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FORUM REVIEW ARTICLE

Reassessing the Structure and Function Relationship of the O_2 Sensing Transcription Factor FNR

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Abstract

Significance: The *Escherichia coli* regulatory protein fumarate nitrate reduction (FNR) mediates a global transcriptional response upon O_2 deprivation. Spanning nearly 40 years of research investigations, our understanding of how FNR senses and responds to O_2 has considerably progressed despite a lack of structural information for most of that period. This knowledge has established the paradigm for how facultative anaerobic bacteria sense changes in O_2 tension.
Recent Advances: Recently, the X-ray crystal structure of *Aliivibrio fischeri* FNR with its [4Fe-4S] cluster cofactor was solved and has provided valuable new insight into FNR structure and function. These findings have alluded to the conformational changes that may occur to alter FNR activity in response to O_2.
Critical Issues: Here, we review the major features of this structure in context of previously acquired data. In doing so, we discuss additional mechanistic aspects of FNR function that warrant further investigation.
Future Directions: To complement the [4Fe-4S]-FNR structure, the structures of apo-FNR and FNR bound to DNA or RNA polymerase are needed. Together, these structures would elevate our understanding of how ligation of its [4Fe-4S] cluster allows FNR to regulate transcription according to the level of environmental O_2. *Antioxid. Redox Signal.* 29, 1830–1840.

Keywords: FNR, transcription factor, O_2 sensor, regulation, structure

O_2 Sensing in Facultative Anaerobic Bacteria

IT IS WELL KNOWN THAT O_2 availability exerts considerable influence on the physiology of most organisms. In the case of bacteria that thrive in both aerobic and anaerobic environments (facultative anaerobes), O_2 tension dictates the use of distinct metabolic pathways usually directed to optimize energy conservation. Aerobic respiration is the more energetically favorable pathway, yielding maximal adenosine 5′-triphosphate (ATP) when O_2 serves as terminal electron acceptor. Upon O_2 deprivation, ATP is generated by alternative pathways, such as fermentation or anaerobic respiration, which utilizes alternative terminal electron acceptors (*e.g.*, fumarate, nitrate, dimethyl sulfoxide, and trimethylamine N-oxide) (9, 16). The expression of stress response pathways is also affected by the presence of O_2, which along with its toxic derivatives (*e.g.*, hydrogen peroxide, superoxide, and hydroxyl radical) can inadvertently damage cellular components (28). Therefore, facultative anaerobes have developed strategies to sense abrupt changes in O_2 concentration and rapidly respond by altering gene expression. Often, this regulation occurs through O_2 sensing transcription factors that function to modify levels of messenger RNA transcripts.

Escherichia coli has served as a model organism for understanding O_2 mediated transcriptional regulation. *E. coli* routinely cycles between environments of varying O_2 tension, such as the anaerobic mammalian gut and aerobic niches outside the host, so it is not surprising that this enterobacterium has evolved an intricate transcriptional network to adapt to such changes. To directly sense O_2, *E. coli* has exploited transcription factors containing protein cofactors that inherently react with O_2, such as Fe-S clusters and Fe^{2+} [*e.g.*, fumarate nitrate reduction (FNR), Fur] (4, 9, 16, 24). O_2 may also be detected indirectly by monitoring changes in the quinone pool (*e.g.*, ArcA) (9, 16). Perhaps the most extensively studied O_2 sensing mechanism has been that of FNR, which mediates

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an adaptive response upon anaerobiosis. The findings of numerous studies to elucidate this mechanism have been the subject of many reviews (*e.g.*, see references 13, 34, 72). Although these studies identified a $[4Fe\text{-}4S]^{2+}$ cluster as central to FNR's ability to sense O_2, the lack of a structure limited further mechanistic understanding.

Recently, Volbeda *et al.* solved the X-ray crystal structure of FNR from *A. fischeri* (which shares 84% of amino acid sequence identity with *E. coli* FNR) with the [4Fe-4S] cluster intact (Fig. 1A) (73). Not only does this structure support many of the earlier findings, it has also uncovered several exciting and novel facets of FNR function. Here, we highlight specific features of this structure and that of the previously solved FNR homolog $FixK_2$ bound to DNA (8). In doing so, we dissect the major functional regions of FNR (Fe-S cluster binding, dimerization, DNA binding, and RNA polymerase (RNAP) interaction) and relate structural information with previously and newly acquired data. For ease of comparison, we will refer to amino acid residues using *E. coli* FNR numbering because of the wealth of FNR mutants that have been studied.

General Functional and Structural Properties of FNR

The first evidence to link a functional interaction between FNR and anaerobic respiration emerged from the discovery that *fnr* mutants were incapable of fumarate and nitrate reduction, hence the name FNR (38). This observation launched decades of research from several laboratories to define FNR's role in transcriptional regulation of respiratory pathways. More recently, genome-wide analyses have defined the scope of the direct FNR transcriptional network that encompasses ≥63 genes, many of which mediate adaptation to O_2 limitation (12, 20, 22, 31, 56, 58, 63). For instance, FNR activates genes involved in anaerobic oxidation of carbon sources and reduction of alternative terminal electron acceptors and represses some genes specifically used in aerobic respiration. Although biochemical and genetic studies of *E. coli* FNR are reviewed here, it should be noted that FNR homologs are widely distributed in Proteobacteria and Bacilli and a core FNR regulon appears to be conserved across many facultative anaerobes (17, 37, 47).

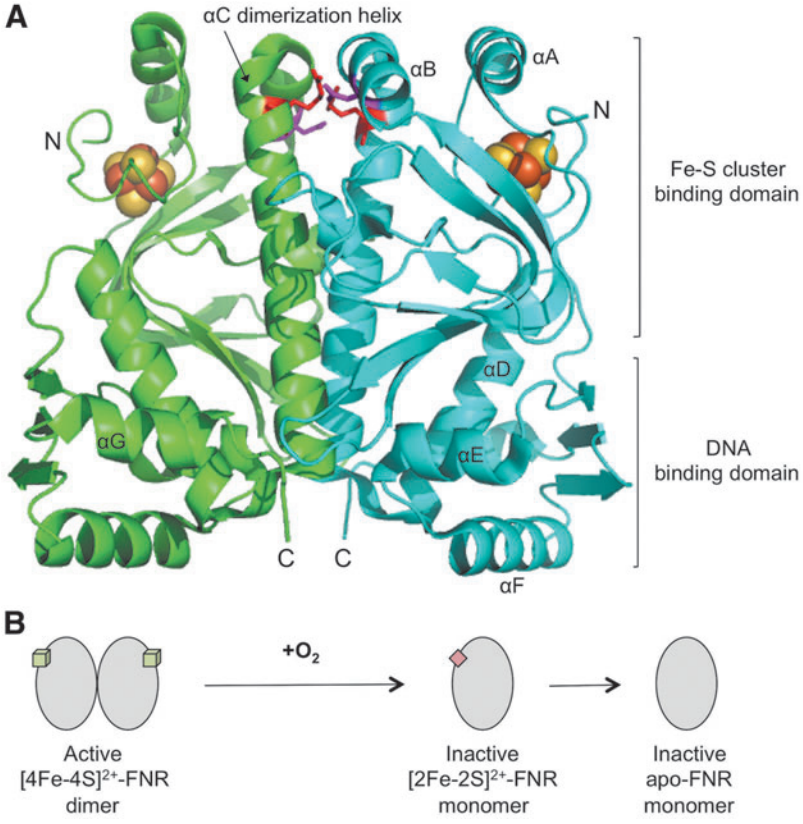

FIG. 1. The role of the [4Fe-4S] cluster in FNR structure and function. (A) Crystal structure of [4Fe-4S]-FNR. The protein dimer is shown in cartoon representation, with individual protein subunits shown in *cyan* and *green*. Present in each subunit is a [4Fe-4S] cluster shown as *yellow* and *orange* spheres. Labeled are the N-terminal Fe-S cluster binding domain, the C-terminal DNA binding domain, and the seven α-helices present in FNR (designated with *letters* A–G), including the αC dimerization helix. Also shown are the side chains of residues R140 (*red*) and D130 (*magenta*), which form intersubunit salt bridges that contribute to dimerization. This figure was prepared with MacPyMOL using the structure available in the Research Collaboratory for Structural Bioinformatics PDB under the accession code 5E44 ref. (73). **(B)** Model for the regulation of FNR activity by O_2. In the absence of O_2, FNR contains a $[Fe\text{-}4S]^{2+}$ cluster (*green cube*), which promotes subunit dimerization, site-specific DNA binding, and transcriptional regulation. When O_2 is introduced, the $[4Fe\text{-}4S]^{2+}$ cluster is rapidly converted to a $[2Fe\text{-}2S]^{2+}$ cluster (*red square*), resulting in FNR inactivation through loss of dimerization. In aerobic cells, the $[2Fe\text{-}2S]^{2+}$ cluster is further degraded, generating clusterless apo-FNR. FNR, fumarate nitrate reduction; PDB, protein data bank.

The capacity of FNR to globally regulate gene expression depends on the presence of its $[4Fe\text{-}4S]^{2+}$ cluster cofactor. The integrity of this cluster is intricately linked with FNR function since it promotes a protein conformation necessary for FNR dimerization, site-specific DNA binding, and transcriptional regulation (23, 32, 42). However, in the presence of O_2, the $[4Fe\text{-}4S]^{2+}$ cluster is rapidly converted to a $[2Fe\text{-}2S]^{2+}$ cluster *via* sulfur-based oxidation, resulting in FNR inactivation through loss of dimerization (15, 30, 33, 60, 82). In aerobic cells, the $[2Fe\text{-}2S]^{2+}$ cluster is further degraded, generating clusterless, apo-FNR (70) (Fig. 1B). Ultimately, due to sensitivity of the $[4Fe\text{-}4S]^{2+}$ cluster toward O_2, FNR-dependent transcriptional regulation occurs primarily during anaerobic growth despite similar FNR protein levels in aerobic and anaerobic cells (69).

Before the *A. fischeri* FNR structure, gaining mechanistic insight into how O_2 mediated cluster conversion influenced the conformational alterations in FNR that must occur for regulated activity was somewhat challenging. It was known that FNR is structurally related to the cyclic adenosine monophosphate (cAMP) cAMP receptor protein (CRP) family of transcription factors whose members are broadly distributed in bacteria (37, 47, 65). These proteins characteristically contain an N-terminal effector binding domain that encompasses a β-roll motif, a long α-helix that mediates subunit dimerization, and a C-terminal DNA binding domain that comprises a helix-turn-helix (HTH) motif (Fig. 1) (79).

Structural similarities aside, individual CRP family members are distinguished by their respective effector molecules, which allow for adaptive responses to diverse environmental cues (*e.g.*, cAMP, CO, O_2, NO, 2-oxoglutarate, aromatic compounds) (37). The structures of CRP and other family members have been solved and have provided the initial framework for understanding how FNR operates as a transcription factor (6, 79). Despite the valuable information acquired from these structures, many questions regarding FNR activity remained. For instance, although most CRP family members are constitutive homodimers that bind DNA target sites upon association with their cognate effectors (26, 36, 79), FNR appears unique in that ligation of one $[4Fe\text{-}4S]^{2+}$ cluster per subunit induces the protein dimerization that is required for DNA site recognition. Thus, the FNR structure has been highly anticipated to fully understand how FNR is mechanistically regulated by O_2.

Ligation of the [4Fe-4S] Cluster Appears to Organize the FNR N-Terminal Region

A major question in the field has been how the N-terminal cluster binding domain of FNR differs from the analogous domain of other CRP family members. Although secondary structure of the effector binding domain is conserved within the CRP family, it is perhaps not surprising that the effector ligands and neighboring residues display significant variability, likely accounting for the broad range of effector molecules recognized by family members (37). Furthermore, FNR contains an N-terminal extension of 26 amino acid residues that are not present in CRP, and *in vivo* and *in vitro* experiments established that C20, C23, and C29 within this extension, along with C122 of the β-roll motif, serve as ligands for the $[4Fe\text{-}4S]^{2+}$ cluster (48, 64, 67, 68). In addition, susceptibility of the N-terminal region to limited trypsin digestion suggested some conformational flexibility within this domain (55).

Although electron density is not visible for the first 18 residues, the X-ray crystal structure demonstrates that the FNR cluster binding region is near the protein surface and is indeed more disordered than the rest of the protein. In this region, the N-terminal residues of the protein (encompassing C20, C23, and C29) are unstructured and appear to partially wrap the [4Fe-4S] cluster and position it adjacent to the top of the β-roll motif (containing C122; Figs. 1 and 2). Although the β-roll and preceding αA helix are common to CRP family members, in FNR, these elements and the unstructured N-terminal residues form a topology that is similar to a protein fold shared by some Fe-S proteins (53). The structure also reveals that proximal to the [4Fe-4S] cluster is an unpredicted network of hydrophobic interactions involving residues of the αA, αB, and αC helices (I30, L34, L42, I45, I46, I124, I128, L129, I132, L139, I143; Fig. 2A). As discussed later, these interactions likely provide a signaling relay between O_2 mediated cluster conversion and the loss of FNR dimerization.

In addition to [4Fe-4S]-FNR, Volbeda *et al.* solved the X-ray crystal structure of *A. fischeri* FNR with a partially degraded cluster. In this form, the electron density of the first 42 amino acids is not visible, suggesting that the cluster binding loop becomes disordered upon cluster degradation. Therefore, the $[4Fe\text{-}4S]^{2+}$ cluster likely organizes this N-terminal region of FNR, but remains accessible to O_2, a property presumed to be pivotal for FNR's physiological role.

The Structure Yields Additional Insight into Subunit Interactions That Are Necessary for FNR Dimerization

As discussed earlier, cluster binding is a major regulatory checkpoint for FNR since presence of the $[4Fe\text{-}4S]^{2+}$ cluster dictates its oligomeric state. Only in the $[4Fe\text{-}4S]^{2+}$ cluster-containing form is FNR capable of dimerization, which is required for affinity and specificity in DNA binding and hence transcriptional regulation (23, 32, 42). Conversely, keeping FNR apoprotein in a monomeric, inactive state under aerobic conditions is important for limiting gene expression to anaerobic conditions. Given the significance of this regulated oligomerization, biochemical and genetic studies were employed to characterize residues involved in subunit interaction. Specifically, the role of FNR residues 140–159 was evaluated by alanine mutagenesis as these correspond to the αC helix along which CRP dimerizes *via* a coiled coil motif (Fig. 1). It was found that mostly hydrophobic residues predicted to be on the same face of the αC helix (M144, M147, I151, I158) are important for dimerization (Fig. 3A). The isoleucine at position 151 appeared particularly compelling since substitution of this residue with alanine causes the most severe dimerization defects (54). In contrast, these studies also demonstrated that two negatively charged residues near the subunit interface deter dimerization of apo-FNR *via* subunit charge repulsion. Substitution of either D154 or to a lesser extent E150 with alanine results in dimeric, active FNR even in the absence of the cluster (55). Together, these findings suggested that cluster binding might induce a conformational rearrangement such that I151 would shield the negative charges of D154 and E150, thereby allowing

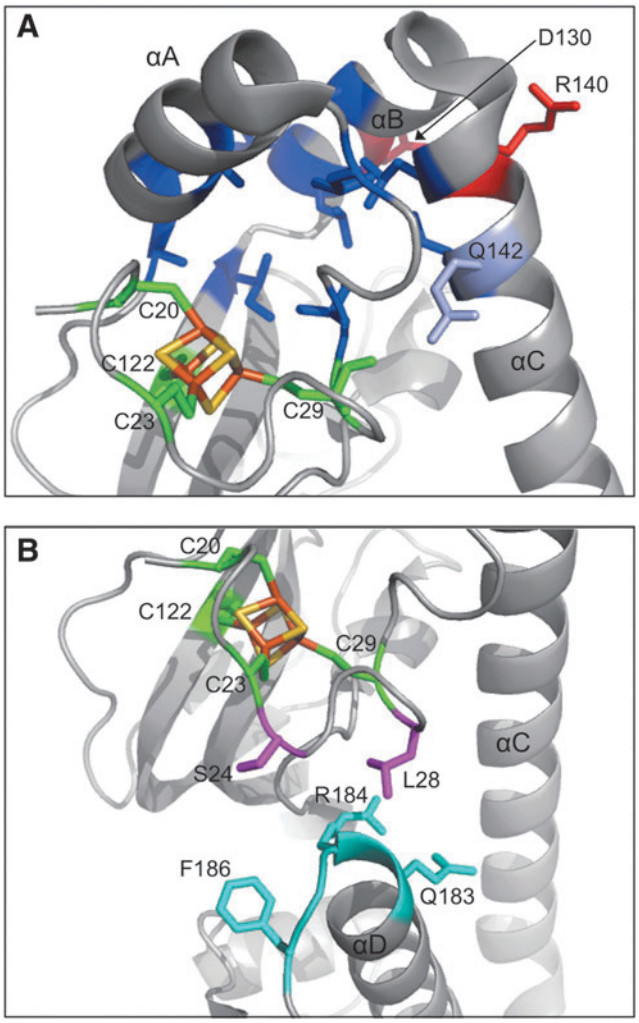

FIG. 2. The [4Fe-4S] cluster binding region within FNR. The cluster is shown as *orange* and *yellow* sticks, and the protein is depicted in cartoon representation, with relevant α helices and amino acid side chains marked. Shown in *green* are the four cysteine cluster ligands (C20, C23, C29, and C122). **(A)** Proximal to the cluster is a patch of hydrophobic residues (shown in *blue*) that form a network of interactions. This hydrophobic patch likely provides communication between the cluster binding domain and the intersubunit R140–D130 salt bridges that contribute to dimerization (R140 and D130 are shown in *red*). **(B)** Facing away from the cluster are the side chains of residues S24 and L28 (*magenta*), which are proximal to residues 183–186 (*cyan*) of FNR AR1. AR, activating region.

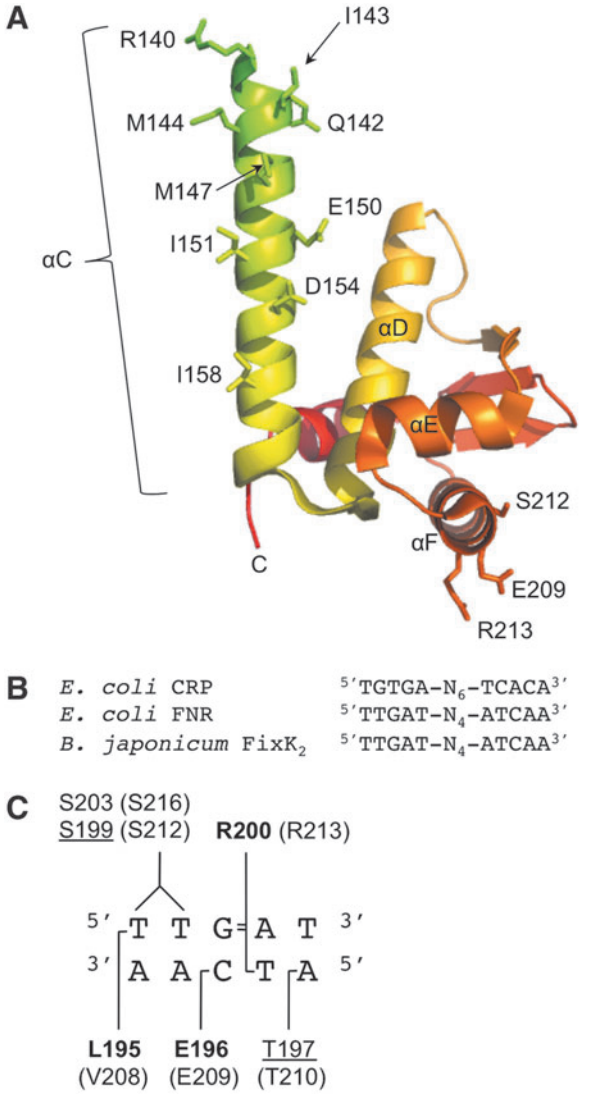

FIG. 3. FNR residues important for dimerization and specific DNA recognition. (A) The FNR dimerization interface and DNA binding domain. Only shown are residues 140–246 of one FNR subunit, depicted in cartoon representation with relevant amino acid side chains shown as *sticks*. **(B)** The consensus DNA target sites recognized by CRP and FNR from *E. coli*, and $FixK_2$ from *B. japonicum*. **(C)** The $FixK_2$ residues involved in consensus half-site interactions. These residues form specific interactions with nucleotides (indicated in *bold*) or interact with the DNA phosphate backbone either directly or through water (*underlined*). Indicated in *parentheses* are residues at equivalent positions in *E. coli* FNR.

energetically favorable subunit interactions between hydrophobic side chains. However, since the D154A substitution only partially rescues the I151A mutant phenotype, this indicated that I151 may have an additional role in FNR dimerization (54).

The structure has allowed refinement of this oligomerization model. As discussed by Volbeda *et al.*, residues D154 and E150 from both subunits create a negatively charged pocket in [4Fe-4S]-FNR. Rather than shielding this negative charge as previously hypothesized, the I151 residues of each subunit establish van der Waals contacts. The proximity of these contacts to the inhibitory D154 residues may explain why I151 is especially critical for FNR oligomerization. Furthermore, the partial activity of the FNR-I151A-D154A double mutant suggests that even after removing negativity at the subunit interface, the van der Waals contacts between I151 residues make a significant contribution to maintaining dimer stability.

The structure has also clarified the role of R140 in FNR dimerization. A previous study showed that substitution of this positively charged residue with alanine, leucine, or glutamate results in substantial FNR activity defects (54).

Consequently, it was hypothesized that R140, being at the start of the αC helix, may promote subunit interaction through formation of interhelical hydrogen bonds. Instead of making contacts at the coiled coil interface, however, the structure indicates that R140 of one subunit forms a salt bridge with D130 belonging to the αB helix of the opposite subunit (Figs. 1 and 2A) (73). Volbeda *et al.* propose that this salt bridge may be a critical determinant in the mechanism by which the $[4Fe\text{-}4S]^{2+}$ cluster mediates FNR oligomerization. In light of this new structural information, it is surprising that when R140A is expressed at increased cellular levels, it exhibits activity similar to that of wild-type FNR (54). These findings suggest that at higher protein concentrations, the hydrophobic interactions and the I151 van der Waals contacts along the αC helix are sufficient to provide the energetic driving force needed for dimerization. However, at wild-type protein levels, the R140–D130 salt bridges, along with the aforementioned interactions, play a vital role in enabling the O_2 sensitivity of the FNR monomer–dimer equilibrium.

The Structure Uncovers the Mechanism by Which O_2 Mediated Cluster Conversion Leads to Loss of Dimerization

Before the structure, the cluster-induced conformational changes that propagate FNR oligomerization remained largely a mystery. Most CRP family members are constitutively dimeric. Furthermore, circular dichroism spectroscopy and limited proteolysis assays did not indicate any broad scale changes upon FNR cluster ligation (55). Indeed, these latter observations are corroborated by the structure, which suggests that conformational alterations may be more fine tuned. Volbeda *et al.* propose that the patch of hydrophobic interactions proximal to the $[4Fe\text{-}4S]^{2+}$ cluster provides a communication relay between the cluster and the intramolecular R140–D130 salt bridges (Fig. 2A). Upon exposure of the $[4Fe\text{-}4S]^{2+}$ cluster to O_2, local rearrangements that take place to accommodate the resulting $[2Fe\text{-}2S]^{2+}$ cluster may disseminate through this hydrophobic network to subsequently break the salt bridges. The resulting loss of binding energy would weaken stability at the top of the dimerization helix, which, in turn, may propagate unzipping of the coiled coil, and hence the loss of dimerization.

According to this model, disruption of the hydrophobic patch proximal to the $[4Fe\text{-}4S]^{2+}$ cluster would likely alter FNR's ability to oligomerize. Consistent with this hypothesis is the phenotype of the previously characterized FNR-M143A mutant, which exhibits a significant dimerization defect (54). As shown in the structure, the residue at position 143 in *A. fischeri* FNR is an isoleucine that participates in the hydrophobic network (Figs. 2A and 3A) (73). In addition, the presence of a hydrophobic residue at this position is conserved among FNR homologs (54). Curiously, a different *E. coli* FNR mutant, FNR-Q142A, displayed slightly increased activity relative to wild-type FNR (54). Although the basis for this phenotype remains to be elucidated, it is tempting to speculate that Q142 may also play a role in linking $[4Fe\text{-}4S]^{2+}$ cluster ligation and dimerization as the structure demonstrates that the Q142 side chain potentially interacts with the C29 cluster ligand main chain (Fig. 2A). Future work to solve the structures of [2Fe-2S]-FNR or apo-FNR will likely provide valuable information as to how this network of interactions is disrupted in the absence of the $[4Fe\text{-}4S]^{2+}$ cluster, thereby inactivating FNR function.

The Structural Position of Residues S24 and L28 Emphasizes the Correlation Between Conformational Flexibility and Proper O_2 Sensing

Tremendous insight into the functional form of FNR was delivered from genetic and biochemical characterization of FNR variants that retain activity in the presence of O_2. In the case of substituting leucine at position 28 with histidine, it was found that the $[4Fe\text{-}4S]^{2+}$ cluster was more resistant to O_2 mediated conversion than wild-type FNR. Given its proximity to the cluster ligands, it was hypothesized that the imidazole ring of histidine may form a hydrogen bond with a cluster sulfide ion (3). A similar phenotype was observed upon substitution of S24 with F, R, H, W, or Y, suggesting that the presence of bulky amino acid side chains at this position may shield the $[4Fe\text{-}4S]^{2+}$ cluster from O_2 (29). However, based on the position of S24 and L28 in the FNR structure, which displays their side chains facing away from the [4Fe-4S] cluster, it is apparent that these residues affect cluster stability by a different mechanism (Fig. 2B).

Volbeda *et al.* propose that substitution of S24 or L28 with larger amino acids may hinder conformational flexibility of the cluster binding region such that increased rigidity would impede O_2 mediated cluster destruction by restricting the rearrangement from binding a $[4Fe\text{-}4S]^{2+}$ cluster to a $[2Fe\text{-}2S]^{2+}$ cluster. In addition, S24 and L28 are in proximity to residues 183–186, which are part of an amino acid loop implicated in making contacts with α-C-terminal domain (CTD) of RNAP (Fig. 2B) (75, 76). The presence of smaller side chains at positions 24 and 28 is likely necessary to avoid steric hindrance with residues 183–186. Substitution of S24 or L28 may also establish new interactions with this amino acid stretch, potentially affecting plasticity of the structure. Ultimately, these findings suggest that for FNR, conformational flexibility of the cluster binding domain is important for its O_2 sensing function, in addition to $[4Fe\text{-}4S]^{2+}$ cluster accessibility.

Aside from the cluster ligands, the neighboring residues within the cluster-binding domain display variability even among FNR homologs. Considering the characteristics of the S24F and L28H mutants, this variability may account for the altered cluster sensitivities observed for FNR homologs, in which their clusters are either more susceptible (*Azotobacter vinelandii* CydR) or resistant (*Neisseria meningitidis* FNR) to destruction by O_2 compared with *E. coli* FNR (18, 80). Indeed, it has been proposed that the cluster binding domain has evolved to tailor the appropriate O_2 response according to the environmental niche (29).

Interestingly, although S24F and L28H mutants exhibit increased cluster stability, previous *in vitro* experiments have demonstrated that these variants display some defects in DNA binding and/or transcription activation (2, 57, 74). Together, these observations raise the question as to whether conformational flexibility is important for DNA and RNAP recognition, in addition to efficient O_2 sensing. We address this question for the L28H mutant in further detail later in this review.

The $FixK_2$-DNA Structure Has Broadened Our Understanding of FNR–DNA Interactions

Given that DNA binding is often a property among transcription factors that is critical for their function, several strategies have been used to map the contacts between the FNR HTH motif and its DNA recognition site (11, 41, 66). This work was initially guided by knowledge of CRP–DNA interactions, since primary and secondary structures of the HTH motif are relatively well conserved between CRP and FNR (65). In addition, these two proteins bind symmetrical DNA sites that share sequence similarity (Fig. 3B) (68). In fact, substitution of just two amino acids in the FNR–DNA binding domain with those at corresponding positions in CRP enables FNR to activate a CRP-dependent promoter (66). Both *in vivo* and *in vitro* assays revealed that FNR residues E209, S212, and R213 play a major role in DNA binding (41, 66). These residues belong to the αF helix of the HTH and were predicted to be surface exposed for making contacts with the DNA major groove. Indeed the FNR structure demonstrates that the side chains of E209, S212, and R213 are poised for potential DNA interactions (Fig. 3A) (73).

In recent years, the X-ray crystal structure of the protein–DNA complex containing the FNR homolog $FixK_2$ from *Bradyrhizobium japonicum* was solved (8). Since the consensus DNA binding sequences for FNR and $FixK_2$ are identical (Fig. 3B) (11), this cocrystal structure has provided deeper insight into putative FNR–DNA interactions. The $FixK_2$–DNA complex reveals protein-induced DNA bending, a property presumed to be shared by FNR as suggested by DNA mobility shift assays and DNA sites hypersensitive to DNase I-mediated cleavage when FNR is present (51, 77, 83). The $FixK_2$–DNA structure also demonstrates binding of each protein subunit to its cognate DNA half site by way of direct and indirect interactions. Specifically, the side chains of the E209 and R213 amino acid equivalents in $FixK_2$ contact DNA bases through hydrogen bonds, whereas the side chain of the S212 equivalent makes interactions with the DNA phosphate backbone *via* water molecules (Fig. 3C). Additional $FixK_2$–DNA contacts involve other residues of the αF helix, as well as nearby residues of the αE helix and the short loop between the dimerization αC helix and the αD helix. Based on this observation, it may be likely that the side chains of other FNR residues (*e.g.*, R169, R197, T207, V208, T210, G216, G228) interact, either directly or through water, with DNA bases or the phosphate backbone. In the case of V208, the equivalent residue in $FixK_2$ (L195) forms a hydrophobic interaction with the first T of the TTGAT half site (Fig. 3C). Interestingly, substitution of this valine in FNR with an arginine residue, which is present at the equivalent position in CRP, did not disrupt DNA binding. Rather, this FNR variant was able to activate both FNR- and CRP-dependent promoters (66). Together, these findings suggest that the hydrophobic interaction mediated by V208 may be critical in discriminating FNR and CRP recognition sites.

Although the $FixK_2$–DNA complex has shed light on how FNR may specifically interact with DNA, differences in positioning of the HTH motif between these two proteins remain a possibility. Indeed, structures of other CRP family members suggest that upon effector binding, changes in αF helix orientation may occur to promote DNA recognition (79). $FixK_2$ may be an exception since DNA binding activity is not controlled by the presence of an effector molecule, but rather the oxidation of a unique cysteine residue near its HTH motif (49). Furthermore, as already noted, FNR is unique in that cluster binding does not directly regulate DNA binding activity but instead induces the subunit dimerization required for DNA recognition. Thus, additional knowledge may be gained from an FNR–DNA cocrystal structure. This desired structure would not only confirm presumed FNR–DNA contacts, but it would also reveal alterations in protein conformation that may occur subsequent to DNA binding. These putative conformational changes may explain why FNR bound to DNA exhibited an increased rate of O_2 mediated cluster degradation as compared with unbound FNR *in vitro* (14).

Structural Differences Between FNR and CRP Likely Contribute to Distinct Interactions with RNAP

For the CRP/FNR family of transcription factors, direct contacts with RNAP are necessary for transcription activation. Identifying these contacts has been the focus of several studies, which were guided by known CRP–RNAP interactions elucidated from experimental data and the CRP–α-CTD cocrystal structure (5, 10). Researchers established that like CRP, FNR contains three individual activating regions (ARs) that mediate contacts with RNAP (Fig. 4A). These include AR1, AR2, and AR3, predicted to recognize the α-CTD, the α-N-terminal domain, and σ^{70}, respectively (43). Furthermore, the influence of each AR on transcription activation appears to depend on promoter architecture (Fig. 4B). At class I promoters in which the FNR recognition site is centered at −61.5 bp or further upstream of the +1 transcription start site (TSS), AR1–α-CTD interactions are required for transcription activation (44, 75, 76, 78). However, the mode of RNAP recognition differs for class II promoters, which constitute the most frequently occurring FNR-dependent promoters and contain the FNR target site ~41.5 bp upstream of the TSS. At class II promoters, all three ARs have the potential to make contacts with RNAP, with AR1 and AR3 having predominant roles and AR2 making only a minor contribution (7, 39, 40, 45, 75, 77, 78). Consistent with this notion, the structure demonstrates that AR3 forms a surface exposed loop that is poised to make interactions with σ^{70} as previously predicted by alanine mutagenesis (40).

Although some contacts with RNAP appear to be conserved between FNR and CRP, evidence suggests that significant functional differences exist for the ARs of these two transcription factors. For instance, in contrast to that of FNR, CRP AR2 appears to play a larger role and CRP AR3 does not normally appear to be functional in class II promoter activation unless a lysine at position 52 is mutated (10, 62). In addition, FNR–AR1 displays a significantly broader interacting surface than CRP (Fig. 4A). The ARs may also exhibit distinct transcription initiation mechanisms for FNR and CRP. CRP AR1 and AR2 enhance binding of RNAP to class II promoters and the rate of open complex formation, respectively (10). In contrast, FNR AR1 accelerates RNAP isomerization from a closed to an open complex at an FNR-dependent synthetic class II promoter (77). Although further work is needed to solve the FNR–RNAP cocrystal structure to elevate our understanding of the protein–protein

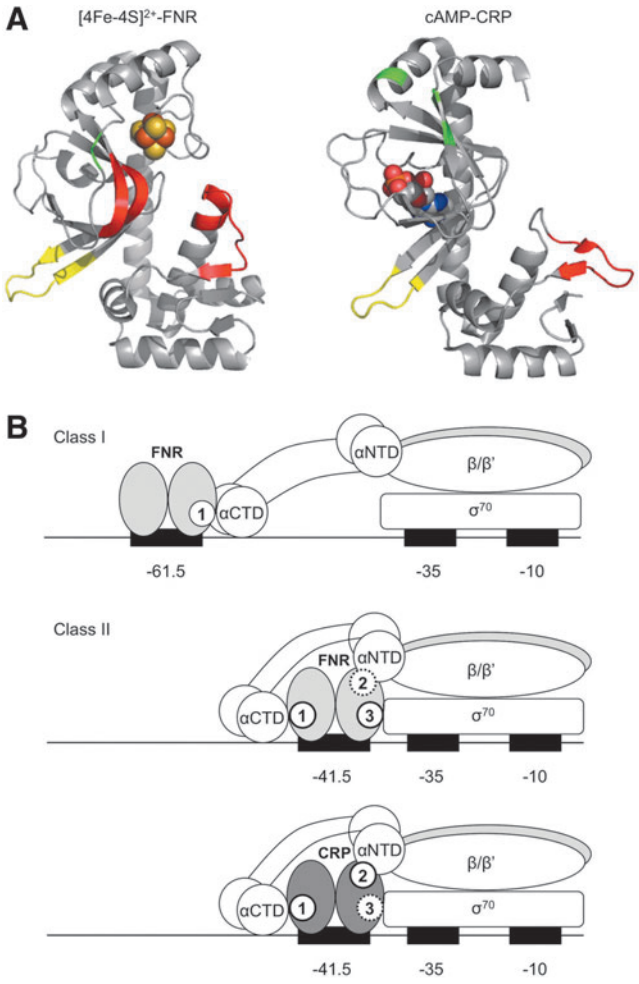

FIG. 4. Developing a model for FNR transcription activation. (A) The ARs within FNR and CRP that are involved in interacting with RNAP. Shown are the monomeric crystal structures for [4Fe-4S]-FNR [PDB code 5E44, Ref. (73)] and cAMP-CRP [PDB code 1G6N, Ref. (59)] in cartoon representation with their respective cofactors shown as *spheres* and ARs highlighted: AR1 (*red*), AR2 (*green*), AR3 (*yellow*). **(B)** Promoters that are activated by FNR can be categorized into two main classes. At a class I promoter, the FNR binding site is centered ~61.5 bases or further upstream of the transcriptional start site (+1), allowing FNR to make contacts with RNAP through AR1 in the downstream subunit. At a class II promoter, the FNR binding site is centered ~41.5 bases upstream of the +1, and FNR is poised to make multiple contacts with RNAP through AR1 in the upstream subunit and AR2 and AR3 in the downstream subunit, although AR2 plays only a minor role. In contrast to that of FNR, in class II promoter activation mediated by CRP, AR2 plays a larger role and AR3 does not normally appear to be functional. cAMP-CRP, cyclic adenosine monophosphate-cAMP receptor protein.

interactions required for transcription activation, it is intriguing that the FNR cluster binding domain is in proximity to AR1, specifically the 183–186 amino acid stretch (Fig. 2B). It is possible that this proximity would permit communication between the cluster binding domain and AR1 upon cluster ligation. This is in contrast to CRP whose structure shows that the cAMP binding pocket is distally located from AR1 (Fig. 4A). This variation may at least partially explain the mechanistic differences between FNR and CRP transcription activation.

The Structure Reveals an Unexpected Link Between the Cluster Binding Domain and AR1

In a previous study, FNR residue R184 was proposed to be an AR1 determinant based on the observation that alanine substitution at this position resulted in decreased class II promoter activation but no change in DNA binding (75). According to the structure, the R184 side chain does not appear to be in a position to make direct contacts with RNAP (Fig. 2B). Notably, this residue is part of the 183–186 amino acid stretch that is in proximity to the cluster binding domain. As discussed earlier, this domain encompasses L28, which upon substitution with histidine results in increased $[4Fe\text{-}4S]^{2+}$ cluster stability. Volbeda *et al.* hypothesize that a reduction in conformational flexibility of the cluster binding domain may account for this phenotype. Conformational inflexibility of the FNR-L28H mutant may also explain its general defect in activating transcription compared with wild-type $[4Fe\text{-}4S]^{2+}$-FNR (3, 35, 57, 74). Data in Figure 5 show that anaerobic expression of a synthetic class II promoter is decreased nearly eightfold when FNR-L28H is present. Although this may be explained, in part, by a decrease in FNR-L28H DNA binding affinity, *in vitro* experiments from our laboratory indicate that this mutant is indeed less capable of activating transcription (2, 74). Interestingly, we found that introducing the R184A substitution results in an approximate threefold increase in FNR-L28H activity (Fig. 5). To explain this finding, we hypothesize that a histidine at position 28 may

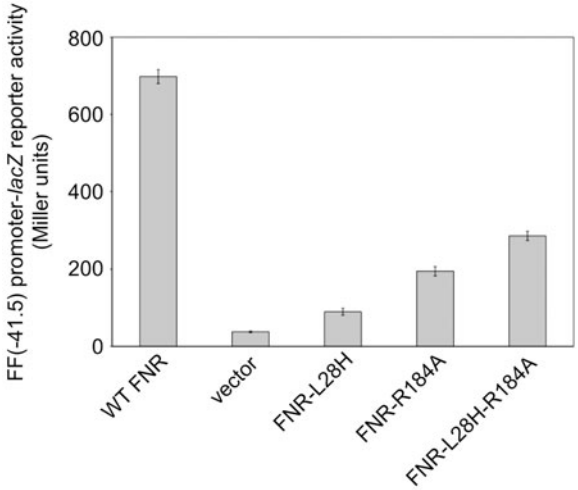

FIG. 5. The transcription activation defect displayed by the FNR-L28H mutant is partially restored by the R184A substitution. β-Galactosidase activity (given in Miller units) from the FNR-dependent synthetic class II FF(–41.5) promoter-*lacZ* reporter was measured in strains expressing wild-type FNR, FNR-L28H, FNR-R184A, or FNR-L28H-R184A from plasmid pET11a. These strains lack the chromosomal copy of *fnr*. Cultures were grown under anaerobic growth conditions in M9 minimal medium containing 0.2% glucose and 50 μg/mL ampicillin. *Error bars* represent the standard deviation of β-galactosidase activity measured from three independent strain isolates.

either interact or cause steric hindrance with R184, thereby affecting FNR-L28H transcription activation. By replacing R184 with a smaller amino acid, some conformational flexibility in the cluster binding region of FNR-L28H may be restored, thereby partially complementing the defect in activity. Together, these findings not only stress the significance of conformational flexibility in FNR's ability to carry out its physiological role, they also reveal an unexpected link between the cluster binding region and AR1 such that presence of the $[4Fe\text{-}4S]^{2+}$ cluster may promote AR1 and RNAP interactions. This link may also explain why anaerobic transcription activation by the constitutive dimer mutant FNR-D154A is more than eightfold higher than that of double mutant variants (FNR-D154A-C23S and FNR-D154A-C122S) that lack the $[4Fe\text{-}4S]^{2+}$ cluster (data not shown). Future investigation is needed to dissect the mechanism by which cluster binding may influence interactions between AR1 and RNAP.

Future Directions

Despite the wealth of information provided by decades of research, many aspects of FNR function are still unresolved. This is largely true for the N-terminal cluster binding domain, in which electron density was not visible for the first 18 residues. In fact, residues within this amino acid stretch have been shown to play a critical role in regulating FNR activity. For instance, a mutant FNR lacking residues 2–15 surprisingly exhibited increased cluster stability toward O_2 compared with the wild-type protein (81). It is possible that these residues are needed to maintain the cluster ligands in an orientation for appropriate O_2 sensing. This region also encompasses one of the two target sequences in FNR that are recognized by the ClpXP protease (residues 5–11 and 249–250) (21). Although monomeric FNR is degraded by this protease, dimeric FNR escapes proteolysis (50). Thus, it is not clear how the ClpXP target sequences would be protected in the dimerized form. Addressing these questions will likely be challenging given the conformational flexibility of the FNR N-terminal region.

Future cocrystal structures will be helpful in further elucidating FNR contacts with RNAP, ClpXP, or with other proteins implicated in associating with FNR. For example, FNR must interact with carrier proteins of Fe-S biogenesis pathways that facilitate delivery of its $[4Fe\text{-}4S]^{2+}$ cluster (52). FNR has also been implicated in binding the NarL transcription factor for activation of *nirB* (71). Lastly, there is evidence that at some promoters, repression of transcription depends on specific interactions between two tandem-bound FNR dimers (1, 25, 46). The latter point simultaneously stresses how an FNR–DNA cocrystal is likewise needed, not only for further defining FNR–DNA contacts but also because the mechanism(s) of FNR-mediated repression is not well understood.

Finally, we would greatly benefit from structures of FNR homologs from other bacteria. These structures would provide further insight as to how variation in the cluster binding domain might tune FNR's O_2 sensing function to a particular bacterial niche. Furthermore, they would address the extent to which the cluster-induced dimerization mechanism proposed by Volbeda *et al.* is conserved. Indeed, variation of FNR regulation is readily apparent in Gram-positive *Bacillus subtilis* and *Bacillus cereus*, in which FNR utilizes different cluster ligands than that of *E. coli* FNR and is dimeric even in the apoprotein form (19, 27, 61). In summary, there is still much to discover regarding the versatility of the FNR transcription factor.

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Abbreviations Used

AR = activating region
ATP = adenosine 5′-triphosphate
cAMP = cyclic adenosine monophosphate
CO = carbon monoxide
CRP = cAMP receptor protein
CTD = C-terminal domain
DNA = deoxyribonucleic acid
FNR = fumarate nitrate reduction
HTH = helix-turn-helix
NO = nitric oxide
PDB = Protein Data Bank
RNA = ribonucleic acid
RNAP = RNA polymerase
TSS = transcription start site