

Heme binds to and inhibits the DNA-binding activity of the global regulator FurA from *Anabaena* sp. PCC 7120

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Abstract Heme is an iron-containing cofactor that aside from serving as the active group of essential proteins is a key element in the control of many molecular and cellular processes. In prokaryotes, the family of Fur (ferric uptake regulator) proteins governs processes essential for the survival of microorganisms such as the iron homeostasis. We show that purified recombinant FurA from *Anabaena* sp. PCC 7120 interacts strongly with heme in the micromolar range and this interaction affects the *in vitro* ability of FurA to bind DNA, inhibiting that process in a concentration-dependent fashion. Our results provide the first evidence of the possible involvement of heme in the regulatory function of cyanobacterial Fur.

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1. Introduction

Heme is a near planar coordination complex obtained from iron and the dianionic form of porphyrin [1]. Fe-protoporphyrin IX is the most frequently occurring heme. It carries out a wide range of biological functions and participates in a number of key biological processes, including respiration and energy transfer. This diversity of functions is a consequence of the versatility of the heme group and its ability to interact with protein frameworks generating different heme environments [2]. Even though heme is an essential cofactor for many proteins, it is poorly soluble in aqueous solution under physiological conditions. Moreover, it is also highly toxic because of its ability to catalyze free radical formation and cells must protect themselves against an excess of free heme [3]. In this way, heme is usually associated with proteins, and rarely dissociates completely in the environment and in normal healthy cells [4]. However, this heme–protein association can be dele-

terious for the protein and sometimes this compound operates as an effector molecule for the protein degradation [5].

With a few exceptions, a property of ligand binding interactions is that there is a single binding site for a particular ligand on each molecule of a polypeptide chain. The magnitude of the affinity determines whether a particular interaction is relevant under a given set of conditions. Heme binds very tightly, sometimes covalently, through protein sequences that commonly contain a histidine/methionine pair or bis-histidine as happens in globins and cytochromes [2]. Heme can also transitorily interact with proteins and sometimes this interaction potentiates the binding of a protein to DNA serving a regulatory function. This is the case of the yeast transcriptional activator HAP1 [6], where the interaction of heme with the protein occurs through a heme regulatory motif (HRM) that contains the amino acid sequence Cys–Pro–Val [7]. This motif has shown to be an important structural element for the direct binding of heme by mitochondrial heme lyases [8]. Heme binds specifically as well to the dipeptide cysteine–proline (CP) motif in the mammalian transcription factor Bach1 and regulates its DNA-binding activity [9]. Therefore, CP motifs prove to play an important role in the binding of heme by various proteins even though histidines are the residues normally involved in the binding of heme by the polypeptide chain in the majority of hemoproteins [2]. In particular, histidine has shown to be essential in the reversible heme–protein binding that takes place in HasA protein from *Serratia marcescens* [10], a hemophore that binds free or hemoprotein bound heme with high affinity and delivers it to a specific outer membrane receptor HasR [11]. It has been suggested that histidine residues might be involved as well in the binding of heme by Fur (ferric uptake regulator) from *Escherichia coli* [12]. Fur is a DNA-binding protein that regulates iron responsive genes [13]. The current model of regulation mediated by Fur proposes that, when complexed to ferrous ions, a dimer of Fur binds a specific DNA sequence known as the Fur box that is located in the promoter region of iron responsive genes and affects their expression, causing a cascade of negative and positive regulatory responses [14,15]. The protein functions as regulator of genes involved in a variety of cellular processes such as iron uptake and storage, virulence determinants, intermediate metabolism, oxidative stress defense, acid shock response, electron transport, redox cofactor synthesis, chemotaxis or photosynthesis [16,17]. This repressor is also involved in the regulation of systems required for heme and hemoglobin uptake in different species [3]. This is the case of *Pseudomonas aeruginosa*, where expression of the *phuR* receptor gene (*Pseudomonas* haem

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Abbreviations: Heme, ferriprotoporphyrin IX; Fur, ferric uptake regulator; DTT, 1,4-dithiothreitol; K_d , complex dissociation constant; EMSA, electrophoretic mobility shift assay; BSA, bovine serum albumin; bp, basepair(s); FMN, flavin mononucleotide; HRM, heme regulatory motif; P_{isiB} , promoter region of the *isiB* gene; P_{furA} , promoter region of the *furA* gene; Ntc, nitrogen control; P_{glnA} , promoter region of the *glnA* gene

uptake) and the *phuSTUVW* operon encoding a typical ABC transporter (ATP-binding cassette) occurs under iron-restricted growth conditions and is directly controlled by the Fur protein [18]. In *Serratia marcescens*, different data suggest that HasR, an extracellular heme binding protein which alone enables an *E. coli* *hemaA* mutant to grow on heme or hemoglobin as a porphyrin source, is also iron regulated in a Fur-dependent manner [19]. Moreover, Fur titration assays indicate that expression of *rhuI* (regulator of heme uptake) from *Bordetella avium* is probably Fur dependent [20].

The presence of a CP motif, which is absent in Fur from *E. coli*, and a histidine rich region in FurA from the cyanobacterium *Anabaena* sp. PCC 7120 [21] with a higher content of this residue than *E. coli* Fur, as well as the involvement of Fur in the regulation of different heme metabolism pathways, has prompted us to investigate the binding of heme to this protein and the possible consequences of this interaction from the regulatory point of view.

In this paper, we show that heme binds to FurA from *Anabaena* sp. PCC 7120 in the micromolar range and this interaction produces a bathochromic effect in the heme absorption spectrum. We also investigate several chemical aspects affecting this interaction and its influence on the binding of the protein to different promoters. In vitro results suggest that FurA from *Anabaena* sp. PCC 7120 could act as a heme-responsive transcription factor that would regulate gene expression by altering its DNA-binding activity in response to the intracellular free heme concentration in the presence of its metal co-repressor.

2. Materials and methods

2.1. Proteins and reagents

Recombinant FurA from *Anabaena* sp. PCC 7120 was purified according to Hernández et al. [22] and stored at -20°C in 10 mM acetic acid/acetate, pH 4, containing 10% glycerol. The concentration of the protein was determined spectrophotometrically using a molar extinction coefficient of $13,760\text{ M}^{-1}\text{ cm}^{-1}$ at 276 nm [22].

Heme solutions were prepared by dissolving heme (purchased from Sigma, ferriprotoporphyrin IX) in a (1:9 v/v) 0.1 N NaOH/ethanol solution to approximately 0.5 mM concentration. After vigorous shaking, the solution was filtered through a $0.2\text{ }\mu\text{m}$ filter. Heme concentration was determined spectrophotometrically by diluting 50 μl of this solution in 950 μl of NaOH 0.1 N using an extinction coefficient of $58,44\text{ mM}^{-1}\text{ cm}^{-1}$ at 385 nm [23]. All other reagents used in this study were of analytical grade.

2.2. UV–Vis absorption spectroscopy

The UV–Vis measurements were carried out using UV–Vis double beam Kontron Uvikon 860 or 942 spectrophotometers.

2.3. Complex formation

The heme–FurA complex was prepared in one milliliter final volume of the corresponding buffer (50 mM Tris/HCl, pH 8, or 10 mM acetic acid/acetate, pH 5) by directly mixing a 2 μM heme solution with aliquots of concentrated FurA at different heme/FurA ratios. The heme solution used was obtained by dilution of a concentrated one in the corresponding buffer.

Since FurA tends to precipitate as its concentration increases at pH 8, instead of performing stepwise additions of FurA to the same heme solution, independent mixtures for each FurA addition (from 1 to 40 μl) were prepared in this case. After addition of equal volumes of buffer to the reference cuvette and FurA to the sample cuvette, difference spectra were obtained by subtracting heme signals from those of heme–FurA samples. Spectra were recorded immediately after addition of FurA.

When the reduction of the heme–FurA complex was studied, 5 mM final concentration of sodium dithionite was added to the sample cu-

vette after the formation of the complex at pH 8 (1:1 heme/FurA (3.5 μM) concentration ratio). The correction of volume in the reference cuvette was performed by addition of a volume of water equivalent to the volume of sodium dithionite solution added to the sample cuvette.

In the case of analyzing the influence of the ionic strength, the heme solution contained 200 mM NaCl prior to the addition of the protein.

The heme–FurA complex was also prepared in the presence of DNA by performing stepwise additions of DNA and FurA to a heme solution as previously described. The difference spectrum was recorded immediately after addition of each component. The final concentrations were 3.3 μM heme, 0.7 nM *P_{isiB}* (348 bp DNA fragment containing the *isiB* gene promoter) and 3.3 μM FurA.

2.4. Dissociation constant

The dissociation constant (K_d) was calculated from the difference absorption spectra in the Soret region plotting the spectral changes between 382 and 416 nm vs. FurA concentration. Experimental data were fit to the theoretical equation for 1:1 stoichiometry by means of non-linear regression using the program KaleidaGraph 2.1 from Albebeck.

2.5. Electrophoretic mobility shift assays

Binding assays with FurA were carried out as described in [22] using a modified binding buffer which contained 10 mM Bis–Tris, pH 7.5, 40 mM KCl, 0.1 mM MnCl_2 , 1 mM DTT, 0.05 mg/ml BSA and 5% glycerol. Samples were supplemented with heme from a stock solution prepared as previously explained or with flavin mononucleotide (FMN) from a stock solution prepared by directly dissolving this cofactor in water. As DNA targets, we chose a 348 bp DNA fragment containing the *isiB* gene promoter and a 398 bp DNA fragment containing the *furA* gene promoter. DNA fragments to be used in electrophoretic mobility shift assays (EMSA) were obtained by PCR and further purified using the GFX PCR DNA and Gel Band purification kit from Amersham Pharmacia. Reactions were carried out in the presence of a 224 bp non-specific competitor DNA (fourth exon of the human *apoE* gene) in order to demonstrate the specificity of the DNA-binding activity of the FurA protein.

As a control of protein–heme unspecific binding, we performed binding assays with purified NtcA protein and a 489 bp DNA fragment containing the *glnA* promoter region in the presence of increasing amounts of heme. These experiments were carried out as described by Montesinos et al. [24].

3. Results

3.1. FurA from *Anabaena* sp. PCC 7120 binds heme

Since the absorption spectrum of heme is perturbed when it becomes incorporated into proteins, we examined the effects of FurA on the absorption spectrum of this compound by using recombinant wild type FurA. Considering that histidine residues are common heme ligands that have been implicated in iron binding by this protein [12], and this iron binding is affected by the pH [25], we performed our studies at two different pHs (8 and 5). Upon addition of purified recombinant FurA to a solution of heme at pH 8, appearance of a peak at 416 nm in the difference spectrum was indicative of a complex formation between heme and FurA, since maximum absorbance of free heme under the same conditions is 384 nm (Fig. 1). This peak corresponds to the Soret band of heme which suffers a shift to a longer wavelength by 32 nm (from 384 to 416 nm). The amount of heme capable of interacting with the protein was determined by measuring the difference spectrum at various concentrations of FurA by UV–Vis spectroscopy. Results of the titration at pH 8 are presented in Fig. 2. It shows that FurA from *Anabaena* sp. PCC 7120 interacts with heme in a 1/1 molar ratio of heme/protein. Binding of heme to FurA from *Anabaena* was found to occur in a concentration-dependent and saturable manner at pH 8. Lysozyme, basic protein in the

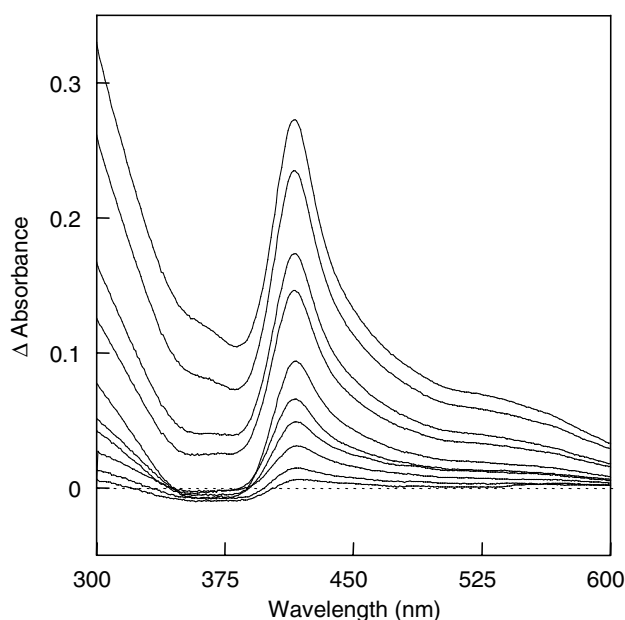


Fig. 1. FurA-heme complex. Difference spectra on addition of increasing amounts of FurA from *Anabaena* sp. PCC 7120 to a 2 μ M solution of heme. The experiment was performed at 25 $^{\circ}$ C in 50 mM Tris/HCl buffer at pH 8. Aliquots of a concentrated FurA solution were added to the heme solution, as described in Section 2.

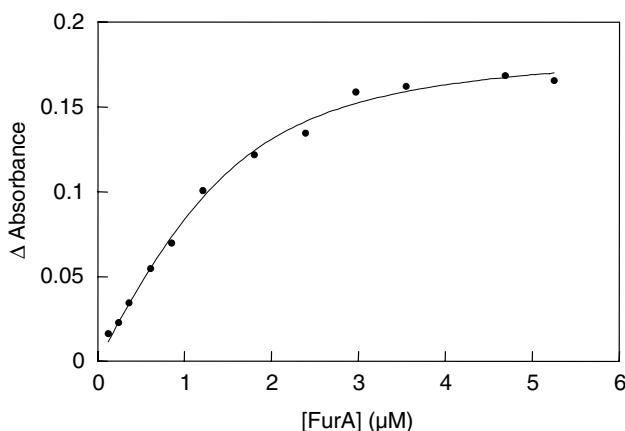


Fig. 2. Saturation curve of heme with FurA. Best fit of the change in absorbance (416 nm minus 382 nm) after addition of increasing amounts of FurA from *Anabaena* sp. PCC 7120 to a 2 μ M heme solution. Solid line corresponds to a 1:1 complex with a K_d of 0.4 ± 0.1 μ M.

range of size (14.3 kDa) of FurA (17.2 kDa), was used as a control of non-specific binding of heme to a protein. As shown in Fig. 3, addition of lysozyme to a solution of free heme did not alter the difference spectrum, excluding that possibility.

When the above mentioned measurements were performed with FurA at pH 5 instead of pH 8, a small perturbation in the differential spectrum was observed that seemed to correspond to a residual interaction more than a real heme–protein complex. In this way, cross-linking experiments with glutaraldehyde performed at pH 5 (data not shown) evidenced that the presence of heme did not affect the general status of the

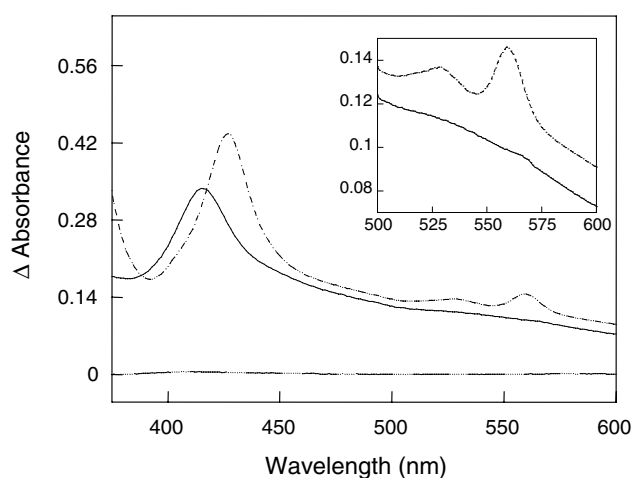


Fig. 3. Reduction of the heme–FurA complex. (—) Heme was mixed with FurA from *Anabaena* sp. PCC 7120 in 1:1 concentration ratio (3.5 μ M) in 50 mM Tris/HCl buffer at pH 8 and the difference absorption spectrum measured. (-----) Difference absorption spectrum of the previous heme–Fur complex after reducing with 5 mM final concentration of sodium dithionite. The shift in the Soret band from 416 to 427 nm and appearance of α (559 nm) and β (529 nm) bands (Fig. 3, inset) were indicative of the reduction of the complex. (---) Difference absorption spectrum for the control with lysozyme. As a control of non-specific binding of heme to a protein, heme was mixed with lysozyme in 1:1 concentration ratio (3.5 μ M) in 50 mM Tris/HCl buffer at pH 8, as indicated in Section 2.

protein population. FurA exists in vitro in several discrete oligomeric forms stabilized by hydrophobic interactions and disulfide bridges, the dimer being the prevalent one, although the protein is mainly a monomer at concentrations below 15 μ M [22].

Reduction of the heme–FurA complex with sodium dithionite at pH 8 resulted in a complex with a sharp Soret peak at 427 nm (Fig. 3) and well-resolved β - and α -bands (529 and 559 nm, respectively), which implied a low spin iron (Fig. 3, inset). Spontaneous oxidation was not immediate and the complex remained stable, preserving the measured spectrum fifteen minutes after reduction of the oxidized form.

In order to analyze the influence of the ionic strength on the heme–FurA complex formation, titration of a heme solution with increasing amounts of FurA was performed at pH 8 in the presence of 200 mM NaCl. We observed that the heme–protein interaction was not abolished when this salt amount was included (data not shown), although it was affected in some degree as the absorbance changes measured in the difference spectra were slightly smaller than the values obtained in the absence of salt. This suggested that, in vitro, electrostatic forces could be involved in the stabilization of the complex between FurA from *Anabaena* sp. PCC 7120 and heme, although they are not the major factor controlling the binding. At the assayed FurA concentrations (<15 μ M), the presence of hydrophobically stabilized oligomers, whose relative ratio can be altered in the presence of salt, is almost negligible [22].

3.2. K_d measurement

The complex formed between FurA from *Anabaena* sp. PCC 7120 and heme can be detected and quantified from the spectral changes that are observed in the visible absorption spectrum of the mixture (Fig. 1). Difference absorption spectra can

be used to measure the binding constant of a complex when it has a well defined stoichiometry.

Plotting the spectral changes observed between 382 and 416 nm at pH 8 vs. protein concentration (Fig. 2) yields a saturation curve. The binding constant (K_d) of the heme–FurA complex has been calculated to be $0.4 \pm 0.1 \mu\text{M}$ at that pH, assuming a 1:1 stoichiometry. FMN, prosthetic group found in flavoproteins and involved in biological oxidation and reduction processes, was used as control of non-specific binding of heme by FurA. When added to a 3.5/1 (FMN/FurA) ratio, the protein was unable to produce any perturbation on the FMN spectrum.

3.3. Influence of heme on in vitro DNA-binding activity of FurA from *Anabaena* sp. PCC 7120

The strength of the binding of heme to FurA from *Anabaena* sp. PCC 7120 suggested the possibility that heme affected the ability of the protein to bind DNA. In order to assess the influence of heme on the DNA binding activity of FurA from *Anabaena* sp. PCC 7120, we performed EMSA. In these experiments, we used two different DNA fragments. On the one hand, the above mentioned *isiB* promoter (Section 2), on the other, the promoter region of the *furA* gene, since the protein FurA from *Anabaena* sp. PCC 7120 seems to be autoregulated [21].

Both DNA fragments were incubated with FurA in different conditions and the resulting DNA-binding complexes were

analyzed by EMSA using conventional polyacrylamide gels (Fig. 4). Fig. 4A and B shows the results obtained for the *isiB* promoter and Fig. 4C and D for the *furA* promoter. As shown in Fig. 4A and C (lanes 2 and 3), in the absence of heme, the presence of metal slightly improved this activity in the case of *isiB* (Fig. 4A, lanes 2 and 3). Addition of heme to the binding reactions inhibited the FurA–DNA interaction with both promoters in a concentration-dependent manner (lanes 3, 4, 5 and 6 in Fig. 4A and C). Inhibition of the FurA–DNA interaction seemed to be produced as a consequence of the heme–FurA complex formation and not of a heme–DNA complex formation, at least in the case of the interaction of FurA with *P_{isiB}*. In fact, we analyzed spectrophotometrically if the presence of DNA affected the heme–FurA interaction. Addition of *P_{isiB}* to a heme solution did not perturb the spectrum of heme, suggesting that there was not significant interaction between both species in the assayed conditions (data not shown). After addition of purified recombinant FurA to the solution of heme previously treated with *P_{isiB}* at pH 8, a peak with maximum at 416 nm and similar absorbance to the one measured for the complex in the absence of DNA appeared in the difference spectrum (data not shown).

Heme at 1:8 (protein/heme) ratio almost completely inhibited the DNA-binding activity of FurA on the *isiB* promoter (Fig. 4A, lane 5), whereas there was a partial inhibition of

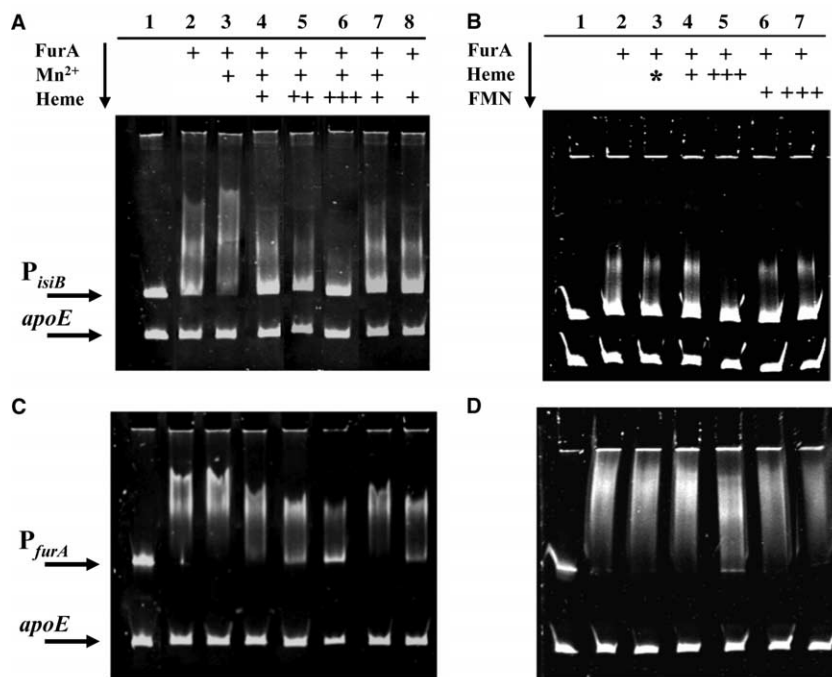


Fig. 4. Inhibition of in vitro DNA binding of FurA by heme. Results obtained for the binding (EMSA) of FurA to a 348 bp DNA fragment containing the *Anabaena* sp. PCC 7120 *isiB* promoter (A, B) and to a 389 bp DNA fragment containing the *Anabaena* sp. PCC 7120 *furA* promoter (C, D). Complexes were separated through 7% polyacrylamide gels. In A and C, 700 nM FurA was incubated with 20 ng of DNA, 40 mM KCl, 1 mM DTT, 0.05 mg/ml BSA, 5% glycerol, 0.1 mM MnCl₂ and heme. *apoE* (non-specific competitor DNA) was present in all the assays. Lane 1: free DNA (without FurA). Lane 2: reaction mixture lacking added metal and heme. Lane 3: reaction mixture without heme. Lanes 4, 5 and 6: reaction mixture with increasing amounts of heme (1:5, 1:8 and 1:14 FurA/heme concentration ratio, respectively). Lane 7: reaction mixture in the presence of 1:5 FurA/heme concentration ratio. Addition of DTT was made after addition of heme in order to compare to lane 4 where addition of DTT was made prior to the addition of heme. Lane 8: reaction mixture without metal in the presence of 1:5 FurA/heme concentration ratio. In B and D, 500 nM FurA was incubated with target DNA as previously described. Lane 1: free DNA (without FurA). Lane 2: reaction mixture without heme. Lane 3: reaction mixture without heme, but in the presence of an equivalent amount of heme solvent as in the 1:14 FurA/heme experiment. Lanes 4 and 5: reaction mixture with increasing amounts of heme (1:5 and 1:14 FurA/heme concentration ratio, respectively). Lanes 6 and 7: reaction mixture with increasing amounts of FMN (1:5 and 1:14 FurA/FMN concentration ratio, respectively).

DNA binding by FurA on its own promoter at this FurA/heme ratio (Fig. 4C, lane 5). We have observed that the excess of heme necessary to inhibit the DNA binding activity of FurA can be reduced when albumin is absent in the reaction mixture, since this is a protein that binds heme very tightly. In fact, a reported strong albumin-heme complex had a K_d of 5×10^{-7} M [26]. However, we have kept this reagent in the binding buffer because its presence stabilizes the protein [27] and provides the conditions of crowding or confinement peculiar to the reaction in its physiological milieu [28].

Addition of 1,4-dithiothreitol (DTT) to the reaction mixture is necessary to avoid the presence of FurA covalent oligomers and to favor the protein–DNA affinity [22]. Since DTT can reduce heme as well, the sequence of addition of both reagents was analyzed concluding that it did not affect significantly the final result (lanes 4 and 7 in Fig. 4A and C). In consequence, the fact that reduction of heme iron to the ferrous form (Fe^{2+}) by DTT did not alter the current inhibition efficacy indicates that the electronic state of the iron center is not crucial. When competition between heme and metal for FurA binding was established, we observed that the presence of metal in the reaction mixture improved the yield at least in the case of the *furA* promoter (Fig. 4C, lanes 4 and 8). This effect was negligible in the case of the *isiB* promoter (Fig. 4A, lanes 4 and 8), suggesting a different strength in the interaction of the heme–Fur complex with both promoters.

Heme is a hydrophobic molecule that dissolves very poorly in an aqueous medium, therefore the preparation of the heme solution was made using an organic solvent. Taking into account that most of the proteins precipitate in organic solvents, it raises the question whether the observed inhibitory behavior is a consequence of the inactivation of the protein in the presence of the solvent enclosed in the pool of heme. Control assays performed using the same volume of organic solvent in the reaction mixture but in the absence of heme concluded that the observed inhibitory behavior was only a consequence of the presence of heme in the reaction mixtures (Fig. 4B and D, lane 3).

In the same way, we performed control experiments to confirm that the reduced binding activity of FurA was specifically caused by the presence of heme. On the one hand, we replaced heme by FMN in the reaction mixture and obtained no effect on the binding activity of FurA at the assayed concentrations (Fig. 4B and D, lanes 6 and 7). On the other hand, a band shift assay was performed with the protein–DNA complex NtcA-*P_{glnA}* from *Anabaena* sp. PCC 7120 (Fig. 5). NtcA is a DNA binding protein that acts as a global regulator of nitrogen homeostasis in cyanobacteria by promoting the expression of various genes important in nitrogen metabolism [29]. Results did not show any alteration of the DNA-binding activity of this protein in the presence/absence of heme in the assayed conditions (Fig. 5, lanes 4 and 5). This control indicated that the protein–DNA interaction was specifically affected by heme.

4. Discussion

Proteins involved in heme–protein complex formation show commonly high affinity for this cofactor. The use of spectroscopic methods to measure the affinity of both species, when the fraction of reversible dissociated heme molecules is too

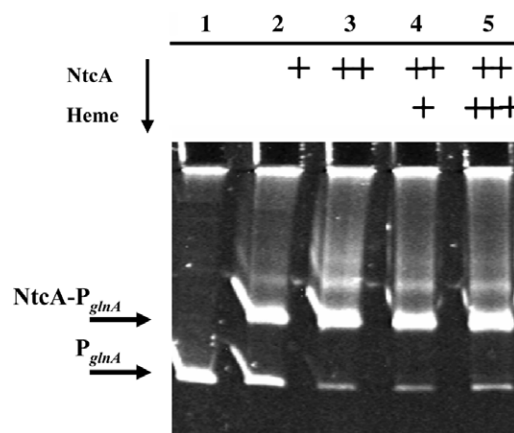


Fig. 5. Binding assay of NtcA to DNA in the presence of heme. Results obtained for the binding (EMSA) of NtcA to a 489 pb DNA fragment containing the *Anabaena* sp. PCC 7120 *glnA* promoter. Complexes were separated through 7% polyacrylamide gels. Lane 1: free DNA (without NtcA). Lanes 2 and 3: reaction mixture with increasing amounts of NtcA (400 nM and 800 nM, respectively) in the absence of heme. Lanes 4 and 5: reaction mixture with 800 nM NtcA and increasing amounts of heme (1:5 and 1:14 NtcA/heme concentration ratio, respectively).

small, can become very difficult [30]. Nevertheless, these methods have been used to determine K_d values for heme–protein complexes in the micromolar range [12,31,32]. Using differential spectroscopy, we have been able to identify a complex between heme and FurA from *Anabaena* sp. PCC 7120. Our results show that the protein exhibits considerable affinity for heme, rendering a heme–protein complex which causes partial inhibition of its ability of binding to promoters *in vitro*.

The binding of a relatively large molecule to a protein is the result of several simultaneous interactions that favorably contribute to the final complex. According to our results and the analysis of the FurA amino acid sequence, we can speculate in the nature of the residues that take part in the binding of heme to FurA. The heme-binding site of FurA from *Anabaena* sp. PCC 7120 seems to involve some histidine residue. This is supported by the data obtained from the complex formation at two different pHs and the red shift displacement in the heme Soret band, typical for globins and cytochromes, observed after binding of this compound to FurA. However, the contribution of some cysteine residue to the complex formation should not be excluded. FurA from *Anabaena* sp. PCC 7120 contains five cysteine residues in its amino acid sequence, where Cys 141 is placed in a CP amino acid sequence located at the C-terminus of the protein (residues 141–142). This domain has been identified as responsible for metal binding and dimerization in Fur proteins from *E. coli* [33] and *Pseudomonas aeruginosa* [34]. CP motifs have been proposed as commonly used structural elements for non-covalent binding of heme to various proteins, where the cysteine is critical for binding and the proline aids the affinity of binding [7]. Several functions could be attributed to this motif in FurA from *Anabaena* sp. PCC 7120. On the one hand, it could act as secondary binding site. In fact, the presence of different heme binding sites in the same protein molecule has been already reported in the case of the bacterial iron response regulator (Irr) protein [5]. It could contribute as well to the complex formation by magnifying the

concentration of heme around the binding site or also properly positioning the hydrophobic group to form the complex, as has been proposed for heme lyases [8]. Although we determined a 1:1 stoichiometry for the FurA-heme complex, the possible contribution of the CP motif to the heme–FurA interaction is currently being tested. It is worth to note that the appearance of a CP motif is a characteristic of the amino acid sequence of most Fur proteins found in database searches in cyanobacteria, photoautotrophic prokaryotes that perform oxygenic photosynthesis. In the case of the cyanobacterium *Anabaena* sp. PCC 7120, this motif is present in two Fur family members (FurA and FurB) out of the three identified to date [35]. This motif is usually absent in the reported Fur homologs from other origins.

Fur is a constitutive bacterial iron sensor/regulator whose DNA-binding activity is, in most cases, dependent on iron [13]. Our results show that the binding of heme to FurA from *Anabaena* sp. PCC 7120 affects specifically the affinity of this repressor for DNA in vitro. In particular, its ability to interact with two DNA targets, the *isiB* and *furA* promoters, diminishes in a concentration dependent fashion, even in the presence of metal. According to our observations, although heme impairs the DNA-protein complex formation with both promoters when Mn^{2+} is in the reaction mixture, it does not prevent it completely even at high concentration of heme in the case of *P_{furA}* (Fig. 4C, lanes 3 and 6). In fact, metal is necessary to improve the yield of the complex formation in the presence of heme (Fig. 4C, lanes 4 and 8). This agrees only partially with previous observations of Smith et al., suggesting that heme most likely binds at or near the same site as Mn^{2+} rather than to an independent site, since binding of heme to Fur from *E. coli* prevents Mn^{2+} binding in vitro [12]. From our results, we cannot infer that both species compete for the same site although it seems that the binding of one of them affects the affinity of the complex for the other. This point could be explained as a consequence of a conformational change produced within or around the metal binding site after binding of heme that hinders the DNA-complex formation in the presence of metal.

The two assayed promoters correspond to genes that encode proteins expressed in different iron conditions in the medium. The *isiB* is a gene that encodes flavodoxin, a protein that in vivo is completely repressed by FurA in an iron-replete medium as proves the presence of negligible amount of transcript in these conditions [36]. The *furA* gene encodes the FurA repressor, a constitutive protein whose synthesis slightly increases in the absence of iron in *Anabaena* sp. PCC 7120 (unpublished results). According to our results, we can speculate in the possibility that in vivo heme might modulate the binding of FurA to DNA, even in the presence of metal, lowering the Fur–operator interaction. The differences observed in the ability of heme to inhibit the FurA–DNA interaction with both promoters indicate that *isiB* is derepressed to lower concentration of heme than *furA*. This is in accordance with the K_d measured for the interaction of the protein with both promoters, around 100-fold higher for the *isiB* promoter than for the *furA* one (unpublished data).

In this work, binding assays have been performed in the presence of free heme, a potential cause of oxidative stress inside the cell [37]. Recently, it has been reported that transcription of major iron regulated genes, such as *isiA* and *isiAB* in the cyanobacterium *Synechococcus elongatus* PCC 7942 is

induced by oxidative stress [38]. At the same time, studies performed by Zheng et al. [39] have demonstrated that regulators of the *E. coli* responses to oxidative stress, OxyR and SoxRS, activate the expression of Fur. In particular, a transcript encoding Fur is induced by hydrogen peroxide in a wild type strain. Therefore, the in vitro inhibitory effect of heme on the DNA–FurA interaction vs. both promoters (*furA* and *isiB*) that we report in this study is in accordance with these results.

The possible role of heme as FurA–DNA binding modulator in the presence of the metal co-repressor could reside in the transient character of the heme–FurA interaction, demonstrated by the fact that the protein is purified as a free heme species [22] and by the strong heme–protein association. In fact, the K_d values measured in *E. coli* for the Mn^{2+} Fur complex (85 μM) [40] and the heme–Fur one (<1 μM) [12] indicate that a smaller concentration of heme than Mn^{2+} is sufficient to induce the complex formation.

Inhibitory effects on the in vitro DNA-binding activity of Fur exerted by intermediates distinct from heme at micromolar concentration have been reported. This is the case of NO, an exogenous molecule which when used in threefold excess can switch off Fur binding to the aerobactin promoter in *E. coli* [41].

If the in vitro experiments are physiologically relevant, *isiB* and *furA*, as well as probably other iron-repressible genes in cyanobacteria, would be modulated by heme, whose presence would affect the expression of those genes. This behavior has been first described in bacteria for the *hmuO* gene, a member of the DtxR regulon that is required for the utilization of heme and hemoglobin as iron sources by *Corynebacterium diptheriae* C7 [42]. In this case, expression studies with a *hmuO-lacZ* fusion construct in a *dtxR* mutant of *C. diptheriae* C7 and in a *hmuO* mutant of *C. diptheriae* HCl provided evidence that transcription of the *hmuO* promoter is repressed by DtxR and iron and activated by heme. Ongoing experiments aim to elucidate critical residues for heme binding to FurA from *Anabaena* sp. PCC 7120 and the possible involvement of this interaction in the oxidative stress metabolism.

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