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Iron-containing transcription factors and their roles as sensors

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Abstract

Iron-binding transcription factors are widespread throughout the bacterial world and to date are known to bind several types of cofactors, such as Fe^{2+} , heme, or iron-sulfur clusters. The known chemistry of these cofactors is exploited by transcription factors, including Fur, FNR, and NsrR, to sense molecules such as Fe^{2+} , gases (e.g. oxygen and nitric oxide), or reactive oxygen species. New structural data and information generated by genome-wide analysis studies has provided additional details about the mechanism and function of iron-binding transcription factors that act as sensors.

Introduction

Iron is an essential element for almost all bacteria. Yet, because low pH and oxygen (O_2) promote iron insolubility, iron acquisition is a major challenge for most bacteria depending on their habitat. In addition, iron plays a role generating toxic reactive oxygen species, demanding a tightly regulated system for controlling free iron pools. Thus, it is not surprising that iron-containing transcription factors have evolved roles in metal homeostasis and the cellular response to O_2 , reactive oxygen species, nitric oxide (NO), and other such species. Iron-binding transcription factors bind several types of cofactors, such as Fe^{2+} , heme, or iron-sulfur (Fe-S) clusters, and these transcription factors exploit the known chemistry of their cofactors to sense molecules such as Fe^{2+} , gases (e.g. O_2 , NO, and carbon monoxide), or reactive oxygen species, as seen in Figure 1. Here, we highlight iron-containing transcription factors where recent structural data or information generated by genome-wide analysis studies has provided new details about sensor mechanism or function.

Metal sensors

The regulation of metal homeostasis is critical to cellular growth and survival; while cells require metals to enable the function of many proteins, unbound metals at high concentrations are generally toxic. In bacteria, metal sensing transcription factors that reversibly bind metals are responsible for sensing the cellular levels of metals to achieve metal homeostasis. In response to changes in the free metal pool, metal sensors differentially regulate the transcription of genes that encode proteins involved in, for example, metal-dependent processes or metal uptake and storage. Bacteria often contain many metal

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sensors, each with specificity to a particular metal (see Waldron and Robinson [1] for a recent review). Here we focus on the iron sensors, including the ferric uptake regulator (Fur) and the iron response regulator (Irr), which function in iron homeostasis by two distinct mechanisms.

The most well studied iron sensor is Fur, which functions in many bacteria as a global transcriptional regulator of iron homeostasis. Fur acts primarily to repress transcription of genes under iron replete conditions through the ligation of Fe^{2+} [2,3], which, as predicted by molecular modeling, likely results in conformational changes that activates the protein for DNA binding [4]. Fur regulates many processes, including iron uptake (*fecIR*, *fecABCDE*, *fepA*, *fhuA*, and *cir*), iron storage (*ftnA*), and oxidative stress (*sodA*) in *E. coli* [5]. In addition, Fur regulates bacterial pathogenesis in *Helicobacter pylori* [3], illustrating the importance of iron acquisition in the host environment. Recent studies indicate that small RNAs, such as RyhB in *Escherichia coli* [6,7] or FsrA in *Bacillus subtilis* [8], are part of the Fur regulon, and, in *E. coli*, RyhB targets the mRNA of a subset of iron-containing enzymes (e.g. *sdhCDAB*, *acnA*, *fumA*, *bfr*, and *sodB*). Thus, when iron is limited and RyhB is derepressed, the levels of these iron-containing enzymes are decreased and iron is “spared” for essential cellular functions in what is known as the “iron-sparing response.” Fur also appears to mediate an iron-sparing response in *H. pylori* [3,9], but in this case, apo-Fur represses *sodB* and *pfr* (involved in iron storage) expression under iron-limiting conditions [9].

Initially, Fe^{2+} had been suggested to ligate in a metal-binding site (Site 1) located in the C-terminal dimerization domain of Fur based on studies of the protein from *Pseudomonas aeruginosa* [10]. Nevertheless, both Site 1 and a second metal-binding site (Site 2), which connects the dimerization domain to the N-terminal DNA-binding domain, are occupied with zinc in the crystal structure of *P. aeruginosa* Fur [10] and in the more recent structure of *Vibrio cholerae* Fur [11], leaving the location of the Fe^{2+} binding site unresolved. However, a review of mutational [2] and molecular modeling studies [4] strongly favor assignment of Site 2 as the Fe^{2+} regulatory site, which would coordinate the Fe^{2+} by five highly conserved residues (three histidines and two glutamates), and this assignment was further corroborated by the structure of the *B. subtilis* Fur paralog, PerR [12]. Furthermore, Site 1 was assigned as the structural zinc site, which in the case of *P. aeruginosa* and *V. cholerae* Fur, Zn^{2+} is coordinated by four highly conserved residues (two histidines, a glutamate, and an aspartate), although cysteine residues are essential for zinc binding to Fur of other bacteria, including *H. pylori* [13]. Thus, although many details still need to be addressed, reversible binding of Fe^{2+} at Site 2 appears to regulate Fur DNA binding.

Fe^{2+} -Fur is not the only system for regulating iron homeostasis in bacteria. Rather, in many α -proteobacteria, Irr, another member of the Fur family, acts as a global regulator of iron metabolism by binding heme instead of free iron [2,14,15]. Under iron limiting conditions *Bradyrhizobium japonicum* Irr activates expression of genes involved in ferric iron transport [16] while in iron-replete conditions, Irr is inactivated by binding heme, either by causing its degradation, as in *B. japonicum* [15], or by suffering a dramatic decrease in affinity for DNA binding, as in *Rhizobium leguminosarum* [17]. In addition, *R. leguminosarum* Irr works in conjunction with RirA, an Fe-S cluster-binding Rrf2 transcription factor that *B. japonicum* lacks, to regulate iron homeostasis [15].

Of particular interest is the recognition that iron homeostasis is integrated with manganese homeostasis in these bacteria using a remodeled metal binding network. For example, in *B. japonicum*, Fur appears to specifically bind Mn^{2+} rather than Fe^{2+} *in vivo* [18,19], and in *R. leguminosarum*, Fur is replaced with a Fur homolog named Mur for its manganese-responsive regulatory activities [2]. *B. japonicum* Fur and *R. leguminosarum* Mur are

involved in the manganese-dependent regulation of the manganese transporters and the expression of *irr*. Thus, in bacteria with an apparently greater dependence on manganese, it appears placing iron homeostasis under control of a heme or Fe-S cluster sensor ensures that Mn^{2+} does not compete with Fe^{2+} at a regulatory site on an iron sensor [20]. These new findings illustrate that multiple regulatory strategies have evolved to achieve iron homeostasis in various bacteria.

Sensors of oxygen and reactive oxygen species

For many bacteria, mechanisms that allow adaptation to changes in the levels of available O_2 are necessary for survival. O_2 has a major impact on metabolism in many bacteria because of its key role as an electron acceptor in aerobic respiration and the inadvertent production of toxic reactive oxygen species. Therefore, many bacteria utilize transcription factors to detect O_2 to transcriptionally remodel levels of various metabolic pathways or to detect reactive oxygen species to reduce oxidative stress. Some sensors utilize heme, such as the recently reviewed *E. coli* Dos protein in which the protein-bound heme directly binds O_2 [21]. In addition, an example of a sensor that binds non-heme iron is *B. subtilis* PerR, in which iron is released upon the oxidation of Fe^{2+} -coordinating histidine residues by the reactive oxygen species H_2O_2 [2]. Here we focus on those sensors that utilize Fe-S clusters since these appear to be versatile sensors that are specialized to respond to either O_2 or reactive oxygen species.

FNR, perhaps the most well studied O_2 sensor, is a global transcriptional regulator found in many facultative anaerobes. *E. coli* FNR modulates the expression of hundreds of genes in response to the availability of O_2 [22–24], and it has recently been shown that the core regulon of FNR-type regulators is conserved across many bacteria [25]. However, differences in the extended FNR regulon are noted even in closely related species that seem to reflect adaptation to ecological niches [25]. Recently in *E. coli*, it has been shown that part of the extended regulon of FNR includes a small RNA, FnrS, which then targets the mRNAs of such genes as *sodB*, *maeA*, *gmpA*, *metE*, *folE*, and *folX* [26,27].

Spectroscopic approaches have elegantly demonstrated that FNR directly senses O_2 through the lability of its $[4Fe-4S]$ cluster, a subject that has been recently reviewed [21]. Under anaerobic conditions, FNR exists as a $[4Fe-4S]^{2+}$ homodimer that binds selectively to its target DNA; site-specific DNA binding is lost upon exposure to O_2 , which converts the protein-bound cluster to the $[2Fe-2S]^{2+}$ form and decreases dimerization [28,29]. The complete *E. coli* FNR cycle and the FNR-regulated transitions between aerobic and anaerobic conditions have been computationally modeled, providing new insights into the design principles of this regulatory network [30]. Despite the wealth of knowledge of FNR, the O_2 -dependent conversion of the $[4Fe-4S]^{2+}$ to the $[2Fe-2S]^{2+}$ form of *E. coli* FNR is not completely understood [21], and it may involve a $[3Fe-4S]^{1+}$ intermediate [31–33]. *Neisseria meningitidis* FNR, which is less sensitive to oxygen than *E. coli* FNR, has also been observed in the $[3Fe-4S]$ form in the presence of O_2 but without loss of DNA binding [34], suggesting that the *in vivo* significance of this form of FNR is still unclear.

Another Fe-S cluster-containing sensor is IscR, a member of the Rrf2 family. Like FNR, IscR differentially regulates the expression of a large number of genes under aerobic and anaerobic conditions [35]. However, unlike FNR, the Fe-S form of IscR is required to regulate only a subset of the IscR regulon. $[2Fe-2S]$ -IscR is required for repression of the *isc* operon, which encodes *iscR* and the genes encoding the housekeeping Isc Fe-S cluster assembly pathway, as well as *erpA* and *nfuA* that also function in Fe-S cluster assembly [35,36]. In contrast, IscR-dependent regulation of operons such as *suf* (a secondary pathway for Fe-S cluster biogenesis) or *hya* (encoding Hydrogenase 1) does not require the ligation

of a [2Fe-2S] cluster to IscR, and the effect of IscR on gene expression is more strongly regulated under aerobic conditions [35,37,38]. Thus, the differential regulation of IscR-dependent genes with respect to O₂ availability may result in part from changes in the fraction of IscR with a bound Fe-S cluster. The fraction of [2Fe-2S]-IscR likely depends on the demand for Fe-S cluster biogenesis, such that less Fe-S cluster is predicted to be inserted into IscR via the Isc pathway when the Fe-S clusters of essential proteins need to be repaired or replaced due to damage caused by O₂. Therefore, instead of directly interacting with O₂ like FNR, IscR likely senses O₂ indirectly. Further, it has been shown recently that IscR regulates biofilm formation in *E. coli* [39] and virulence in *Shigella flexneri*, *Erwinia chrysanthemi*, and *P. aeruginosa* [40–42], conditions under which iron can be limited or the bacteria are stressed, suggesting that IscR may respond to stresses in addition to O₂.

A well-known stress-responsive transcription factor is SoxR, which is activated under oxidative stress conditions by the oxidation of its [2Fe-2S]⁺ cluster [43]. [2Fe-2S]²⁺-SoxR of *E. coli* activates the expression of *soxS*, which in turn activates a large number of genes, such as those encoding superoxide dismutase, DNA repair nucleases, and oxidation-resistant enzymes [44,45]. Based on the function of the genes in the SoxRS regulon and numerous studies in which the SoxRS regulon was induced by superoxide-generating redox-cycling drugs, superoxide was the presumed oxidant of SoxR [43]. However, [2Fe-2S]²⁺-SoxR of non-enteric bacteria does not regulate a superoxide stress response, even though many of these bacteria excrete redox-active drugs [46]. In addition, a recent study of *E. coli* SoxR demonstrated that the protein directly senses the redox drug, not superoxide (Gu and Imlay, *in press*). The recently solved crystal structure of SoxR of *E. coli* illustrates that the Fe-S cluster is solvent-exposed [47], suggesting that SoxR could sense a number of diverse redox active drugs. In addition, the *E. coli* SoxRS regulon includes a number of genes encoding proteins involved in drug efflux and/or resistance [45], and truncation of SoxR, which results in constitutive expression of SoxS, leads to decreased susceptibility to the redox-active drug fluoroquinolone in strains of *E. coli* [48] and *Salmonella* [49]. Therefore, despite the fact that redox-cycling drugs can produce superoxide under aerobic conditions, SoxR does not respond directly to this reactive oxygen species *in vivo*. Rather, sensing of superoxide may be attributed to IscR and Fur [44], although direct evidence is lacking.

NO sensors

NO detoxification pathways appear to be widespread amongst bacteria to defend against NO produced either from endogenous pathways such as denitrification or from mammalian host enzymes such as the inducible NO synthase (iNOS) in phagocytes. NO sensing proteins detect the presence of NO and induce the appropriate transcriptional response to survive NO induced stress. As reviewed previously [50], many NO sensors are iron-binding proteins; NO interacts with heme, Fe-S clusters, and non-heme iron to form nitrosyl-iron complexes, which likely leads to protein conformational changes and/or changes in DNA affinity that modulate gene expression in response to the presence of NO. Recent studies focus on identifying the NO sensing component, and three examples, DNR, NsrR, and NorR, are discussed below.

DNR (*dissimilative nitrate respiration regulator*), which is a member of the FNR-CRP family of transcription factors, regulates genes that function in denitrification in denitrifying organisms, including the *nor* genes encoding NO reductase and the *nir* genes encoding nitrite reductase [50–52]. Recent studies suggest that the *P. aeruginosa* DNR uses a non-covalently bound heme to sense NO. The heme group of DNR was shown to be reactive with NO *in vitro* [53], and *in vivo* assays showed that DNR function requires heme [54], suggesting that heme is the likely NO sensor. Despite this, neither X-ray crystal structure of DNR shows a bound heme [53,55], suggesting that the heme is labile. Thus, further studies

are required in order to determine the mechanism(s) by which DNR is activated and whether other FNR-CRP family members, especially NnrR (*nitrite and nitric oxide reductase regulator*) [56–58], function similarly.

The $[4\text{Fe-4S}]^{2+}$ cluster of a second FNR-CRP family member, ANR (anaerobic regulation of arginine deiminase and nitrate reduction), from *P. aeruginosa* is partially destroyed by NO [59]. Yet, recent whole genome analysis of the ANR and DNR regulons of *P. aeruginosa* showed that DNR regulates genes whose products are involved in NO metabolism, whereas ANR regulates genes involved in the switch from aerobic to anaerobic metabolism [60]. Thus, ANR may play a minor role as a NO sensor, similar to the effect of NO on the O_2 sensor $[4\text{Fe-4S}]\text{-FNR}$ [61] and the oxidative stress sensor $[2\text{Fe-2S}]\text{-SoxR}$ [62].

Nevertheless, Fe-S proteins, such as NsrR, function as physiologically relevant NO sensors. NsrR is a member of the Rrf2 family and is found in a wide variety of bacteria, including β - and γ -proteobacteria as well as Gram-positive *Bacillus* and *Streptomyces* species [51]. The NsrR regulon has been determined [63], and *E. coli* NsrR regulates functions that include the NO stress response (*hmp* and *nrfA*), Fe-S cluster assembly (*sufA*), general stress response (*sodB*), and many other pathways [64–67]. In the denitrifying organisms *Neisseria gonorrhoeae* and *N. meningitidis*, NsrR regulates the denitrification pathway, including *aniA* and *norB* [68,69].

The mechanism by which the Fe-S cluster of NsrR senses NO is unresolved, since, as reviewed recently [63], it is uncertain whether the active sensor is a $[4\text{Fe-4S}]$ cluster or a $[2\text{Fe-2S}]$ cluster. $[2\text{Fe-2S}]\text{-NsrR}$, isolated from either *Streptomyces coelicolor* [70] or *N. gonorrhoeae* [68], bound specifically to target promoters *in vitro*. *N. gonorrhoeae* apo-NsrR and *S. coelicolor* NsrR treated with NO had decreased DNA binding affinity, presumably due to a conformational change in the protein and/or a change in affinity for target DNA. On the other hand, *B. subtilis* NsrR binds a $[4\text{Fe-4S}]$ cluster that forms dinitrosyl iron complexes upon exposure to NO [71], as does *S. coelicolor* $[2\text{Fe-2S}]\text{-NsrR}$. In addition, a new report suggests that *S. coelicolor* NsrR actually binds a $[4\text{Fe-4S}]$ cluster and that the $[2\text{Fe-2S}]$ cluster-binding form observed previously may not simply be the result of inadvertent oxygen exposure [63]. Thus, while recent studies have shown that NsrR binds an Fe-S cluster that appears to be critical for NO sensing, the active state *in vivo* has not been defined.

In addition to heme- and Fe-S cluster-containing transcriptional regulators, proteins that bind non-heme iron are also involved in NO sensing. NorR, a σ^{54} -dependent transcriptional regulator and dedicated NO sensor, regulates the transcription of genes encoding NO-utilizing enzymes, including the *norVW* genes specifying a flavorubredoxin and an associated flavoprotein that exhibit NO reductase activity in *E. coli*, the *norAB* genes specifying a NO reductase in *Ralstonia eutropha*, and the *fhp* gene specifying a flavohaemoglobin in *P. aeruginosa*, as reviewed recently [50]. *E. coli* NorR binds to three essential enhancer sites in the intergenic region between *norR* and the divergently transcribed *norVW* operon, likely as three dimers that oligomerize to a hexamer [72] via its C-terminal DNA-binding domain. The N-terminal GAF (cGMP phosphodiesterase, adenylate cyclase, FhlA) domain contains a mononuclear non-heme iron center which contributes three aspartates, an arginine, and a cysteine as coordinating ligands to Fe^{2+} in *E. coli* [73] and blocks the binding of σ^{54} to the central AAA+ domain [74]. Upon exposure to NO, mononitrosyl iron complexes are formed, activating transcription through the movement of the GAF domain to expose the σ^{54} binding site and stimulating the ATP hydrolysis activity of the AAA+ domain [75]. In addition to NorR, the non-heme iron-

binding transcription factors Fur and PerR have also been implicated in NO sensing, as reviewed recently [50].

Conclusions

There have been many recent advances in the understanding of mechanisms of iron-binding transcription factors that act as sensors. In particular, an aspect of iron-binding transcription factors for which there is an increasing amount of information is in the study of the genes that these sensors regulate. The technology used to determine the regulon of a transcription factor is rapidly improving and becoming more accessible, and these methods are providing detailed information in regards to the function of specific transcription factors. With the utilization of these methods, the complexities of regulatory mechanisms *in vivo* are coming into focus, e.g. the function of small RNAs such as RyhB, FsrA, and FnrS and the surprising regulation of genes by the apo-form of sensors. While only apo-Fur regulation in *H. pylori* and apo-IscR regulation in *E. coli* have been observed, the apo-forms of other transcription factors discussed here could potentially be involved in gene regulation, which will become clearer as the regulons are more thoroughly dissected.

The data obtained thus far regarding iron-binding transcription factors, however, has yet to lead to concrete conclusions about specificity of the transcription factors involved in sensing O₂, NO, and reactive oxygen species. Several iron-binding transcription factors are sensitive to more than one of these species *in vitro*, suggesting that additional data is necessary to determine which species these factors primarily sense *in vivo*. In addition, it will be critical to dissect what protein determinants dictate its specificity for O₂, NO, or reactive oxygen species. Cofactor type does not appear to dictate which stimulus the transcription factor responds to, nor do families of transcription factors sense a common stimulus. For example, IscR and NsrR appear to sense O₂ and NO, respectively, despite the fact that both are Rrf2 homologs that bind [2Fe-2S] clusters. As more data—in particular, structural information—becomes available for various sensors, perhaps it will become more evident which characteristics are most important for sensor specificity.

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Figure 1.

Iron-containing transcription factors bind Fe²⁺, Fe-S clusters, or heme moieties and can act as sensors of various species, including metals, oxygen, oxidative stress (ox. stress), or nitric oxide (NO). Examples of these sensors are shown here and discussed in this review.