cys29) are located together in the N-terminal domain of FNR and the fourth cysteine (Cys122) is located in a region of β-roll structure [20]. Substitution of any of the four cysteine ligands of the iron-sulfur cluster has profound effects upon the in vivo activity of FNR. Altering Cys122 abolished the ability of FNR to inactive transcription from model FNR dependent promoters in vivo. On the other hand, replacing Cys20, Cys23, or Cys29, generated FNR proteins with decreased but detectable in vivo activity [25, 26]. Anaerobically purified N-terminal cysteine substitution (FNR-Cys20Ser) in contrast to wild type FNR, did not contain [4S-4S] clusters, suggesting that FNR-Cys20Ser cannot incorporate a [4Fe-4S] 2+ cluster [5]. FNR-C20S can acquire an oxygen sensitive cluster in vivo and that this is sufficient to mediate anaerobic respiration of a FNR-regulator promoter [24]. Studies with FNRΔ29 , which lacks all N-terminal cysteine residues and thus cannot form iron-sulfur clusters, indicated that two iron-sulfur clusters, one per subunit are required for FNR dimerization [27].

A mutant FNR protein containing a substitution of Leu28 with His (FNR-Leu28His) was characterised and functional under aerobic growth conditions [28]. Mössbauer spectroscopy was used to show that aerobically grown cells overexpressing FNR-Leu28His have as much 4Fe-FNR as those grown under anaerobic conditions. The stability of the [4Fe-4S] 2+ cluster to O2- increases [29]. These data provide compelling evidence that assembly of the Fe-S cluster into FNR can occur under aerobic conditions. Therefore the lack of WT-FNR activity under aerobic growth conditions cannot be simply explained by failure to assemble 4Fe-FNR.

5. CRP-FNR PROTEIN INTERACTIONS WITH DNA

Interactions occur between the regulatory protein and DNA at the corresponding promoter using the consensus CRP binding site containing the imperfect palindrome TGTGA-N6-TCACA [30]. The amino acid residues that are involved in the CRP: DNA interaction has been identified by a combination of structural and mutagenic studies [14, 31] and is located in the DNA recognition helices (αF) of the helix-turn-helix motifs (αE-αF) of the DNA binding domains. The two αF helix dimer complexes each occupy one half of the CRP target site where the individual CRP half-sites are located in adjacent major grooves separated from each other by one complete turn of the double helix, such that the CRP: DNA interactions occur on only one face of the helix. CRP regulator DNA recognition helices (αF) contains R180E181TVGR185ILKMLE amino acid residues. Three charged residues (Arg180, Glu181, and Arg185) located on one face of αF have been shown to interact directly with the nucleotides of the target site. Arg180 and Glu181 are responsible for direct contact with the first and second G-C pair of the binding site respectively and Arg185 contacts the second G-C pair and the third T-A pair.

DNA binding by FNR is oxygen sensitive so the function of FNR is limited to anaerobic conditions. The activation of FNR promotes site-specific DNA binding, and a consensus FNR recognition motif was identified by comparing the sequences of well characterized FNR dependent promoters [6]. The conserved G-C and A-T pairs of the TGA half-site core motif are contacted by Glu209 and Arg213 residues. The recognition helix of FNR (TV208E209TIS212R213LLGRGQ) possesses no equivalent to Arg180 of CRP; instead, a Ser 212 makes contact with the first T-A pair of the FNR site. Thus, Ser212 of FNR and Arg180 of CRP provide the discriminatory contacts between the regulators and their respective targets. Based on this prediction, CRP and FNR homologues have been classified according to the amino acid sequence motifs in their putative DNA-recognition helices, where those containing the FNR motif (E--SR) are regarded as FNR like proteins and those containing the CRP motif (RE--R) are regarded as CRP like proteins [3]. It has also been shown that FNR induces an 82-92^{o} bend in target DNA [32].

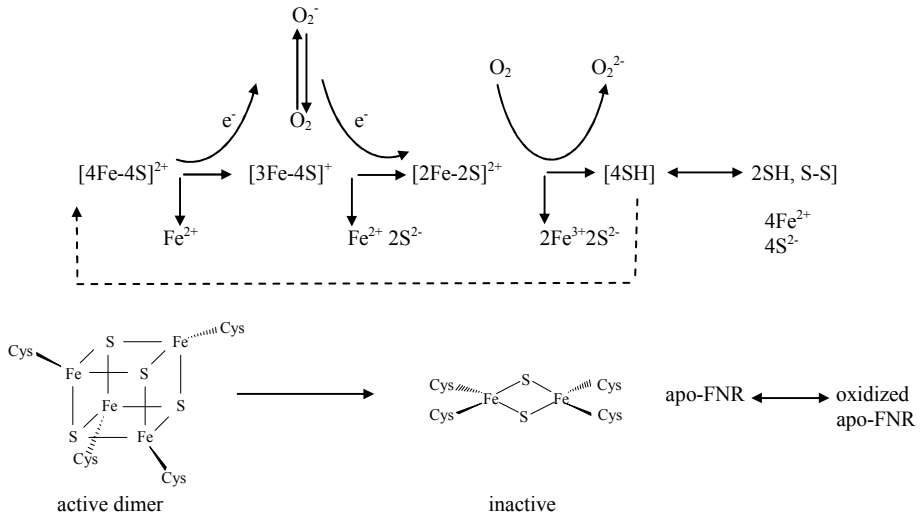

Figure 1. Direct oxygen sensing by the [4Fe-4S] cluster of FNR protein in *E. coli*. The $[4Fe\text{-}4S]^{2+}$ cluster is converted by molecular oxygen to yield a $[3Fe\text{-}4S]^{+}$ cluster, Fe^{2+} and a superoxide anion. The superoxide anion, generated in close proximity to the $[3Fe\text{-}4S]^{+}$ cluster, reduces the $[3Fe\text{-}4S]^{+}$ species to produce a $[2Fe\text{-}2S]^{2+}$ cluster, Fe^{2+} ions and, crucially oxygen which is free to attack another $[4Fe\text{-}4S]^{2+}$ cluster. The $[2Fe\text{-}2S]^{2+}$ form subsequently decays to the monomeric apo protein.

6. FNR-LIKE PROTEINS IN *Lactobacillus casei*

The first member of the CRP-FNR family identified in a gram-positive bacterium was the FNR-like protein (FLP) of Lactobacillus casei [33]. Alignment of FLP with CRP and FNR indicated that FLP is 26% identical to CRP (51% similar) and 22% identical (51% similar) to FNR. Purified and crystallized FLP are homodimeric and contains substoichiometric quantities of Cu and Zn [34]. The essential -E209--S212R combination was found but one of the essential N-terminal cysteines is conserved in FLP [33]. On the other hand, it was predicted to have an FNR-like DNA-binding specificity because its DNA recognition helix contains the E--SR motif which is thought to confer specificity for sites confirming to the FNR site consensus [13]. Isolated FNR did not recognize an FNR site but an FLP sequence (CCTGA----TCAGG) in which the TGA core motifs are separated by four bases [34]. The FLP consensus retains the same TGA/TCA core motifs in each half site but differ from FNR and CRP sequences in having four rather than six intervening nucleotides (TGA-N4-TCA). However, because the core motifs and two residues in the DNA binding motif E--R (Glu184 and Arg188) are conserved relative to CRP and FNR it is predicted that FLP will make contacts with its target. The major difference that might explain the divergent DNA binding specifities of FNR and FLP is the presence of proline as the first residue of the DNA recognizing helix (P183ETVSRTLKRL) in FLP. It is conceivable that in this position proline might affect the conformation of the αE-turn-αF DNA binding motif sufficiently to change the angle between the two helices and thus allow the helices of each subunit to penetrate deeper into the major grove and change the contact with the DNA. The FLP operates a novel redox-responsive switch based on the interconversion of disulfide and dithiol forms of the FLP subunits [34]. Moreover, in contrast to FNR, it was found that the oxidized (disulfide) form of FLP is the DNA-binding form, and that oxidized FLP binds with relatively low affinity to DNA sequences [34].

7. FNR-LIKE PROTEINS IN *L. lactis*

Gostick et al. (1999) used the polymerase chain reaction (PCR) approach to identify two genes (flpA and flpB) encoding FNR like proteins (FlpA and FlpB) with potential for mediating a response to oxygen level or oxidative stress in *L. lactis* [35]. Analysis of the DNA upstream and downstream of flpA revealed that part of an operon consisting of an incomplete orf encoding orfWA, followed by orfXA, orfYA,, flpA, orfZ and then yhbJ. Analysis of the flpB DNA region revealed a similar operon structure consisting of an incomplete orfWB, followed by orfXB, orfYB and then flpA gene. The flpA and flpB gene arrangements are similar and encoded proteins are highly homologous suggesting that the two operons have arisen by duplication. The corresponding promoters (orfXA and orfXB) were each associated with a potential FNR site (TTGAT-N4-ATCAA) at positions +4.5 (flpA operon) and -42.5 (flpB operon) [35]. The FlpA protein with 230 amino acids (Mw 25965 and pI 5.17) has sequence identity of 41% (62% similarity) with Lb. casei FLP, 25% (51% similarity) with FNR and 24% (48% similarity) with CRP. Furthermore, FlpA was predicted to retain all of the characteristic secondary structural features of the CRP-FNR family and according to the criteria suggested by Spiro (1994), represents an FNR-like protein closely resembling the FLP of Lb. casei. Despite their similarity, FlpA and FLP do not sense their respective stimuli by the same mechanism and they may respond to different environmental conditions [36].

The flpB gene encodes a product (FlpB) of 230 amino acid residues with amino acid sequence identity of 61%

(75% similarity) with FlpA, 41% (62% similarity) with Lb. casei FLP, 24% (52%) with FNR and 24% (47%) with CRP. The presence of an FNR-like DNA binding motif (E--SR) in the new Flp proteins suggests that they probably recognize either FNR or Lb. casei FLP sites, because both specificities are associated with this motif.

Two transcriptional regulator rcfA and rcfB genes were identified in *L. lactis* subsp. *lactis* IL1403 and RcfA has significant homology with FlpA and FlpB. Upstream regions of the rcfA gene contains potential FNR binding sites suggesting that the rcfA promoter expression levels may be affected by anaerobic and aerobic conditions [37].

The DNA recognition helices of FlpA (PETISRKLRL), FlpB (PETISRKFKI) and RcfA (PETISRKIKV) contain the E---SR DNA binding motif but two significant differences were observed between FNR (VETISRLLGR) and lactococcal Flp. Valine 208 (Val208) in FNR is replaced by a P residue at the equivalent position in lactococcal Flp and Gly126 in FNR is replaced by a positively charged residue in lactococcal Flp (Lys in FlpB and RcfA and Arg in FlpA) [37]. Comparison of FlpA with FlpB and RcfA indicates that within the last three residues of the helix FlpB (FKI) and RcfA (IKV) have two differences whereas FlpA (LRL) is completely different. FlpB and RcfA are more similar and as the results in this chapter show they appear to interact in the same way with their promoters.

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