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Interaction of FurA from *Anabaena* sp. PCC 7120 with DNA: A reducing environment and the presence of Mn^{2+} are positive effectors in the binding to *isiB* and *furA* promoters*

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Abstract

The Fur (ferric uptake regulator) protein is a global regulator in most prokaryotes that controls a large number of genes. Fur is a classical repressor that uses ferrous iron as co-repressor and binds to specific DNA sequences (iron boxes) as a dimer. Three different genes coding for Fur homologues have been identified in *Anabaena* sp. PCC 7120. FurA controls the transcription of flavodoxin, the product of the *isiB* gene, and is moderately autoregulated. In this work, the promoter of the *furA* gene was defined and the FurA protected regions in the *furA* and *isiB* promoters were identified, showing that the binding sites for *Anabaena* FurA contain A/T-rich sequences with a variable arrangement compared to the conventional 19-base pair Fur consensus. The influence of different factors on the interaction between FurA and the promoters was evaluated *in vitro*. The affinity of FurA for the DNA targets was significantly affected by the redox status of this regulator and the presence of Mn^{2+}. The optimal binding conditions were observed in the presence of both Mn^{2+} and DTT. Those results suggest that, in addition to iron availability, FurA–DNA interaction is modulated by redox conditions.

Abbreviations: bp – Basepair(s); EMSA – Electrophoretic Mobility Shift Assay; *tsp* – Transcription Start Point; P_{furA} – Promoter of the *furA* Gene; P_{isiB} – Promoter of the *isiB* Gene; NEM – *N*-Ethyl maleimide; BSA – Bovine Serum Albumin; DTT – 1,4-Dithiothreitol

Introduction

Among known prokaryotic central regulators, the ferric uptake regulator (Fur) protein governs a wide diversity of processes, including iron homeostasis, oxidative stress responses and central metabolism, as well as more specific functions such as synthesis of redox cofactors, resistance to acidic stress or chemotaxis (Hantke 2001; Andrews *et al.* 2003). In the current model, Fur is a classical repressor that uses ferrous iron as a co-repressor and binds as a dimer to specific DNA sequences (iron boxes) when there is enough iron in the environment. It is rather common to find different *fur* gene homologues in the same organism or other metalloregulators of the Fur and DtxR families (Hernández *et al.* 2002b). In the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120 three open reading frames (*all1691, all2473* and

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alr0957) containing the histidine-rich region, characteristic of the Fur protein family, have been identified (Hernández *et al*. 2004). FurA is the product of the *all1691* gene and it has been shown to be involved in the regulation of the chlorophyll-binding protein CP-43′ and the *isiB* gene product flavodoxin (Ghassemian & Straus 1996).

According to structural studies, footprinting analysis and electrophoretic mobility shift assays (EMSA) of *fur* gene homologues from different sources that have been overexpressed in *Escherichia coli*, there are important differences among Fur proteins. A structural Zn has been found in Fur from several microorganisms such as *E. coli*, *Vibrio anguillarum* and *Bacillus subtilis* (Althaus *et al*. 1999; Bsat & Helmann 1999; Zheleznova *et al*. 2000). However, there are no structural metals in the purified recombinant FurA protein from the cyanobacterium *Anabaena* sp. PCC 7119, which is active in DNA-binding assays (Hernández *et al*. 2002a). More recently, it has been reported that Fur from *Pseudomonas aeruginosa* also lacks structural Zn (Lewin *et al*. 2002), although two metal binding sites have been located in its 3D-structure (resolution 1.8 Å) (Pohl *et al*. 2003). There are also differences in the redox state of cysteines in Fur proteins from diverse sources, which probably has important structural implications (Hernández *et al*. 2002b).

The architecture of iron boxes and other factors governing Fur–DNA interactions have also been considered. Initially, a highly conserved consensus of a 19-bp inverted repeat (5′-GATAATGATAATCATTATC-3′) was found in Fur-regulated promoters from *E. coli*. This sequence has been re-interpreted as three forward-forward-reverse tandem hexamers containing the recognition unit 5′-NAT(A/T)AT-3′ (Escolar *et al*. 1998). Further studies proposed that the Fur-binding sites can also be read as either a 15-bp core region formed by a 7-1-7 heptamer motif, clearly related to the hexamer model (Baichoo & Helmann 2002) or as overlapping 13-bp [6-1-6] motifs (Lavrrar & McIntosh 2003). Analysis of Fur–DNA interactions at the *fepDGC-entS* promoter of *E. coli* (Lavrrar *et al*. 2002) also suggests that the importance of the A and T bases in Fur binding may be overstated. When A–T corrections are introduced by mutagenesis in the Fur target, the affinity of Fur for the new sequence is not changed substantially.

Normally Fur only binds to DNA in the presence of Fe^{2+}, which is usually substituted by Mn^{2+} in *in vitro* experiments because the latter is more stable in the presence of oxygen. However, binding does occur in the absence of metal using the protein from *B. subtilis* (Bsat & Helmann 1999). In *Streptomyces reticuli*, metal ions are not essential but FurS activity depends on the redox state of its cysteine residues (Ortiz de Orue Lucana & Schrempf 2000).

The ability of Fur proteins to form oligomers also affects how it binds to operator sequences. Fur–DNA complexes have been observed with a high degree of Fur oligomers using electron microscopy (Frechon & Le Cam 1994). The protein–protein contacts allow a sequential occupancy of iron-boxes that results in a very fine regulation of the expression of certain genes tuned to different metabolic situations. Higher order oligomers have also been detected in *Anabaena* FurA using cross-linking experiments and MALDI-TOF (Hernández *et al*. 2002a). In addition to disulphide bridges, the formation of oligomers also depends on the FurA concentration and ionic strength, suggesting the existence of hydrophobic interactions among monomers. Probably the conditions that influence the tendency of the protein to form high-molecular weight aggregates determine the quality of the Fur–DNA interaction.

Many iron-stress induced genes have been found in the unicellular cyanobacterium *Synechocystis* sp. using DNA arrays (Singh *et al*. 2003), but the Fur regulon has yet to be identified in cyanobacteria. Putative iron-boxes have been proposed for the *isiAB* and *irpA* promoters in unicellular cyanobacteria, as well as for *isiA*, *isiB* and *furA* genes in *Anabaena* sp. (Straus 1994; Bes *et al*. 2001). However, it has not been shown that Fur binds to those sequences.

This paper provides new insights into the molecular basis of cyanobacterial FurA–DNA interaction. We define the promoter of the *furA* gene and then identify the regions of the *isiB* and *furA* promoters that are involved in that interaction. The iron boxes contain A/T-rich sequences with a variable arrangement compared to the *E. coli* consensus. Gel retardation assays have been used to measure the influence of several effectors in the binding of FurA to P_{isiB} and P_{furA}, showing that the presence of both, divalent metals and a reducing environment, contribute to an optimal

interaction of the *Anabaena* FurA regulator with those targets.

Materials and methods

Overexpression and partial purification of FurA

Cells from *Anabaena* sp. PCC 7120 were grown in BG11 liquid medium (Rippka 1988). Chromosomal DNA from *Anabaena* sp. PCC 7120 was isolated as described previously (Cai & Wolk 1990) and used as a template for amplification of the *furA* gene (*all1691*). The PCR product was sequenced and cloned between the *Nco*I and *Hin*dIII sites of pET28a(+). This construct was used to transform *E. coli* BL21-Gold DE3 cells to overexpress the FurA protein. The recombinant FurA was purified by chromatography through a heparin-Sepharose 6 Fast Flow column (Amersham-Pharmacia), as described previously (Bes *et al.* 2001; Hernández *et al.* 2002a). Purified samples were analysed by SDS-PAGE, and purity was estimated by densitometric analysis of gels to be > 86%.

Electrophoretic mobility shift assays

The 389-bp and 348-bp fragments from the upstream regions of *furA* and *isiB* genes, respectively, were amplified by PCR and used in binding assays performed as described previously (Bes *et al.* 2001). All the assays were performed using 140 nM of DNA probe in the presence of unspecific competitor *apoE* DNA (2:1 molar ratio respect to P_{furA} and P_{isiB}) in a core binding buffer containing 10 mM bis-Tris (pH 7.5), 40 mM KCl, 0.1 mg/ml BSA and 5% glycerol. Depending on the effect analysed, the buffer was supplemented with 100 μM $MnCl_2$ and/or different concentrations of NaCl, DTT, diamide, *N*-ethyl maleimide (NEM) or H_2O_2, as indicated in figure legends. Results were processed with a Gel Doc 2000 image analyser from BioRad. The remaining unbound DNA in the titration assays was estimated with respect to the band area corresponding to free DNA control, taken as 100%.

DNAse I footprinting

The DNase I protection assay used in this study was a modified version of that described previously (Frias *et al.* 2000). The PCR products containing the upstream regions of *furA* and *isiB* used in EMSA were cloned into pGEM-T (Promega). The fragments from both promoters were excised using *Nco*I/*Pst*I and *Sac*II/*Spe*I enzymes and 3′end-labelled with the DNA polymerase Klenow fragment and [α-^{32}P]-dCTP (3000 Ci mmol^{-1}). Binding reactions were performed incubating 0.05 pmol of labelled promoter with increasing concentrations of partially purified FurA in the presence of 1.5 μg of salmon sperm DNA as non-specific competitor. Radioactive bands were visualized with a Cyclone storage phosphor system and OptiQuant image analysis software (Packard).

RNA isolation and analysis

Total RNA from *Anabaena* sp. PCC 7120 was prepared as described previously (Muro-Pastor *et al.* 2002). Primer extension experiments were performed as described in (Muro-Pastor *et al.* 1999) using 25 μg of RNA per reaction. Primers proC (5′-GCCTTGAGCGAAGTATTTGTG-3′) complementary to nucleotides 12–32 of the *Anabaena furA* gene and tsp2 (5′-TTGAGGGG TCAAACGCCAGC-3′) complementary to nucleotides 50–69 of the same gene were used. Results were visualized with a storage phosphor system as above. For sequencing ladders used in primer extension analysis, sequencing was carried out by the dideoxy chain termination method using a T7-sequencing TM kit (Amersham Biosciences) and [α-^{35}S]-thio-dATP.

Results

Mapping of P_{furA} and localization of FurA binding sites on P_{furA} and P_{isiB}

After the primer extension analysis using oligonucleotides proC and tsp2, a single transcription start site was found for the *furA* gene located 26 nt upstream from the ATG start codon (Figure 1). This information was used to identify other potential transcription/translation signals such as a putative ribosome binding site located 10 bp upstream from the start codon (GAGAA), as well as the −10 and −35 promoter hexamers that are

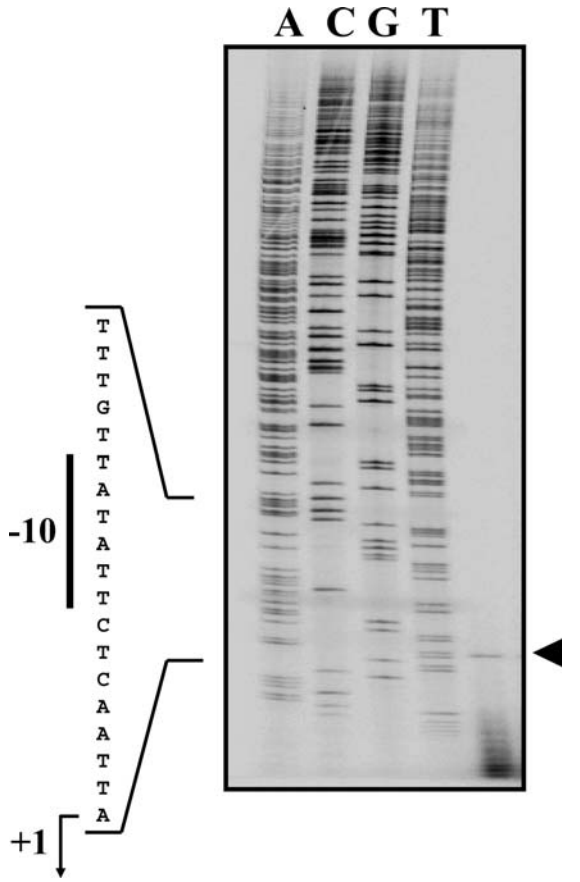
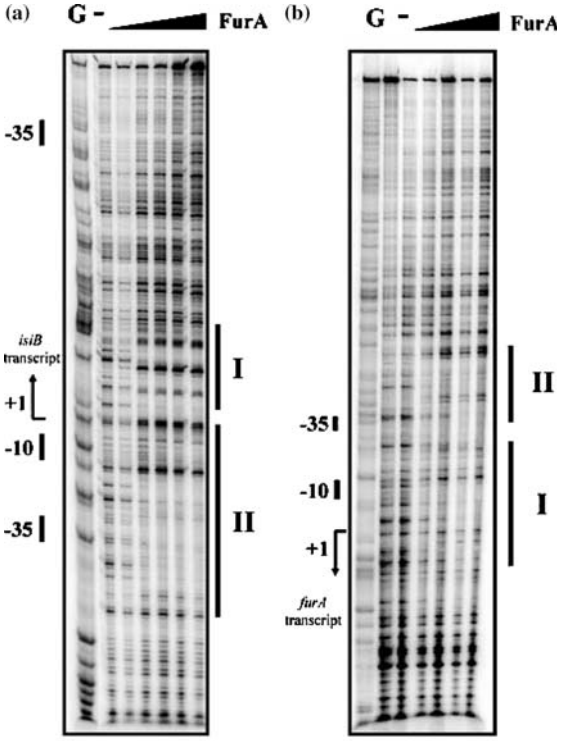


Figure 1. Primer extension analysis of *fur*A in *Anabaena* sp. PCC 7120. RNA was isolated from cells grown in complete BG11 medium containing 8 mM NH_4Cl. The oligonucleotide primer was proC. Similar results were obtained using oligonucleotide tsp2 (not shown). The sequencing ladder was generated with the same oligonucleotide and plasmid pGEM®-T containing the *furA* promoter. The putative transcription start point is indicated by an arrowhead and a putative −10 box is marked.

similar to the *E. coli* σ^{70}-type consensus (see Figure 2c). The transcription start sites in P_{isiB} have been reported previously (Leonhardt 1989).



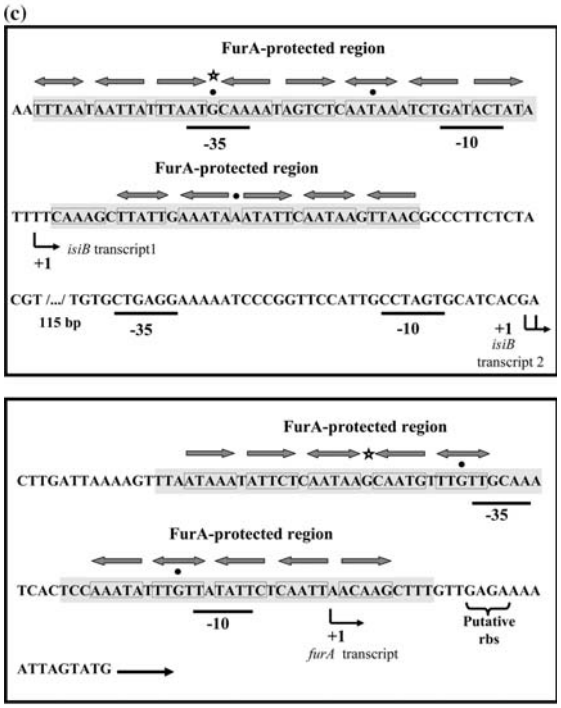


Figure 2. FurA-protected regions in P_{isiB} and P_{furA}. (a) DNase I footprint on the *isiB* promoter incubated with 0, 40, 80, 160, 320 and 640 nM FurA. (b) DNase I footprint on the *furA* promoter incubated with 0, 0.05, 0.25, 0.5, 0.75 and 1 μM FurA. Please, note that different preparations of purified protein were used in (a) and (b). The G lanes correspond to chemical cleavage sequencing (Maxam & Gilbert 1980). The boundaries of the regions protected by FurA are indicated. (c) Sequences of P_{isiB} and P_{furA} showing transcription start points and regulatory elements. FurA protected regions are marked as grey boxes and arranged using the G(ATA/TAT) hexamer model. Arrows indicate a forward (ATA/TAT) or reverse (TAT/ATA) arrangement in the A/T-rich regions. Symmetry axis (●), palindromes (⋆) and GC motifs are indicated.

DNase I footprinting assays were performed using the FurA protein and the promoter regions of the *isiB* and *furA* genes with DNA fragments containing 355 bp upstream of the *isiB* ATG start codon and 350 bp upstream of the *furA* ATG start codon, respectively. Protection by FurA of the *isiB* promoter unraveled two regions of interaction with the repressor. A primary binding site (I) was detected in the presence of 40 nM FurA between the transcription start points (tsp_1 and tsp_2) of *isiB* and covers 36 bp spanning from positions +3 to +38 with respect to the *isiB* tsp_1. A 48-bp secondary binding site (II) that is more evident at higher FurA concentrations spans from −3 to −50 with respect to tsp_1 (Figure 2a). Several sites had an enhanced sensitivity to DNase I in both protected regions. The apparent periodicity of hypersensitive sites was longer than one helical turn of β-DNA, since they are observed every 14–16 nucleotides and are flanked by one protected nucleotide in at least in one position. This could indicate a conformational change in DNA when FurA binds to the operator region.

Footprinting analysis of the *furA* promoter (Figure 2b) showed two protected regions, one covering the transcription start point and the TATA box spanning from −26 to +10 (site I) that shows a fainter protection and a second region from bp −67 to −31 (site II). As with the *isiB* promoter, FurA protected regions are essentially the same in both DNA strands (not shown). The locations of the protected regions in P_{isiB} and P_{furA} are summarized in Figure 2c.

Architecture of iron-boxes in Anabaena promoters

Alignment of DNAse I-protected sequences in P_{isiB} and P_{furA} with the canonical consensus defined for *E. coli* (Figure 3a) shows that all the *Anabaena* putative 19-bp motifs are flanked by A/T-rich sequences. Exact matching of protected sites in the *isiB* promoter with this consensus is 58% and 52.6%, respectively, while identity in the *furA* promoter is 42% for site I and 47 % for site II. However, if A/T mismatches are allowed, the similarity with the *E. coli* consensus sequence increases greatly. This suggests a slacker arrangement of iron boxes in cyanobacteria consisting of several A/T-rich adjacent hexamers with diverse orientations and frequent A–T permutations, compared to the canonical G(ATA/TAT) unit.

In *Anabaena*, the FurA-binding sequences analysed (Figure 2c) have six A/T-rich consecutive motifs in site (I) of the *isiB* operator and eight N(ATA/TAT) units in site (II). There is a high degree of symmetry in both sites (I) and (II), as well as in site (I) of the *furA* promoter with five A/T-rich consecutive arrays. The presence of the GC motif in site (II) of *isiB* and *furA* promoters indicates the existence of more than one 19-bp target. Since stable Fur–DNA interactions require recognition of iron-boxes containing at least three hexamers (Lavrrar *et al*. 2002), those additional motifs could increase the affinity of the repressor for the operator sequences and facilitate recruitment of extra dimers, favouring protein–protein interactions and increasing the stability of the complex.

Divalent metals and/or thiol-reducing agents activate FurA–DNA interaction in vitro

It is generally accepted that Fur acts as a positive repressor that regulates transcription after interacting with its co-repressor Fe^{2+}. However, the DNA binding behaviour of Fur proteins may differ depending on the presence of metal and the redox state of cysteines (Bsat & Helmann 1999; Ortiz de Orue Lucana & Schrempf 2000). To define the binding requirements of the *Anabaena* FurA protein, we carried out EMSA with P_{furA} and P_{isiB} over a range of FurA concentrations in the presence or absence of 100 μM Mn^{2+} and/or 1 mM DTT. Previous work (Bes *et al*. 2001) indicates that FurA binds its own promoter with higher affinity than P_{isiB}. Those results are in good concordance with titrations

		% Identity	(A/T)
isiB (site I)	---------CAAAGCTTATT**GAaAtaaATAtTCAaTAag**TTAACG	58	73
isiB (site II)	---------TTTAATAATTA**ttTAATGcaAAatAgTcT**AATAAATCTGATACTATA	52.6	74
fur (site I)	----------TCC**AaAtattTtgTtATatTC**TCAATTAACAAGCTTT	42	79
fur (site II)	----------TTAATAAAT**atTctcaATAAgCAaTgTt**TGTTGCAAA	47	63
CONSENSUS	**GATAATGATAATCATTATC**		

Figure 3. Alignment of the FurA protected regions in P_{isiB} and P_{furA} with the canonical 19-bp consensus. Percentage identities and homologies allowing A–T mismatches (A/T) of the cyanobacterial sites to the consensus are indicated.

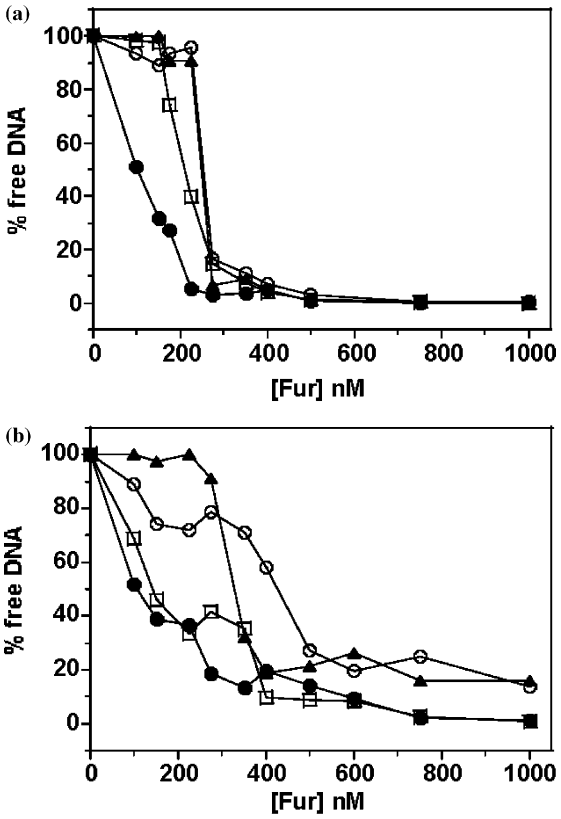


Figure 4. Effects of Mn^{2+} and DTT in FurA binding to P_{furA} (a) and P_{isiB} (b). EMSA assays were performed with equimolar amounts of both promoters and increasing concentrations of FurA. Binding in the presence of 100 μM Mn^{2+} and 1 mM DTT (closed circles), 100 μM Mn^{2+} (open squares), 1 mM DTT (open circles), or in the absence of Mn^{2+} and DTT (black triangles).

assays shown in Figure 4. FurA shows maximum affinity for both promoters in the presence of Mn^{2+} and DTT. The absence of metal had more severe consequences on the interaction of the repressor with both promoters than the absence of DTT. However, the profiles FurA titrations versus P_{furA} or P_{isiB} were slightly different without Mn^{2+} or DTT in the binding mixture. The lack of the metal impaired FurA–P_{isiB} interaction more than FurA–P_{furA}, whereas changes in the titration of FurA–P_{isiB} under non-reducing conditions were rather small. In contrast, the redox status of the cysteines had a greater effect on the binding of FurA to its own promoter, even in the presence of Mn^{2+}.

It can be concluded that, *in vitro*, the presence of divalent metals and/or thiol-reducing agents enhance the affinity of *Anabaena* FurA for its target sequences, although neither Mn^{2+} nor DTT are strictly necessary for FurA–DNA interaction. The shape of the titration curve for P_{isiB} was also significantly different from P_{furA}. This could be a consequence of different repressor affinity. Titration curves are the result of several events which involve FurA–DNA recognition and interaction as well as protein–protein contacts. The latter depend, in turn, on the availability of metal, the presence of DTT, and the concentration of free protein in solution, among other parameters. Protein–protein interactions can either produce active dimers or complexes with less ability to bind DNA. The contribution of protein–protein interactions to the global process is greater in the system with lower affinity (FurA–P_{isiB}), as seen in Figure 4b. The complexity of the system does not allow standard data analysis in curves where metal or DTT are absent. Besides, both promoters have multiple FurA binding sites. From the curve shapes we can infer that FurA binds iron boxes in P_{furA} with similar affinities, while binding to P_{isiB} seems to occur more sequentially. These observations agree with the footprinting results, where the protected regions were more clear cut in P_{isiB} than P_{furA},, suggesting that different mechanisms could be involved in the interaction of FurA with these DNA fragments.

When binding assays were performed in the presence of thiol-modifying agents, there were significant changes in the amount of complex formation (Figure 5). Before incubation with DNA, the interaction was impaired when FurA was treated with the thiol-oxidizing agent diamide (Figure 5, lane 3). Although to a lesser extent, a similar effect was observed when the protein was incubated with NEM (Figure 5, lane 5). However, the effect on the interaction was negligible when the FurA–DNA complex formed in the presence of DTT was challenged with diamide or NEM (Figure 5, lanes 4 and 6). These results indicate that once FurA binds to DNA, the complex is rather stable, possibly because the cysteines are less exposed to the modifying agents.

Peroxide inactivation of FurA is reversed by DTT

Since Fur-like proteins are involved in peroxide regulation in several heterotrophic bacteria (Bsat *et al.* 1998; van Vliet *et al.* 1999; Herbig & Helmann 2001), we investigated the effect of H_2O_2 on

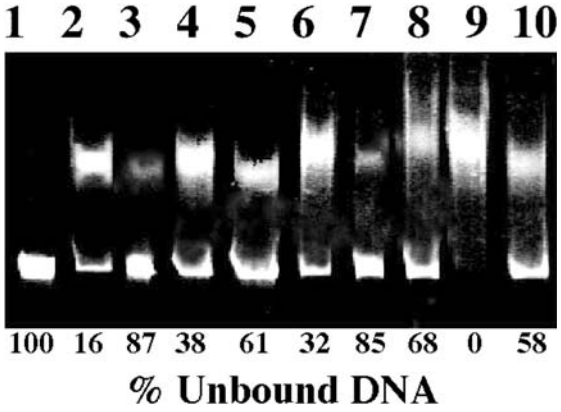


Figure 5. Gel retardation analysis of the binding of FurA to its own promoter in the presence of thiol-modifying agents and H_2O_2. Effects of diamide, NEM and H_2O_2 were assayed by treating the FurA protein before DNA binding or by challenging the complex to test stability. Lane 1: free DNA; lane 2: P_{furA} bound to 500 nM FurA in the presence of 5 mM DTT and 100 μM Mn^{2+}; lane 3: 500 nM FurA preincubated at 37 °C for 1 h with 1 mM diamide and 5 mM DTT before adding DNA; lane 4: FurA–DNA complex formed as in lane 2 was challenged with 1 mM diamide at 37 °C for 1 h; lane 5: 500 nM FurA preincubated with 1 mM NEM and 5 mM DTT for 1 h at room temperature before DNA addition; lane 6: complex formed as in lane 2 was challenged with 1 mM NEM for 1 h at RT; lane 7: complex formed in the absence of DTT treated with 10 mM H_2O_2 for 10 min at RT; lane 8: P_{furA} bound to 500 nM FurA in the presence of 100 μM Mn^{2+} and challenged with 10 mM H_2O_2 for 10 min at RT; lane 9: complex of P_{furA} with 500 nM FurA in the absence of Mn^{2+} challenged with 10 mM H_2O_2 as in lane 8 before addition of DTT 100 mM; lane 10: complex of P_{furA} with 500 nM FurA formed in the presence of 100 μM Mn^{2+} was challenged with 10 mM H_2O_2 for 10 min at RT before addition of DTT 100 mM.

the DNA-binding ability of FurA and Mn^{2+}-FurA (Figure 5). Adding 10 mM H_2O_2 before incubation with DNA completely impaired complex formation (data not shown). When the same concentration of oxidant was added to bound FurA, the presence of Mn^{2+} slightly stabilized the interaction (Figure 5, lanes 7–8). Adding 100 mM DTT after the H_2O_2 challenge completely restored the ability for complex formation in the absence of metal (Figure 5, lane 9). However, in the presence of Mn^{2+}, about 58% of DNA remained unbound after adding DTT (Figure 5, lane 10). This should be interpreted carefully, since the excess DTT could be replacing the metal (destabilizing the FurA–DNA interaction) or Mn^{2+} could be promoting free-radical formation which could damage both the Fur protein and the complex. Nevertheless, it appears that FurA–DNA interactions are clearly affected by the redox state of the environment.

Presence of Mn^{2+} stabilizes FurA–DNA complexes at high ionic strength

To assess the influence of ionic strength in the *in vitro* FurA–DNA interaction, EMSAs were conducted over a range of increasing salt concentrations in the presence or absence of Mn^{2+}. As seen in Figure 6, FurA-Mn^{2+} forms a stable complex with P_{furA} under all conditions, although the FurA–DNA association was higher at 40 and 100 mM KCl. When the binding reaction was performed without Mn^{2+}, the sensitivity of the complex to ionic strength increased significantly. Thus, Mn^{2+} favours the formation of FurA–DNA complexes, but also stabilizes the FurA–DNA contacts mediated by ionic interactions.

Discussion

The regulation of flavodoxin can be considered a paradigm of Fur-modulated genes in cyanobacteria. This repressor exerts a tight control over the expression of the *isiB* gene, resulting in negligible transcript levels in iron-repleted cells (Fillat *et al.* 1991). In contrast, the regulation of FurA seems to be rather complex and the result of different events. In addition to autoregulation, the other *fur* homologues present in *Anabaena* should contribute to determine the final concentration of FurA in the cell (Hernández *et al.* 2004). The aim of the present study was to characterize the interaction of a cyanobacterial FurA with the P_{isiB} and P_{furA} targets by identifying their iron boxes and analysing the influence of different effectors on FurA–DNA binding.

Identification of FurA binding sites on P_{furA} and P_{isiB}

FurA protected sites in P_{furA} and P_{isiB} have been detected by DNase I footprinting analysis, leading to the first experimental identification of cyanobacterial iron-boxes. Rapid development of DNA chips, bioinformatics and proteomic tools have allowed the identification of Fur-binding sites in several microorganisms. The GAT(A/T)AT "unit" of the Fur-binding motif proposed for *E. coli* is

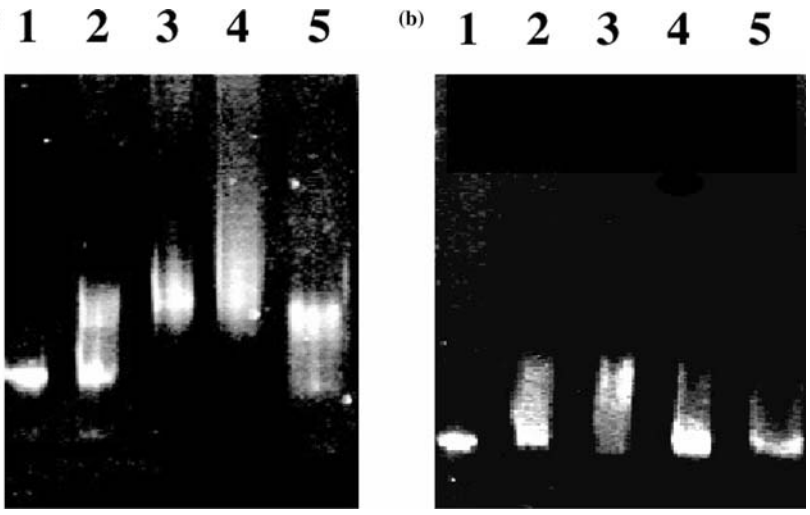


Figure 6. Effect of ionic strength on the binding of FurA to its own promoter. (a) EMSA assays were performed under standard conditions using 250 nM FurA in the presence of 100 μM Mn^{2+} at increasing KCl concentrations. Lane 1: free DNA; lanes 2–5: binding of FurA to DNA in binding buffer containing KCl at 10, 40, 100 and 500 mM respectively. (b) Same as panel (a) but Mn^{2+} was substituted by 1 mM EDTA.

highly conserved in most promoters of Fur regulated genes from *Pseudomonas* sp. and *B. subtilis* (Baichoo *et al*. 2002; Ochsner *et al*. 2002). However, the number of matches with the consensus sequence in *Shewanella oneidensis* is rather low (Thompson *et al*. 2002). Alignment of the Fur binding sites identified in this work with the canonical consensus from *E. coli* (Figure 3a) shows that the exact positions of A or T nucleotides is not very critical. Similarly, a recent study on Fur–DNA affinity at the *fepDGC-entS* promoter in *E. coli* suggests that the architecture of the binding site is more important than individual bases (Lavrrar *et al*. 2002).

The low homology between FurA-binding sites in cyanobacteria with *E. coli* and *Pseudomonas* can be explained by differences in the recognition helix of the repressor that interacts directly with the major groove of the DNA. In *E. coli*, the recognition motif is thought to comprise the α-helix [Y55-F61] and some adjacent amino acids (Gonzalez de Peredo *et al*. 2001). Resolution of the Fur crystal structure in *Pseudomonas* has confirmed the interaction of α-helix H4 [G50-A64] with DNA (Pohl *et al*. 2003). In addition, the N-terminal domain from residues 2 to 9 may be involved in DNA recognition. Aligning the Fur proteins using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) showed that the α-helix H4 [S57-M71] and the N-terminal domain in the *Anabaena* protein are significantly different than the corresponding *E. coli* and *Pseudomonas* domains.

Effectors modulating FurA–DNA interaction

The affinity of Fur for DNA target depends on features from both elements. In addition to the architecture of the iron-boxes, the presence of metals or the redox status of Fur can change its binding properties. Moreover, some proteins of the Fur family, such as FurS or PerR, are involved in peroxide sensing and rely on the state of redox-active cysteines for DNA binding (Ortiz de Orue Lucana & Schrempf 2000; Herbig & Helmann 2001). In unicellular cyanobacteria, a tight interrelationship between iron homeostasis and oxidative stress involving Fur-type transcriptional regulators has been clearly illustrated (Yousef *et al*. 2003; Kobayashi *et al*. 2004; Li *et al*. 2004). Our titration experiments of P_{furA} and P_{isiB} with *Anabaena* FurA show that, even though the affinity of FurA for both promoters clearly decreases in the absence of Mn^{2+}, the presence of divalent metals is not essential for FurA–DNA interaction. As it has been observed previously (Hernández *et al*. 2004), DTT also acts as a positive effector of FurA in DNA binding. The effect of DTT is more evident in P_{furA}–FurA binding, which is more

favoured than P_{isiB}–FurA. Apart from its effect on the FurA–DNA interaction, the redox state of cysteines also influences protein–protein interactions and determines the degree of oligomerization of the repressor (Hernández *et al*. 2002a). In solution, Fur monomers coexist in equilibrium with dimerized protein and high-order oligomers. Those aggregates, as well as dimers formed with a wrong orientation, are less able to bind DNA. The reduction of cysteine residues allows the Fur monomers to reorganize and increases the proportion of active protein available for DNA binding. Since Fur cysteines are involved in metal coordination and protein–protein interactions, their redox status has a direct influence on the binding to Fur targets. As a consequence, the redox status of the cell might help regulate Fur. In support of this hypothesis, FurA-DNA binding activity is lost in the presence of H_2O_2 and restored by thiol-reducing agents. Therefore, like PerR proteins, FurA contains redox-active cysteines that could be involved in peroxide sensing. Further work using *in vivo* approaches is necessary to assess a possible role of FurA in redox-sensing in cyanobacteria.

Another parameter that influences the formation and stability of Fur–DNA complexes is ionic strength. Our results suggest that the FurA–DNA complex reaches a conformation of minimal energy in the presence of Mn^{2+}. The Fur–DNA contacts are more resistant to rupture and this is mediated by ionic interactions. In addition, the formation of trimer and tetramer species of FurA at high ionic strength (Hernández *et al*. 2002a) may impair an optimal interaction with DNA and reduce the number of active molecules in the binding mixture. The formation of high-molecular-mass species detected by cross-linking studies of FurA from *Anabaena* suggests the existence of hydrophobic interactions in protein oligomerization. This is supported by the studies on the 3D structure of *P. aeruginosa* Fur which indicate that dimers are formed as a consequence of a large hydrophobic dimerization interphase and salt bridges between arginine and aspartic residues (Pohl *et al*. 2003). Therefore, like the redox state of cysteines, ionic strength plays a role in protein–DNA interactions and protein–protein complexes.

In summary, the FurA–DNA interaction appears to be the result of a complex overlap of different factors, including the architecture of iron boxes, the availability of divalent metals and redox status, as well as hydrophobic and ionic interactions. Most of these parameters also determine the degree of oligomerization of the repressor and the quality of the dimers (Hernández *et al*. 2002a). Efforts are underway to evaluate the contribution of different amino acid residues to the activity of this regulator and to better understand *in vivo* modulation of FurA–DNA interactions.

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