Published in final edited form as:

J Phys Chem Lett. ; 2012(3): 3503–3508. doi:10.1021/jz301689b.

Iron Binding Site in a Global Regulator in Bacteria - Ferric Uptake Regulator (Fur) Protein: Structure, Mössbauer Properties, and Functional Implication

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Abstract

Fur protein plays key roles in regulating numerous genes in bacteria and is essential for intracellular iron concentration regulation. However, atomic level pictures of the iron binding site and its functional mechanism remain to be established. Here we present results of the first quantum chemical investigation of various first- and second-shell models and experimental Mössbauer data of *E. Coli* Fur, including 1) the first robust evidence that site 2 is the Fe binding site with a 3His/2Glu ligand set, being the first case in non-heme proteins, with computed Mössbauer data in excellent accord with experiment; 2) the first discovery of a conservative hydrogen bonding interaction in the iron binding site based on X-ray and homology structures; 3) the first atomic level hypothesis of active site reorganization upon iron concentration increase, triggering the conformational change needed for its function. These results shall facilitate structural and functional studies of Fur family proteins.

Keywords

Fur; Iron binding; Mössbauer; Mechanism; Quantum chemical calculation

Iron is an important element in living organisms. But excess intracellular Fe concentration can catalyze the formation of reactive oxygen species, leading to various damages to cellular components.¹ In order to maintain a proper intracellular Fe concentration, bacteria have specific mechanisms of sensing and regulating the uptake of Fe.¹⁻³ The most prominent one involves the Fur protein, which is a global regulator. For instance, Fur controls the expression of >90 genes in *E. Coli* (EC).³ Fur is a multimeric protein and coordination of one ferrous ion in each Fur monomer causes conformational changes that allow for binding to a specific 19 bp DNA sequence called the Fur-box.⁴⁻⁶ This prevents the access of RNA polymerase to downstream genes responsible for Fe uptake. Fur is also responsible for a variety of other biochemical activities, such as expression of virulence factors.⁷⁻⁹ Fur binding to certain DNA regions was shown to act as a positive regulator in the expression of Fe-dismutases, catalases, and even further expression of more Fur proteins in order to mitigate the catalytic activity of ferrous ions.¹⁰ In addition, Fur protein was found to be involved in response to NO stress.¹¹

The coordination of one ferrous ion in each Fur monomer indicates that the iron binding is site specific since there are two or three metal binding sites in different Fur homologs.¹²⁻¹⁵

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Supporting Information Available: Computational details and optimized coordinates (Tables S1-S12) are available free of charge via the Internet at <http://pubs.acs.org>.

However, in contrast with other Fur family proteins whose X-ray structures show the clear binding sites of their respective metals,¹⁶⁻¹⁹ all the available Fur crystal structures do not have the direct assignment of the Fe binding site because metal sites are all occupied by Zn.¹³⁻¹⁵ As shown in Figure 1, there are three types of metal binding sites in the published X-ray structures for Fur proteins, which are from *Pseudomonas aeruginosa* (PA),¹³ *Vibrio cholera* (VC),¹⁴ and *Helicobacter pylori* (HP).¹⁵ Although site 3 can be identified as a Zn site, both site 1 and site 2 could bind with Fe, as found from early EXAFS experiments.²⁰ A more recent EXAFS experiment on PA-Fur suggested that site 1 be the Fe-binding site, while site 2 be the structural Zn binding site.¹³ However, recent molecular dynamics simulations of DNA-bound PA-Fur dimer proposed that site 2 be the Fe binding site.²¹ Nevertheless, only two Fe coordination motifs based on one of the three Fur X-ray structures were investigated in that report. It is also puzzling to see that among PA-Fur structures with Fe binding in site 2, the only thermodynamically favorable species involves a vacant site 1 other than the Zn-bound site 1, which is inconsistent with the fact that Zn can readily bind in these metal sites.¹³⁻¹⁵ Unfortunately, mutagenesis experiments of EC-, PA-, VC-, and HP-Fur were all ambiguous as both sites were found to affect Fur functions.²²⁻²⁷ A more recent mutagenesis study in conjunction with a circular dichroism and UV-Visible spectroscopy using Co^{II} showed that although site 2 is important, site 1 (called site 3 in their nomenclature) can also affect DNA binding.¹⁵ Therefore, there is still no robust evidence of the iron binding structure from using direct iron probes. Its functional mechanism at the atomic level also remains to be elucidated.

It is well-known that ⁵⁷Fe Mössbauer spectroscopy is a powerful, sensitive, and direct probe of iron binding sites and DFT calculations have been successful in investigating Mössbauer properties of non-heme Fe proteins such as soybean monooxygenases,²⁸ isopenicillin N-synthase,²⁹ and hydroxylase.³⁰ Our previous combined quantum chemical and Mössbauer spectroscopic investigations have also enabled structure refinement and determination of iron binding sites in a number of iron protein systems.^{29, 31-34} Here, we extend such kind of investigation to various first- and second-shell site 1 and site 2 models as well as the experimental Mössbauer properties of Fe-bound EC-Fur.³⁵

It is interesting to note that all Fur X-ray structures share the same ligand set as 2His/Asp/Glu for site 1, see Figure 1. In contrast, site 2 ligand sets were found to be different: 2His/2Glu in PA-Fur, 3His/Glu in VC-Fur and in B-monomer of HP-Fur (HPb-Fur), and 3His/2Glu in A-monomer of HP-Fur (HPa-Fur). It should be noted that although site 2 of PA-Fur shows a 2His/2Glu motif, the sequence shows that His87 close to the metal center is replaced by Ala in the crystal structure due to disorder. His87 is well conserved in the Fur-Family and is considered to be involved in metal-binding, see sequence alignment in Figure 3. Hence, only the 3His/Glu and 3His/2Glu motifs from the X-ray structures were investigated here for site 2. As shown in Table 1, altogether six first-shell models and six second-shell models were investigated (see Supporting Information for computational details and optimized coordinates) with optimized structures shown in Figure 2. All these models were constructed on the basis of the three X-ray structures¹³⁻¹⁵ by replacing Zn^{II} with Fe^{II} and a few modifications in some cases to facilitate comparisons of different coordination motifs. All first-shell models were fully optimized to explore the dynamic coordination space for Fe, as its binding is known to trigger the conformational change needed for its function.⁴⁻⁶ In contrast, all second-shell models which include residues with hydrogen bonding/van der Waals/hydrophobic interactions with first-shell residues were subject to partial optimization with terminal carbons fixed at the X-ray structure positions to mimic protein environment effects.

The variable nature of site 2 structures suggests that it could be the regulatory Fe site, whose structure (ligand set) could be more easily influenced by the dynamic biological

environment or different crystallization conditions than site 1. To provide a direct evidence for the iron binding site in Fur, the sensitive structural probes - Mössbauer isomer shift (δ_{Fe}) and quadrupole splitting (ΔE_{Q}) for each model were investigated using the previously established method with high accuracy predictions for a broad range of iron proteins and compounds (see Supporting Information for computational details).³⁶ As shown in Table 2, for first-shell models **1-5**, site 2 models show generally better agreement with experimental data than site 1 models, as the average error in δ_{Fe} for site 2 models (0.09 mm/s) is clearly smaller than that for site 1 models (0.15 mm/s), and the average error in ΔE_{Q} for site 2 models (0.04 mm/s) is even more significantly smaller than that for site 1 models (0.28 mm/s). The same trend was also observed for second-shell models **6-11**, as the average error in δ_{Fe} for site 2 models (0.15 mm/s) is smaller than that for site 1 models (0.20 mm/s), and the average error in ΔE_{Q} for site 2 models (0.33 mm/s) is less than half of that for site 1 models (0.77 mm/s). The best agreement occurs with the site 2 model **4**, exhibiting only 0.02-0.03 mm/s errors with respect to experimental isomer shift and quadrupole splitting. Its corresponding second-shell model **10** is also the best among all second-shell models. It is interesting to note that **4** and **10** are the only models here that employ the complete set of five conserved metal-binding residues in site 2 (see Figure 3), which are also the only models that accurately reproduce experimental Mössbauer data. A further comparison of Mössbauer results between the fully optimized first-shell model **4** and corresponding partially optimized second-shell model **10** suggests that, the Fe-bound EC-Fur structure is perhaps more similar to **4** than **10**, which has the imposed structural restraints from Zn-bound HP-Fur structure.

To more rigorously evaluate these site 1 and site 2 models, a reduced χ^2 analysis and Bayesian probability (or Z-surface) technique³⁷ were employed, using both experimental Mössbauer isomer shift and quadrupole splitting (see Supporting Information for details). A small χ^2 value indicates a small deviation from experiment and a large Z value means a high probability of the model corresponding to the experimental system. As shown in Table 2, both statistical analyses show that again site 2 models in general are better than site 1 models, with **4** and **10** being the best first-shell and second-shell models for EC-Fur iron binding site, respectively. **4** was again found to be better than **10**.

Since quite a number of different first-shell coordination motifs and second-shell environments were investigated here, all these calculations, comparisons with experimental data of direct iron probes, and statistical analyses provide the first strong and consistent support for site 2 as the Fe binding site. It is interesting to note that the unique 3His/2Glu ligand set found for site 2 is the first case among iron binding sites in non-heme proteins.³⁸

In order to help understand how the increased Fe concentration drives the Fur conformational change needed to activate its transcriptional repressor function,⁴⁻⁶ the first and second coordination shell residues were investigated in more details. In contrast with the octahedral coordination mode found for Fe in site 2, the Zn bound experimental X-ray structures of site 2 are effectively a tetrahedral coordination mode when their geometric parameters were examined. As shown in Table 3, several Zn-ligand distances (highlighted in bold) are of 2.5-2.7 Å, indicating weak interactions with Zn from such ligands and thus making Zn effectively four-coordinate. Therefore, these results show that when site 2 is occupied by Zn, it perhaps adopts the tetrahedral coordination typically preferred by Zn, with a ligand set of 3His/Glu, based on all non-disordered X-ray structures as shown in Figure 4B-4D. The 2nd Glu that is a part of the 3His/2Glu ligand set for Fe is basically not bound with Zn.

Interestingly, a conservative hydrogen bonding interaction was discovered for the first time to stabilize this 2nd Glu residue near the metal binding site, as shown in Figure 4. This 2nd

Glu residue is well conserved (Glu100 in PA-Fur, Glu101 in VC-Fur, Glu110 in HP-Fur, Glu101 in EC-Fur, highlighted by a black arrow in Figure 3). Its hydrogen bond partner is a polar or positively charged residue present in all crystal structures as well as the homology modeled structure of EC-Fur (see Supporting Information for modeling details): Gln32 for VC-Fur and Arg31 for PA-Fur (see structures in Figure 4A and 4B, and sequence highlighted by a blue arrow in Figure 3), and Lys94 for HP-Fur and Gln85 for EC-Fur (see structures in Figure 4C-4E, and sequence highlighted by a green arrow in Figure 3).

Based on these results, we hypothesize that there may be a built-in mechanism for the dynamic metal binding in site 2: in the “resting state” of zero to low Fe concentration, site 2 structure favors a tetrahedral coordination that is amenable for Zn via 3His/Glu, with the 2nd Glu not coordinated, but kept close via the above-mentioned conserved hydrogen bonding to minimize the reorganization energy for 3His/2Glu motif when Fe binds. Under increased Fe concentrations, the concentration difference drives the site 2 reorganization into an “active state”, wherein this nearby hydrogen bonded 2nd Glu residue becomes ligated to Fe to form an octahedral coordination, which is typically favored by Fe binding in proteins. This kind of active site reorganization from four coordination to six coordination may trigger the conformational change in Fur to induce it to bind with the Fur-box in DNA,⁴⁻⁶ and thus activate its function. It is interesting to note that in EC-Fur, the reverse process, a change of coordination from six to four under NO stress was suggested to account for the Fur activity loss.¹¹ We suggest experimental biochemists in this area to use mutations of these hydrogen bonding residues to further examine this hypothesis.

Overall, this work provides the first evidence from a combined investigation of quantum chemistry results and experimental spectroscopic data of direct iron probes, that site 2 is the Fe binding site, with only models having the complete set of the five conserved metal-binding residues (3His/2Glu) in site 2, namely the first-shell model **4** and its corresponding second-shell model **10**, reproducing experimental Mössbauer data. This ligand set also displays the first case in iron binding sites in non-heme proteins. A conservative hydrogen bonding interaction in the metal binding site was also discovered for the first time, which help offer the first atomic level hypothesis of active site reorganization upon iron concentration increase, triggering the conformational change needed to activate its biological function. These results shall facilitate structural and functional investigations of Fur family proteins, important regulators in bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH grant GM-085774 to Y.Z.

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Figure 1. X-ray crystal structures of PA-Fur, VC-Fur and HP-Fur with first shell residues highlighted in ball and stick representation. Color Schemes: Zn-purple, C-cyan, N-blue, O-red, S-yellow, H-grey.

Figure 2. Optimized structures of first shell (**1-5**) and second-shell (**6-11**) models. Color Schemes: Fe-black, C-cyan, N-blue, O-red, H-grey.

Figure 3.

Sequence alignment of studied Fur proteins here. Colored dots below the sequences represent important residues involved in metal binding. Green refers to site 1 in Fur and Red refers to site 2. Arrows indicate hydrogen bonding residues with the conserved Glu discussed in the text.

Figure 4. The hydrogen bonding residues found in the 2nd shell of site 2 in the crystal structures of **A)** PA-Fur; **B)** VC-Fur; **C)** HPa-Fur; **D)** HPb-Fur and in the homology modeled structure of **E)** EC-Fur.

Table 1

Structural models used in this work

Shell	Model	X-ray structure	Ligand set	Note
First	1	PA-Fur site 1	2His/Asp/Glu/water	Asp is bidentate
	1'	PA-Fur site 1	2His/Asp/Glu/water	Asp modified to be monodentate
	2	VC/HP-Fur site 1	2His/Asp/Glu	Asp is monodentate
	3	VC/HPb-Fur site 2	3His/Glu	One bidentate Glu
	4	HPa-Fur site 2	3His/2Glu	One bidentate Glu
	5	HPa-Fur site 2	3His/2Glu/water	Modified from 4 with both monodentate Glu and an addition of a water molecule
Second	6	PA-Fur site 1	2His/Asp/Glu/water	
	7	PA-Fur site 2	3His/2Glu	Mistaken Ala87 changed back to His 87
	8	VC-Fur site 1	2His/Asp/Glu	
	9	VC-Fur site 2	3His/Glu	
	10	HPa-Fur site 2	3His/2Glu	
	11	HPb-Fur site 2	3His/Glu	

Table 2
Mossbauer Properties for Fe Binding Site Models (unit: mm/s) and Statistical Results

Model	Expt ^a	1	1'	2	3	4	5
δ	1.19	1.13	1.00	0.99	1.05	1.22	1.28
ΔE_Q	3.47	3.22	3.29	3.06	3.50	3.49	3.53
χ^2		0.733	4.431	5.560	2.324	0.108	0.976
Z		0.489	0.021	0.007	0.135	0.910	0.429
Model		6	7	8	9	10	11
	1.19	1.41	1.09	1.01	0.96	1.22	0.97
ΔE_Q	3.47	2.10	2.99	3.31	3.10	3.74	3.27
χ^2		14.95	2.315	3.960	6.933	0.465	5.924
Z		0.000	0.100	0.032	0.002	0.608	0.006

^aRef. 35

Table 3

Zn-ligand Distances in Site 2 X-ray structures (unit: Å)

Ligand	N _{His1}	N _{His2}	N _{His3}	O _{Glu1}	O ['] Glu1	O _{Glu2}
PA-Fur	2.000	2.041	/	2.159	/	2.040
VC-Fur	2.039	2.085	2.182	2.078	2.713	/
HPa-Fur	2.190	2.142	2.162	2.262	2.463	2.642
HPb-Fur	2.184	2.105	2.102	2.039	2.572	