The Competition Between Zn(II) and Iron(II) in Binding E.coli Fur and the effect on structural and conformational changes of Fur upon DNA binding: an MD study

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Binding of the Zn^{2+} ion to ferric uptake regulation protein from *E. coli* and the competition with Fe^{2+} binding: a molecular modeling study of the effect on DNA binding and conformational changes of Fur

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Abstract The three dimensional structure of Ferric uptake regulation protein dimer from *E. coli*, determined by molecular modeling, was docked on a DNA fragment (iron box) and Zn^{2+} ions were added in two steps. The first step involved the binding of one Zn^{2+} ion to what is known as the zinc site which consists of the residues Cys 92, Cys 95, Asp 137, Asp141, Arg139, Glu 140, His 145 and His 143 with an average metal-Nitrogen distance of 2.5 Å and metal-oxygen distance of 3.1–3.2 Å. The second Zn^{2+} ion is bound to the iron activating site formed from the residues Ile 50, His 71, Asn 72, Gly 97, Asp 105 and Ala 109. The binding of the second Zn^{2+} ion strengthened the binding of the first ion as indicated by the shortening of the zinc-residue distances. Fe^{2+}, when added to the complex consisting of $2Zn^{2+}$/Fur dimer/DNA, replaced the Zn^{2+} ion in the zinc site and when a second Fe^{2+} was added, it replaced the second zinc ion in the iron activating site. The binding of both zinc and iron ions induced a similar change in Fur conformations, but shifted residues closer to DNA in a different manner. This is discussed along with a possible role for the Zn^{2+} ion in the Fur dimer binding of DNA in its repressor activity.

Keywords Fur · Iron box · DNA binding · Zinc site · Repressor proteins

Introduction

Escherichia coli Fur (ferric uptake regulation) is a repressor protein. Under high Fe^{2+} concentration Fur acts as a repressor to the transcription of iron chelators and several other genes [1–5]. It has been established that Fur binds DNA as a dimer, especially to the iron box (a 19 bp consensus site with the sequence 5′-GATAATGATAATCATT AT-3′). It has been suggested that Fur recognizes an area consisting of three adjacent hexamers made up of units of sequence 5′-GATAAT-3′ [6, 7]. Fur was reported to bind a 13-mer sequences containing inverted repeats of 5′-GATAAT-3′ as two overlapping dimers positioned on opposite faces of the DNA helix in a similar manner to the DtxR binding [6–8]. Variation in Fur affinities from the consensus "iron box" was also reported. In these reports Fur binds DNA on unrelated target sites with different affinities and Fur polymerization was found to take place in addition to DNA conformational changes to aid binding [9].

In addition to the naturally occurring corepressor Fe^{2+}, Fur is activated by Mn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} and to a lesser extent by Zn^{2+} [2, 10–12]. There are reports of the presence of Zn^{2+} ion bound to the Fur dimer as a structural support [10, 11, 13–17]. Fur has recently been considered a zinc protein with one Zn^{2+} strongly bound to Fur in a Zn-site [11, 14, 15, 18, 19]. Contrary to EC Fur which was reported to bind DNA in the presence of a Structural Zn^{2+} [14, 15], zinc ion was reported to be insufficient in V. A. Fur to activate Fur binding to DNA [18]. The crystal

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structure of P. A. Fur has been resolved; it provides the first structural information on a member of the Fur family at the molecular level [20]. The structure contains the zinc ion in the two metal-binding sites. On the other hand, both B. J. Fur and P. A. Fur were found not to contain the Zn^{2+} ion in vivo, but both responded to Fe^{2+} in vitro and were able to demonstrate the presence of zinc in E. C. Fur [14, 21]. Bearing in mind that the Zn^{2+} ion could activate E. C. Fur in vitro and to a lesser extent in vivo [10, 11, 15, 17–20] it has been worthwhile testing any changes in conformation that the Zn^{2+} ion induces when added to the Fur DNA complex.

In this work we employ molecular modeling techniques to investigate the binding of Zn^{2+} ion to E. C. Fur and explore the possible effect it would have on activating the Fur dimer to bind DNA "iron box". The study is based on introducing one Zn^{2+} ion per Fur dimer followed by another ion and studying the Zn^{2+} binding, conformational changes induced by the Zn^{2+} ion and the possible effect it would have on Fur/DNA binding. Apo Fur activity was explained as due to the presence of a structural Zn^{2+} ion [10, 15, 17]. there are reports which state that Structural zinc is necessary yet insufficient for DNA binding [18]. In a recent report on P. A. Fur [22] the structural zinc was found to have lower stability constant ($K_A = 3.2 \times 10^4\ M^{-1}$), while the sensory binding site showed greater affinity towards Zn^{2+} with K_A value of $5.7 \times 10^6\ M^{-1}$). This result disagrees with the exceptional strength of the zinc binding in the structural zinc site [11, 15].

In view of all these findings, we considered it worthwhile to investigate the possibility of replacing Zn^{2+} ion by Fe^{2+} and study the effect on Fur dimer and its DNA binding. The possibility of the other metal ion to bind the activating site was studied previously and Co^{2+} was found to be incapable of replacing the Zn^{2+} ion in the structural zinc site [23], in a recent report Zn^{2+} could replace Co^{2+} in the Fur structural zinc site [22]. In view of these reports, we studied the possibility of Fur binding to one Zn^{2+} ion and one Fe^{2+} ion at the same time and the possible replacement of Zn^{2+} in both Fur sites by Fe^{2+}.

In a previous report [24] we found that Fur dimer binds two Fe^{2+} ions, and the increase in Fe^{2+} concentration enhanced both conformational changes and DNA binding of Fur dimer. In the present work we study the fine tuning of Fur dimer and the nature of Fur DNA contacts and effects of Zn^{2+} and Fe^{2+} on the process.

The metal ion sites on the Fur dimer are discussed in order to reveal information about the structure function relationship of E. C. Fur and the role of metal binding in the activation mechanism. In addition, we discuss the possibility of Fe^{2+} acting as antagonist to an inhibitor of Fur DNA binding (i.e., another metal ion competing for the Fe^{2+} binding site) [17].

Computations

All the molecular modeling simulations were performed using Amber 9 package [25, 26] and docking was performed using AUTODOCK 2.4 [24]. All calculations were performed on a Dell Precision 490 workstation supplied with two dual core-3.2 GHz CPU processors, 256 MB NIVIDA Quadro Fx 3450 graphic card, and 4 GB ram, running RED Hat Linux platform.

Homology modeling of Fur protein

The Fur model was based on homology modeling and multiple alignment with known crystal structure [20, 24] pdb reference 1 mzb. The known Fur sequence (from *E. coli*) was submitted to different servers in order to predict the three-dimensional structure. Several templates for Fur protein were generated while the sequence with high similarity served as a reference sequence. The superposition of each atom was optimized by maximizing *Cα* in the common core while minimizing their relative mean square value deviation (RMSD) at the same time. Spare part algorithm was used to search for fragment that can be accommodated into the frame work of the Brookhaven protein Data Bank (PDB). The coordinates of central backbone atoms (N, O, and C) were averaged. The side chains were added according to the sequence identity between the model and the template. Idealization of the geometry for bonds and removing any unfavorable non-bonded contacts was performed using AMBER9 package, which is an improved version of Amber7 that has QMM/MM facility and can also calculate the minimum energy for α helices with an improved library. Energy minimization was performed, all hydrogen atoms were added and the apo Fur was subjected to a refinement protocol with constraints on the Fur structure gradually removed. Hundred steps of steepest descent, followed by 300 steps of conjugate gradient algorithm were applied during energy minimization. The energy minimization process on the apo Fur model was performed in H_2O as solvent and nine Na^+ ions were added to the model to neutralize the system. From the output which gave a minimum energy of the structure after 10 ps we concluded that this structure reached the desired global minima.

Building the Fur dimer

Two molecules of the previously determined structure for the apoFur monomer were docked on each other using AUTODOCK, and the best docking sites were predicted. Monte Carlo (MC) simulated annealing (SA) algorithm was used for exploring the Fur configuration by a rapid energy evaluation technique using a grid-based molecular

affinity potential. The energy of interaction, affinity, and the grid for electrostatic potential were evaluated using the Poisson-Boltzmann finite difference method and were assigned to each other.

Docking apo Fur dimer onto iron box

AMBER 9 suite program (Nucgen) was used to build the Cartesian coordinates for canonical B- model of the iron box (5′-GATAATGATAATCATTATC-3′) which is the proposed recognition site of the Fur on the DNA. The right-handed B-DNA duplex conformation was used for the model. The Fur-dimer was docked on the iron box using AUTODOCK program. The resultant model was energy minimized in order to refine the Fur dimer-DNA complex.

Adding Zn^{2+} and Fe^{2+} ions

The parameters files for the zinc and iron were prepared, refined and inserted into the AMBER9 library file. The Zinc parameters are present in the Amber library while we had to prepare the iron parameters file (See iron parameters in supplementary file) and place it in the Amber library. The first scenario was using $1Zn^{2+}$ ion per Fur dimer-DNA complex in water environment and adding Na^{+} ions. Explicit solvent model TIP3PBOX water was used as solvent. The model was solvated with 10 A water cap from the center of mass of the ligand. Energy minimizations were carried out at 300 k. In the second scenario, energy minimization was repeated using $2Zn^{2+}$ per Fur dimer-DNA model. Third scenario, $2Zn^{2+}$ and $1Fe^{2+}$ were added to the model and last scenario $2Zn^{2+}$ and $2Fe^{2+}$ were added and the same calculation method was applied.

Results and discussion

The three dimensional structure of the ferric uptake regulation protein from *E. coli* (Fur E. C.) was determined using homology modeling and energy minimization. The Fur monomer consists of turn-helix-turn motif on the N-terminal domain, followed by another helix-turn-helix-turn motif, and two β strands separated by a turn which forms the wing. The C-terminal domain, separated by a long coil from the N-terminal, and consisting of two anti parallel β strands, and a turn-helix-turn-helix-turn motif.

Residues in central domain were found to aid the dimer formation, residues 45–70 as evident in the calculated distances; this region is rich in hydrophobic residues. Most interactions occur between residues Val(55), Leu(53), Gln(52), Glu(49) and Tyr(56) with closest contacts occurring at residues 49–56. These residues are part of an α-helix (α_4) near the N-terminal.

Effect of DNA on apo Fur dimer and conformational changes induced by DNA in the Fur dimer

There are three major contact areas close to DNA on the Fur dimer before adding any metal ion. These areas consist mainly of hydrophobic residues (see Fig. 1 in supplements). The first area (a) near the N-terminal domain consists of Ala11, Gly12, Leu13 and Pro18 (hydrophilic) (part of α1), while the second area (b) consists of the residues His86, His87, His88, Asp89 and His90 (part of the coil T7) [24]. The third area (c) near the C-terminal domain consists mainly of hydrophilic residues, Asp137, Arg139, Glu140, Asp141, with the exception of His132, His143 and His145 (α6). Addition of the Fe^{2+} ion shifts these residues

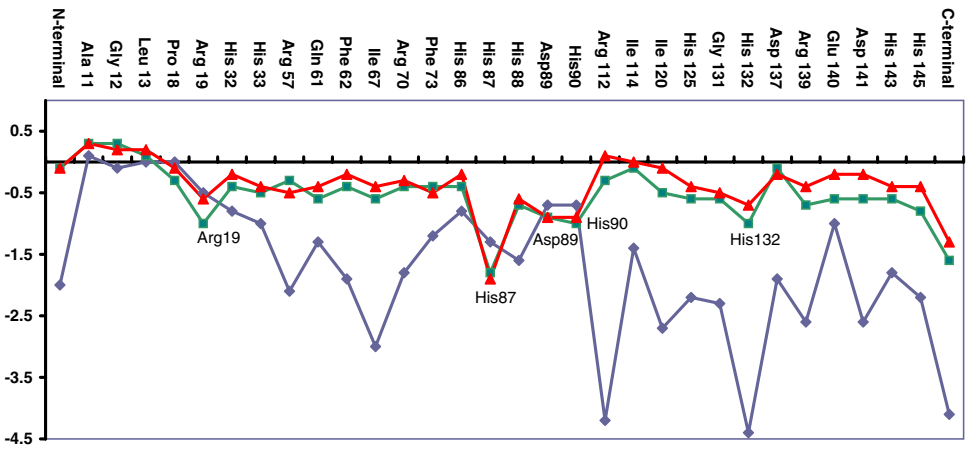

Fig. 1 Effect of metal ions on shifting the Fur dimer residues closer to DNA. Plots were taken as difference between the position of apo Fur dimer with no metal ion present and those of Fur/DNA with metal ion present (M^{2+}/Fur/DNA–ApoFur/DNA): *Red triangle* (▲) after adding $1Zn^{2+}$. *Green squares* (■) after adding $2Zn^{2+}$ ions. *Blue diamond* (◆) after adding $4Fe^{2+}$ (distances measured were between Fur residues and closest contacts on DNA and the Y axis units are in Å), For detailed values and numbers see supplementary material

closer to the DNA as evident in the negative shifts relative to apo Fur/DNA position in (Fig. 1).

A significant change in Fur dimer conformation took place upon docking the apo Fur dimer onto the DNA, i.e., DNA itself induces conformational changes in Fur dimer (see Fig. 2a). This is evident in the increase in separation between Fur subunits at positions Val25, Pro29, Glu85, helix5 and at the contact positions Ala53–Ile 107 and Thr54–Glu108. At the same time Fur subunits close down on each other at positions α1, α2, α3, α4, and α6. Indeed, Coy [27] reported that Fur binds DNA independently of the metal ion and explained the role of the metal ion as to induce high affinity binding to the DNA.

Effect of metal ion binding

E. C. Fur protein is activated by Zn^{2+} ion. The activity of apo Fur was explained as due to the presence of Zn^{2+} ion and was given a structural role [10, 15, 17–19, 22, 23]. We have established that Fur dimer changes conformation upon DNA binding and the process was sensitive to Fe^{2+} concentration [24]. Fe^{2+} enhanced the DNA binding and, at higher concentrations it mediated the DNA binding by bond formation to the AT region of DNA. Fe^{2+} was found to bind in two major sites (cavities) on the Fur dimer. To unravel the effect of Zn^{2+} on the conformational changes of Fur and its DNA binding, we performed the modeling in the presence of Zn^{2+} ions at various concentrations, then we studied the effect of Fe^{2+} on the Zn^{2+} bound Fur dimer in the presence of DNA, and the overall effect on the Fur conformation and its DNA binding.

Some residues appeared to be more sensitive to the metal ion than others as can be seen in (Fig. 1). The most significant shifts in the case of adding Fe^{2+} were for residues His86, His87, His88, His132, His125, His143, His145, Arg112, Arg139, Asp137, Asp141, Ile114, Ile120 and Glu140 on the C-terminal domain. The significant shifts on the N-terminal domain were for Arg57, Phe62, Ile67, Arg70 and Phe73. It was also clear that residues in the middle area of the Fur were shifted to a lesser extent

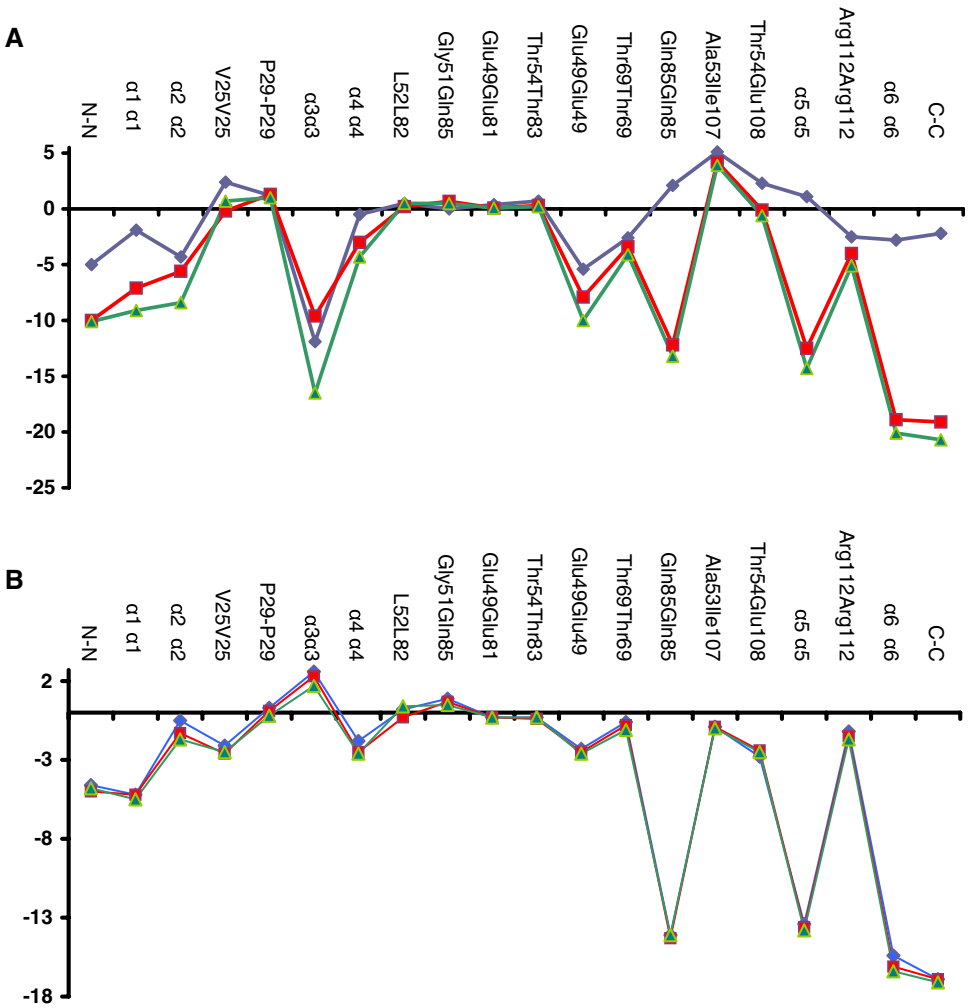

Fig. 2 **a** Conformational changes in the Fur dimer induced by: DNA (◆), by Zn^{2+} (■) and by Fe^{2+} (▲). Conformational changes presented as residue–residue distances in Å. Plots were taken as difference (Fur dimer/DNA–apoFur dimer) *grey diamond* (◆). ($2Zn^{2+}$/ Fur/DNA–apoFur dimer) *red squares* (■). ($8Fe^{2+}$/ Fur dimer/DNA–apoFur dimer) *Green triangle* (▲). (changes were measured between residues on Fur monomers in units of Å, Y-axis), For more details see supplementary material. **b** Conformational changes induced by adding Fe^{2+} and Zn^{2+} in various concentrations to the Fur dimer DNA complex. Differences were taken between M^{2+}/Fur/DNA complex and apoFur/DNA complex: [$4Fe^{2+}$/Fur/DNA–Fur/DNA] (▲). [$2Zn^{2+}$/Fur/DNA–Fur/DNA] (■). [$1Zn^{2+}$/Fur/DNA–Fur/DNA] (◆). Distances on the Y axis are in Å. Closest residue contacts were chosen for representation

than residues on both terminals. Also the C-terminal residues shifted to a greater extent towards DNA than the N-terminal residues. Some of these residues are well known in taking part in helices which bind DNA, especially Arg, Asp, Glu and Ileu.

The effect of adding Zn^{2+} ion (see Fig. 1) produced small shifts in residue positions towards DNA compared to those produced by Fe^{2+}. The most important shift was for His87 followed by Asp89, His90 and His132. Both Arg19 and His33 also shifted closer to the DNA. It is noticeable that the sensitivity of residue position to the Zn^{2+} concentration was considerably less than that for Fe^{2+}. The Zn^{2+} ion was given the role of inducing and preserving the Fur dimer three dimensional structure [2, 10, 15, 17, 19, 22]. Residues on the Fur dimer can be classified according to their sensitivity to metal ions as follows:

Residues sensitive to both Zn^{2+} and Fe^{2+}(on the C-terminal domain)	Residues which appeared to have more sensitivity to Zn^{2+} more than Fe^{2+}	Residues which has stronger sensitivity to Fe^{2+}
His145, Asp141, Glu140, Arg139, His132, Arg112, Ile120	His87 > Asp89 > Arg19 > His90	His125 (largest shift) Asp137, His143, Ile114, His88, His86, Phe73, Arg70, Ile 67, Phe62, Arg57, His32, His33

Conformational changes induced in the Fur dimer by Zn^{2+} ion compared to those induced by Fe^{2+}

The effect of adding the first Zn^{2+} and second Zn^{2+} ions is shown in (Fig. 2a, b). The conformational changes induced by the zinc ion can be measured either relative to apo Fur dimer conformation in absence of DNA (Fig. 2a) or relative to it in presence of DNA, (Fig. 2b). The zinc ion brings the Fur subunits closer together in the following manner. The $\alpha1-\alpha1$ separation showed great sensitivity to Zn^{2+} ion in a similar manner to the effect of Fe^{2+}. All other residues and helices moved closer together except for $\alpha3-\alpha3$ separation which increased by 2.6 Å after adding the first Zn^{2+} ion. The second Zn^{2+} ion did not have a considerable effect. The dimerization region (Leu52–Leu82, Gly51–Glu85, Glu 49–Glu81) did not show considerable change in separation with Zn^{2+}.

The addition of metal ions brings the Fur subunits closer together even further. The role of $\alpha1$ and $\alpha2$ agrees with what was reported as evident in the large shift in separation which took place upon addition of the Zn^{2+} ion [17]. It is worth noting that the $\alpha1$ and $\alpha2$ shift showed great sensitivity to the metal ion concentration in the case of Fe^{2+} as well.

All residues and helices moved closer together upon addition of the first Zn^{2+}, except for the $\alpha3-\alpha3$ distance which increased by 2.6 Å after adding the first Zn^{2+}. Increase in Zn^{2+} concentration did not have a drastic effect on the $\alpha3-\alpha3$ separation (this is part of the HTH DNA binding domain [24, 27, 28]). Addition of more Zn^{2+} ions produced less change in the Fur dimer conformation compared to that induced by adding high Fe^{2+} concentration [24]. The dimerization region (leu52–leu82, gly51–gln85, glu49–glu81, thr54–thr83) did not show considerable change in position upon addition of Zn^{2+}, as the case was for Fe^{2+}. These observations are in agreement with earlier results reporting the secondary structure remained intact on the N-terminal domain when Zn^{2+} ion was added to Fur except for $\alpha1$ [17]. The C-terminal domain of the Fur dimer is well structured as was proven by NMR studies [17]. In general, the trend in induced conformation caused by both Zn^{2+} and Fe^{2+} is almost identical especially in the C-terminal domain (Fig. 2a, b).

In conclusion, Fe^{2+} increases the $\alpha3-\alpha3$ separation while excess Fe^{2+} decreases this separation to the extent that they became closer than in apo Fur dimer /DNA. Considering the fact that $\alpha3$ is part of the HTH motif, this finding agrees well with the role of iron in enhancing the DNA binding of Fur by closing down on the DNA [1, 3, 24, 27]. On the other hand, the first portion of Zn^{2+} increases this separation, but addition of more Zn^{2+} could not induce a greater shift in $\alpha3-\alpha3$ like the one produced by Fe^{2+}.

Competition between Fe^{2+} and Zn^{2+} for binding sites on the Fur dimer

The first Zn^{2+} ion, when added to the Fur dimer/DNA complex occupied the first site (Site 1 or the zinc structural site), which is the cavity formed from Cys92, Cys95, Glu140, His145, and His143 in addition to Asp137 (3.1 Å) Asp141 (3.0 Å) and Arg 139 (4.1 Å) (see Figs. 3, 4; Table 1) .This result agrees well with EXAFS studies which showed that zinc ion is coordinated by two cysteins, one aspartate and one histidine [10, 15], the Zn^{2+} binding carries more resemblance to that reported for P.A. Fur [22]. The second Zn^{2+} ion occupied the second site (site 2 or iron activating site) (see column three Table 1) and strengthened the binding of Zn^{2+} to the first site. This is evident in the metal ion-amino acid distances in Table 1 and Fig. 4. The M–N is 2.1 for sensory site and 2.4 for zinc structural site while M–O distance ranges from 2.1 to 2.3 Å for sensory site and 3.0 Å for structural zinc site taken for closest Asp residues which is close to what was found for Zn^{2+} binding to P. A. Fur [20] with a difference in residue locations yet consisting mainly of His, Asp and Glu residues. The differences are due to structural and functional differences between P. A. Fur and E. C. Fur. However, we

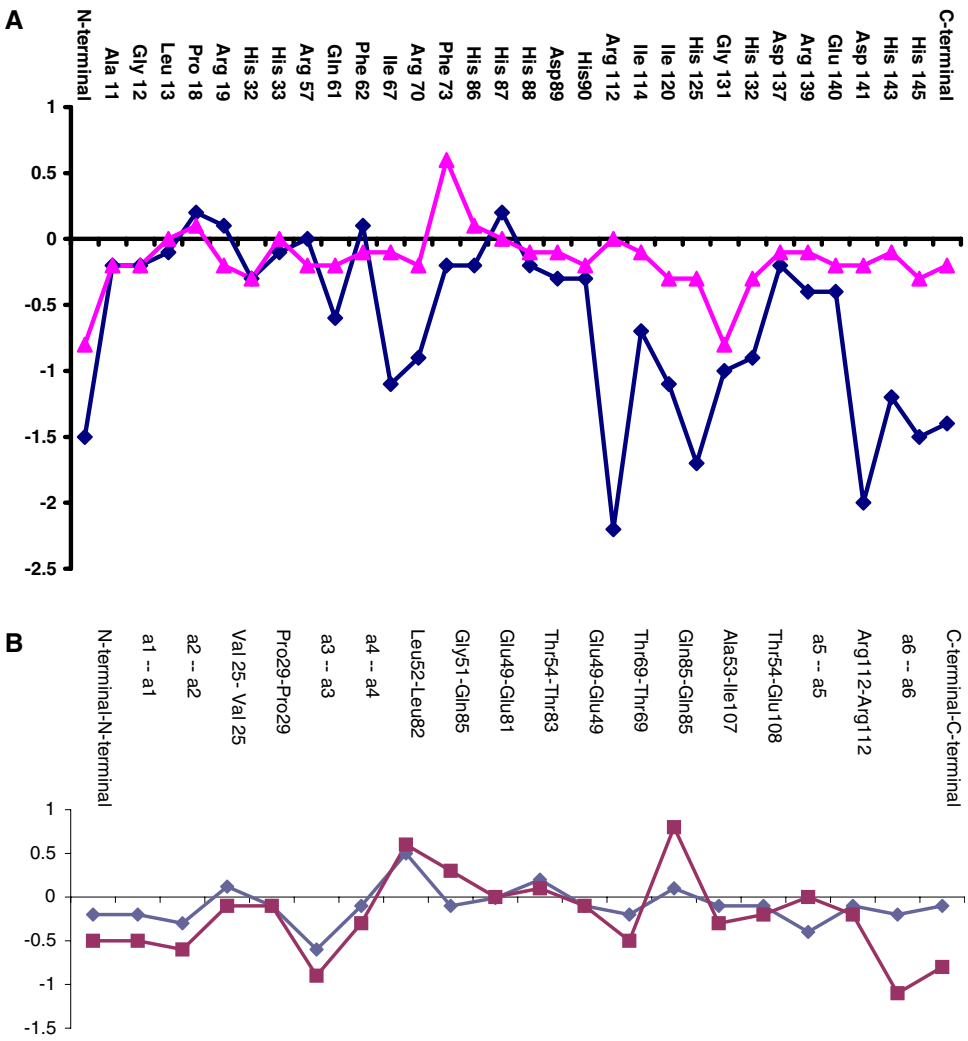

Fig. 3 **a** Effect of adding Fe^{2+} to the Zn^{2+}/Fur/DNA complex. The shift in distances measured in Å of Fur dimer residues from DNA upon adding 1Fe $^{2+}$ to the [2Zn^{2+} /Fur/DNA] complex (*pink triangle*▲). After adding 2Fe $^{2+}$ (*navy blue diamond* ◆). The shifts were measured relative to the [2 Zn^{2+}/ Fur/ DNA] complex positions. Y axis units in Å. **b** The effect induced on the Fur dimer conformation upon adding Fe^{2+} to the Zn^{2+}/Fur/DNA complex(changes measured by residue-residue separation between monomers in the Fur dimer) by adding 1 Fe^{2+} (*blue diamond* ◆). After adding 2Fe^{2+} (*brown square* ■) to the [2Zn^{2+} Fur/DNA]. 2Zn^{2+}/Fur dimer/DNA was taken as position zero. All units in Å and closest contacts were chosen for representation

did not find any zinc at close proximity to Cys132 or Cys137, in agreement with previous experimental reports for P. A. Fur [17, 22] .

From the metal ion-residue distances (Fig. 4e; Table 1) it appears that the binding of the Zn^{2+} ion is weaker than the Fe^{2+} binding. This is expected for Zn^{2+} ions, since it does not have the crystal field stabilization energy as in Fe^{2+} which has a considerable CFSE, also the possibility of H_2O mediated metal residue binding cannot be ruled out.

The Zn^{2+} ion induced a considerable conformational change in the Fur dimer as previously discussed, at the same time it shifted the amino acid residues closer to DNA but less than in the case of Fe^{2+}. However, the trends in conformational changes in both iron and zinc systems are similar.

The Fe^{2+} ion could replace the Zn^{2+} ion in the Fur dimer, as shown in Fig. 4 and Table 1. The first added Fe^{2+} replaced the Zn^{2+} ion in the Zn^{2+} site and resulted in dissociation of the Zn^{2+} ion and the second Fe^{2+} replaced the second Zn^{2+} ion in the iron activating site. Binding of Fe^{2+} to Fur dimer enhanced the Fur DNA binding and induced large conformational changes in the Fur dimer. It seems that the Zn^{2+} ion cannot produce the amount of shift in distances of amino acid residues in order to bring them closer to DNA, except for His87. The close areas of apo Fur to DNA become closer but the change is very small compared to that caused by Fe^{2+}, see (Fig. 1).

In order to compare the Fur dimer binding affinity to both zinc and iron binding energy calculations of metal ions to the Fur sites were performed. The results showed that the zinc binding affinity for the structural zinc site 28 μM and for the second sensory site was 20 μM (with an error of 5–7%), this result is in parallel with a reported experimental result for P. A. Fur [22]. Zinc binding requires a C-terminals basic region to stabilize its interaction. Since we add iron metal to the Fur/protein which induced conformational changes in the C-terminal and as consequence affected the zinc-binding stability because the stabilization of zinc binding is dependent on the C-terminal residues.

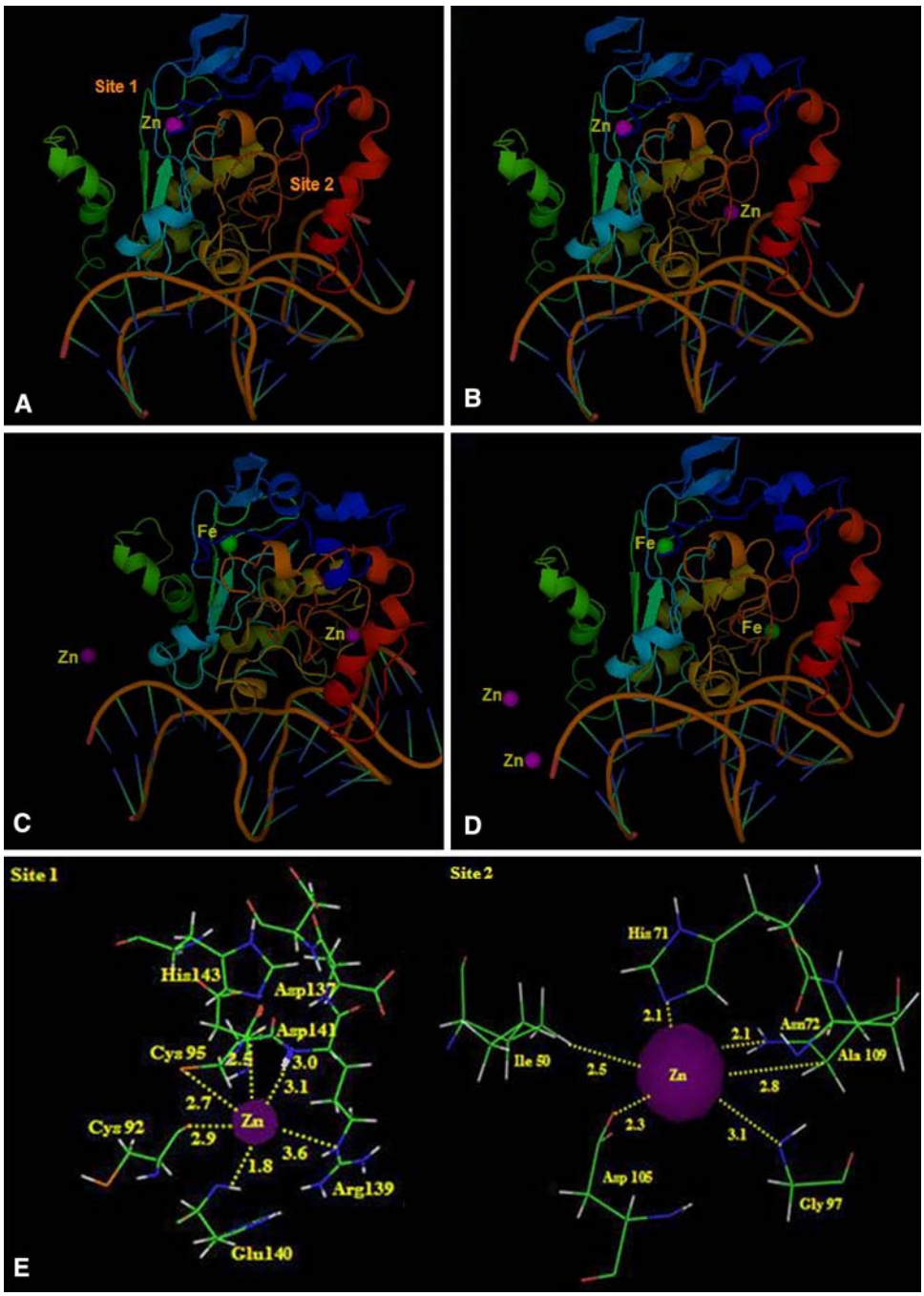

Fig. 4 **a** The Zn^{2+} complex with Fur dimer/DNA, it shows that the first Zn^{2+} binds in the Zinc site and site 2 (the iron site) is vacant (see Supplementary Table 3). **b** The coordination of $2Zn^{2+}$ ions to the Fur dimer/DNA. **c** The addition of $1Fe^{2+}$ to the complex in part b, it shows that Fe^{2+} replaced the first bound Zn^{2+} ion in the structural zinc site. **d** The complex after adding $2Fe^{2+}$, the second Fe^{2+} replaced the zinc ion in the iron activating site. **e** Close up view of Zn^{2+} sites on Fur dimer showing residues bound to zinc ion in the zinc structural site and distances (*left*), zinc bound to the iron activating site and distances (*right*)

On the other hand the calculated binding affinity of Fur to Fe^{2+} indicated more stability than zinc binding, this can be explained as due to the greater conformational changes in C-terminal. These conformational changes affected the zinc binding negatively and enabled selective iron binding with greater affinity (dissociation constant for the first site was 26 μM).We can conclude that adding Fe^{2+} seems to decrease the binding affinity of Fur towards the Zinc ion and allows iron to replace it easily.

The tuning of Fur upon replacing Zn^{2+} with Fe^{2+}

Amino acid residues shifted drastically closer to the DNA upon adding Fe^{2+} to the Zn^{2+}-Fur/DNA complex and, at the same time, iron replaced zinc in the Fur sites (see Figs. 3, 4; Table 1). Some residues like Arg19, His87, His125, Asp141 and His145 shifted to a larger extent upon adding $2Fe^{2+}$ to the Zn^{2+} Fur/DNA complex than in the case of $4Fe^{2+}$ alone to apo Fur/DNA system. His32 moved

Table 1 Metal ion binding sites on the Fur dimer: competition between Zn^{2+} and Fe^{2+} for binding of Fur dimer

Residue	M2+ Fur (Å) [24]	Fur dimer/ DNA + $1Zn^{2+}$	Fur dimer/ DNA + $2Zn^{2+}$	Fur dimer/ DNA + $2Zn^{2+}$ + $1Fe^{2+}$	Fur dimer/ DNA + $2Zn^{2+}$ + $2Fe^{2+}$
Site 2					
M-His 71^{++}	Fe^{2+} 1.3		Zn^{2+} 2.1	Zn^{2+} 3.4	Fe^{2+} 2.6
M-Ile 50^{++}	Fe^{2+} 2.3		Zn^{2+} 2.5	Zn^{2+} 5.8	Fe^{2+} 2.1
M-Asn 72^{++}	Fe^{2+} 1.5		Zn^{2+} 2.1	Zn^{2+} 6.5	Fe^{2+} 3.1
M-Gly 97	Fe^{2+} 2.3		Zn^{2+} 3.1	Zn^{2+} 7.7	Fe^{2+} 2.4
M-Asp 105	Fe^{2+} 1.4		Zn^{2+} 2.3	Zn^{2+} 6.2	Fe^{2+} 2.7
M-Ala 109	Fe^{2+} 2.1		Zn^{2+} 2.8	Zn^{2+} 5.9	Fe^{2+} 1.9
Site 1 (Zn site)					
M-Cys 92[a]	Fe^{2+} 2.2[b]	Zn^{2+} 3.2	Zn^{2+} 2.9[c]	Fe^{2+} 2.5	Fe^{2+} 2.2[b]
M-Cys 95[a]	Fe^{2+} 1.6[b]	Zn^{2+} 2.9	Zn^{2+} 2.7[c]	Fe^{2+} 2.6	Fe^{2+} 2.3[b]
M-Asp 137	Fe^{2+} 1.3	Zn^{2+} 3.1	Zn^{2+} 3.1[c]	Fe^{2+} 2.5	Fe^{2+} 2.7
M-Asp 141	Fe^{2+} 1.5	Zn^{2+} 3.2	Zn^{2+} 3.0[c]	Fe^{2+} 2.9	Fe^{2+} 3.1
M-Arg 139	Fe^{2+} 1.7	Zn^{2+} 4.1	Zn^{2+} 3.6[c]	Fe^{2+} 3.0	Fe^{2+} 2.8
M-Glu 140[a]	Fe^{2+} 1.3	Zn^{2+} 2.1	Zn^{2+} 1.8[c]	Fe^{2+} 1.2	Fe^{2+} 1.7
M –His 145[a]	Fe^{2+} 1.2	Zn^{2+} 2.4	Zn^{2+} 2.1[c]	Fe^{2+} 2.0	Fe^{2+} 1.9
M-His 143[a]	Fe^{2+} 1.5	Zn^{2+} 2.5	Zn^{2+} 2.5[c]	Fe^{2+} 2.3	Fe^{2+} 2.1

[a] Most likely binding sites reported for zinc ion in the structural zinc site [22]

[b] Note the large change in metal-Ligand distance for the same bonds, when starting with apo Fur and adding Fe^{2+} than when adding the Fe^{2+} to the Zn^{2+}/Fur complex

[c] The first Zinc ion to be replaced by Fe^{2+} which agrees with the reported lower affinity for structural site which gave lower $K = 3.2 \times 10^4$ M^{-1} compared to higher affinity for metal ion sensing site for with $K = 5.7 \times 10^6$ M^{-1}, agrees with P. A. Fur binding to Zinc [22]

$^{++}$ Reported for Metal ion (Fe^{2+}) sensing site [22]

to a comparable extent in all systems. This finding agrees with the proposed structural role found for Zn^{2+} ion, i.e., to preserve the three dimensional structure of the Fur dimer until arrival of the Fe^{2+} ions [11, 17, 22]. It is worth noting that the presence of Zn^{2+} in the Fur dimer seems to produce stronger shifts than those when Fe^{2+} was added to apo Fur/DNA.

Upon adding the first Fe^{2+} to the Zn^{2+} Fur/DNA complex (Fig. 4a) the process was accompanied by a considerable shift in positions of the residues closer to DNA with the exception of, His87, Phe73, Phe62, Arg57. Also, Pro18 and Arg19 moved further away from the DNA upon adding two Fe^{2+} ions to the Zn^{2+} Fur/DNA complex (Fig. 4a). These shifts are in opposite direction to those found for the iron system alone [24] which means that when Zn^{2+} was released from Fur upon Fe^{2+} binding these residues moved away from DNA, i.e., they play an important role in DNA binding in the combined zinc-iron system.

His87 is the key for Zn^{2+} activating the Fur DNA binding. When the first Fe^{2+} replaced Zn^{2+} in the zinc site, His87 stayed in position. But, when the second Zn^{2+} was replaced by Fe^{2+} in the iron activating site, His87 moved away from DNA. This finding proves that the His87 residue plays a key role in the Zn^{2+} activation of Fur. This finding agrees with experimental reports which proved that His87 to be essential to Fur activity in the Fur family [14, 22].

The contact areas between the two Fur subunits open up upon the approach of the first Fe^{2+} as indicated by positive shifts, see (Fig. 4b), forcing the helices ($\alpha2-\alpha2$, $\alpha3-\alpha3$, $\alpha4-\alpha4$, $\alpha6-\alpha6$) to close down on DNA in a reversed motion. In fact, this was observed by NMR measurements as previously reported [17]. It seems the Fur dimer engulfs the DNA using both the C-terminal and N-terminal domains with the N-terminal playing the more important role in the DNA binding process [17, 22, 24, 27, 28]. This helps to understand the role of Zn^{2+} in maintaining the Fur dimer structure around the DNA until the first Fe^{2+} attaches itself to the Fur replacing the structural zinc, at which time the contact areas open up (indicated by positive shifts in (Fig. 4b). The Fur dimer changes conformation in a way to engulf the DNA. When the second Fe^{2+} comes in and replaces Zn^{2+} in the iron activating site, the contact areas (Gly51–Gln85, Thr54–Thr83 and Gln85–Gln85) moved further apart forcing both ends of Fur subunits to close down on DNA in a scissor like motion. Most negative shifts caused by Fe^{2+} were for, $\alpha2-\alpha2$, $\alpha3-\alpha3$, and $\alpha6-\alpha6$.

Conclusion

Zn^{2+} binds E. C. Fur on two sites: site 1 is the one assigned previously [11, 15] as the structural zinc site and site 2 as the iron activating site. Metal ion binding to Fur dimer proved to be weak with dissociation constants ranging from 20 μM^{-1} in case of Co^{2+} to 80 μM^{-1} in the case of Mn^{2+}[24], This is consistent with the reversible metal ion-binding (K_d value for iron 55 μM [24]). Surprisingly, Zn^{2+} showed lower affinity towards the structural site than that for the sensory site in an equilibrium study reported for P. A. Fur [22] which contradicts previous experimental reports that zinc is tightly bound in the structural zinc site [11, 15]. This finding on P. A. Fur binding to zinc agrees with our finding that Fe^{2+} could replace Zn^{2+} in the structural site before it replaced zinc in the sensory site. It seems that the metal ion goes into the pre-designed cavity on the Fur dimer with several amino acids necessary to build that cavity of which are most vital are C92, C95 and H87 and Aspartate residues in addition to water molecules.

The shift in residue positions closer to DNA induced by Zn^{2+} is very small compared to that induced by Fe^{2+} with the exception of His87 which plays a key role in the activation of Fur especially by Zn^{2+} ion. The sensitivity of amino acid residues to the Zn^{2+} concentration is low when compared to the sensitivity to Fe^{2+} concentration. The conformational changes in the Fur dimer induced by both Zn^{2+} and Fe^{2+} are parallel and the monomer subunits moved in a similar manner in both systems. The finding that Fe^{2+} could replace Zn^{2+} in the zinc site agrees with experimental reports which stated that Zn^{2+} plays a structural role [11, 18, 19, 22]. Indeed, a recent study confirms the role of Zn^{2+} ion in stabilizing the Fur dimer in a DNA binding process [11].

There is a possibility that Fe^{2+} plays the role of antagonist to a Zn^{2+} inhibitor, this hypothesis needs a more extensive investigation [17]. The metal ions, as is the case for many other DNA binding proteins, appear to act as a tuning factor to support the helices in the protein in the right direction to bind the DNA grooves.

References

1. Escolar L, Perez-Martin J, de Lorenzo V (1999) Opening the iron box: transcriptional metalloregulation by Fur protein. J Bacteriol 181:6223–6229
2. Bagg A, Neilands JB (1987) Ferric uptake regulation protein acts as a repressor, employing Iron(II) as a cofactor to bind the operator of an Iron transport operon in *Escherichia coli*. Biochemistry 26:5471–5477. doi:10.1021/bi00391a039
3. Escolar L, Perez-Martin J, de Lorenzo V (1997) Metalloregulation in vitro of the aerobactin promoter of *Escherichia coli* by the fur (ferric uptake regulation) protein. J Mol Microbiol 26:799–808. doi:10.1046/j.1365-2958.1997.6211987.x
4. Bsat N, Helmann JD (1999) Interaction of *Bacillus subtilis* Fur (Ferric Uptake Repressor) with the *dhb* operator in vitro and in vivo. J Bacteriol 181:4299–4307
5. Lee JW, Helmann JD (2007) Functional specialization within the fur family of metalloregulators. Biometals 20:485–499. doi:10.1007/s10534-006-9070-7
6. Baichoo N, Helmann JD (2002) Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J Bacteriol 184:5826–5832. doi:10.1128/JB.184.21.5826-5832.2002
7. Lavrrar JL, McInosh MA (2003) Architecture of a Fur binding site: a comparative analysis. J Bacteriol 185:2194–2202. doi:10.1128/JB.185.7.2194-2202.2003
8. Lavrrar JL, Christoffersen CA, McIontosh MA (2002) Fur–DNA interactions at bidirectional fep DGC-entS promoter region in *Escherichia coli*. J Mol Biol 322:983–995. doi:10.1016/S0022-2836(02)00849-5
9. Frechon D, Le Cam E (1994) Fur (Ferric uptake Regulation) protein interaction with target DNA: comparison of gel retardation, footprinting and electron microscopy analysis. Biochem Biophys Res Commun 201:346–355
10. Jacquamet L, Aberdam D, Adrait A, Hazemann JL, Latour JM, Michaud-Soret I (1998) X-ray absorption spectroscopy of a new zinc site in the fur protein from *Escherichia coli*. Biochemistry 37:2564–2571. doi:10.1021/bi9721344
11. D'Autreaux B, Pecqueur L, de Peredo AG, Diederix REM, Caux-Thang C, Tabet L, Bersch B, Forest E, Michaud-Soret I (2007) Reversible redox- and Zinc-dependant dimerization of the *Escherichia coli* Fur protein. Biochemistry 46:1329–1342. doi:10.1021/bi061636r
12. Barondeau DP, Getzoff ED (2007) Structural insights into protein-metal ion partnerships. Curr Opin Struct Biol 14:765–774
13. Gabella A, Helmann JD (1998) Identification of a Zinc-specific metalloregulatory protein, Zur, controlling Zinc transport operons in *Bacillus subtilis*. J Bacteriol 180:5815–5821
14. Lewin AC, Doughty PA, Flegg L, Moore GR, Spiro S (2002) The ferric uptake regulator of *Pseudomonas aeruginosa* has no essential cystine residues and does not contain a structural zinc ion. J Microbiol 148:2449–2456
15. Althaus EW, Outten CE, Olson KE, Cao H, O'Halloran TV (1999) The ferric uptake regulation is a zinc metalloprotein. Biochemistry 38:6559–6569. doi:10.1021/bi982788s
16. Lucarelli D, Russo S, German E, Milano A, Meyer-Klaucke W, Pohl E (2007) Crystal structure and function of the zinc uptake regulator FurB from *Mycobacterium tuberculosis*. J Biol Chem 282:9914–9922. doi:10.1074/jbc.M609974200
17. Pecqueur L, D'Autreaux B, Dupuy J, Nicolet Y, Jacquamet L, Brutcher B, Michaud-Soret I, Bersch B (2007) Structural changes of *Escherichia coli* ferric uptake regulator during metal-dependant dimerization and activation explored by NMR and X-ray crystallography. J Biol Chem 281:21286–21295. doi:10.1074/jbc.M601278200
18. Zheleznova EE, Crosa JH, Brennan RG (2000) Characterization of the DNA- and metal-binding properties of *Vibro anguillarum* Fur reveals conservation of a structural Zn^{2+} ion. J Bacteriol 182:6264–6267. doi:10.1128/JB.182.21.6264-6267.2000
19. De Peredo AG, Saint-Pierre C, Adrait A, Jacquamet L, Latour JM, Michaud-Soret I, Forest E (1999) Identification of the two zinc-bound cysteine in the ferric uptake regulation protein from *Esherichia coli*: chemical modification and mass spectroscopy analysis. Biochemistry 38:8582–8589. doi:10.1021/bi9902283
20. Pohl E, Haller JC, Mijovilovich A, Meyer-Klancke W, German E, Vasil ML (2003) Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the

ferric uptake regulator. Mol Microbiol 47:903–915. doi:10.1046/j.1365-2958.2003.03337.x
21. Freidman YE, O'Brian MR (2004) The ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicun* is an iron-responsive transcriptional repressor in vitro. J Biol Chem 279:32100–32105. doi:10.1074/jbc.M404924200
22. Bai E, Rossel FI, Lige B, Mauk MR, LelJ-Garolla B, Moore MR, Mauk AG (2006) Functional characterization of dimerization domain of the ferric uptake regulator (Fur) of *Pseudomonas aeruginosa*. Biochem J 400:385–392. doi:10.1042/BJ20061168
23. Adrait A, Jacquamet L, Le Pape L, de Peredo AG, Aberdam D, Hazeman J, Matour J, Michaud-Soret I (1999) Spectroscopic and saturation magnetization of the manganese and cobalt-substituted Fur protein from *Escherichia coli*. Biochemistry 38:6248–6260. doi:10.1021/bi9823232
24. Hamed M, Al-Jabour S (2006) Iron triggered conformational changes in *Escherichia coli* fur upon DNA binding: a study using molecular modeling. J Mol Graph Model 25:234–246. doi:10.1016/j.jmgm.2005.12.010
25. Case DA, Pearlman DA, Caldwell JW, Cheatham TEIII, Wang J, Ross WS, Simmerling CL, Darden TA, Merz KM, Stanton RV, Cheng AL, Vencent JJ, Crawley M, Tsui V, Gohlke H, Radmer RJ, Duan Y, Pietera J, Massova I, Seibel GL, Singh UC, Weiner PK, Kollman PA (2006) Amber9. University of California, San Francisco
26. Pearlman DA, Case DA, Caldwell JW, Ross WR, Chealtham TE, DeBolt S, Ferguson IIID, Seibel G, Kollman P (1995) AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculation to simulate the structural and energetic properties of molecules. Comput Phys Commun 91:1–41. doi:10.1016/0010-4655(95)00041-D
27. Coy M, Neilands JB (1991) Structural dynamics and functional domains of the fur protein. Biochemistry 30:8201–8210. doi:10.1021/bi00247a016
28. Coy M (1995) The interaction of the ferric uptake regulation protein with DNA. Biochem Biophys Res Commun 212:784–792. doi:10.1006/bbrc.1995.2037