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Ferric uptake regulators (Fur) from *Vibrio cholerae* and *Helicobacter pylori* bind a [2Fe–2S] cluster in response to elevation of intracellular free iron content

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Abstract Intracellular iron homeostasis in bacteria is primarily regulated by ferric uptake regulator (Fur). Since its discovery, Fur has been assumed to bind ferrous iron and regulate expression of target genes. However, the iron-bound Fur has never been isolated from any bacteria. In previous studies, we have shown that *Escherichia coli* Fur and *Haemophilus influenzae* Fur bind a [2Fe–2S] cluster via the conserved Cys-93 and Cys-96 when expressed in the *E. coli* mutant cells in which intracellular free iron content is elevated. Here we report that Fur homologs from *Vibrio cholerae* and *Helicobacter pylori* which contain Cys-93 and Cys-96 can also bind a [2Fe–2S] cluster. On the other hand, Fur homolog from *Magnetospirillum gryphiswaldense* MSR-1 which has no cysteine residues fails to bind any [2Fe–2S] clusters. Interestingly, different Fur proteins with the conserved Cys-93 and Cys-96 have distinct binding activities for the [2Fe–2S] cluster, with *H. influenzae* Fur having the highest, followed by *E. coli* Fur, *V. cholera* Fur, and *H. pylori* Fur. Binding of the [2Fe–2S] cluster in the Fur proteins is significantly decreased when expressed in wild-type *E. coli* cells, indicating that binding of the [2Fe–2S] clusters in Fur proteins is regulated by the levels of intracellular free iron content. Finally, unlike the [2Fe–2S] clusters in *E. coli* ferredoxin, the [2Fe–2S] clusters in the Fur proteins are not stable and quickly release ferrous iron when the clusters are reduced, suggesting that Fur may undergo reversible binding of the [2Fe–2S] cluster in response to intracellular free iron content in bacteria.

Keywords Iron–sulfur cluster binding · Ferric uptake regulator Fur · Intracellular iron homeostasis

Introduction

Ferric uptake regulator (Fur) is a global transcription factor that regulates intracellular iron homeostasis and pathogenesis in bacteria (Troxell and Hassan 2013; Fillat 2014; Pinochet-Barros and Helmann 2018). Since the discovery of Fur in *Escherichia coli* (Hantke 1981), it has been postulated that Fur binds its co-repressor Fe(II) to regulate expression of target genes in response to elevation of intracellular free iron content (Bagg and Neilands 1987; Hantke 2001; Pinochet-Barros and Helmann 2018). The crystallographic studies of Fur proteins from *Pseudomonas aeruginosa* (Pohl et al. 2003), *Vibrio cholerae* (Sheikh and Taylor 2009), *Helicobacter pylori* (Dian et al. 2011), *E. coli* (Pecqueur et al. 2006), *Mycobacterium tuberculosis* (Lucarelli et al. 2007), *Campylobacter jejuni* (Butcher et al. 2012), *Magnetospirillum*

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gryphiswaldense MSR-1 (Deng et al. 2015), and *Francisella tularensis* (Perard et al. 2018) revealed a highly conserved structure model for Fur. Overall, Fur may exist as a homodimer or tetramer with each monomer containing three putative metal binding sites. Site 1 locates in the dimerization domain and consists of His-87, Asp-89, Glu-108, and His-125 (residue numbers in *E. coli* Fur). Site 2 connects the DNA binding domain and dimerization domain, and is coordinated by His-33, Glu-81, His-88, and His-90 (Pecqueur et al. 2006; Sheikh and Taylor 2009). Site 3 is within the C-terminal domain, and contains at least Cys-93 and Cys-96 (Sheikh and Taylor 2009). While site 1 and site 2 in purified Fur proteins are often occupied by Zn(II) (Althaus et al. 1999; Pecqueur et al. 2006; Sheikh and Taylor 2009; Perard et al. 2018), the iron-bound Fur has never been isolated from any bacteria.

In attempts to search for the iron-bound Fur in bacteria, we took advantage of an *E. coli* mutant in which the iron–sulfur cluster assembly proteins IscA and its paralog SufA are deleted (Lu et al. 2008; Tan et al. 2009; Mapolelo et al. 2012). IscA and SufA have a strong iron binding activity and can transfer its iron center for iron–sulfur cluster assembly in vitro (Ding and Clark 2004; Landry et al. 2013). Deletion of IscA and SufA results in deficiency of [4Fe-4S] cluster assembly but has very little or no effect on [2Fe–2S] cluster assembly in *E. coli* cells (Lu et al. 2008; Tan et al. 2009). Using the deferoxamine/electron paramagnetic resonance (EPR) approach (Woodmansee and Imlay 2002), we found that the *E. coli* mutant with deletion of IscA and SufA has an elevated intracellular free iron content (Fontenot et al. 2020), as previously reported in eukaryotic cells with deletion of the IscA homologs (Jensen and Culotta 2000). We reasoned that *E. coli* Fur may become iron-bound in the *E. coli iscA*/*sufA* mutant cells due to elevation of intracellular free iron content. Indeed, while *E. coli* Fur expressed in wild-type *E. coli* cells is colorless as reported previously (Althaus et al. 1999), *E. coli* Fur expressed in the *E. coli iscA*/*sufA* mutant cells has a bright red color. Additional biochemical, spectroscopic, and site-directed mutagenetic studies revealed that *E. coli* red Fur binds a novel [2Fe–2S] cluster (but not a mononuclear iron) at site 3 (via Cys-93 and Cys-96) (Fontenot et al. 2020).

Binding of a [2Fe–2S] cluster in Fur proteins appears to be conserved as Fur homolog from *Haemophilus influenzae* also binds a [2Fe–2S] cluster when expressed in *E. coli* cells (Fontenot et al. 2020). Here, we report that Fur homologs from two pathogenic bacteria *V. cholerae* (Sheikh and Taylor 2009) and *H. pylori* (Dian et al. 2011), both contain the conserved cysteine residues (Cys-93 and Cys-96), can also bind a [2Fe–2S] cluster when expressed in the *E. coli iscA*/*sufA* mutant cells. In contrast, Fur homolog from magnetotactic bacterium *M. gryphiswaldense* MSR-1 (Deng et al. 2015) which has no cysteines, fails to bind any [2Fe–2S] clusters in the *E. coli iscA*/*sufA* mutant cells. Interestingly, different Fur proteins with the same conserved Cys-93 and Cys-96 have distinct binding activities for the [2Fe–2S] cluster, with *H. influenzae* Fur having the highest, followed by *E. coli* Fur, *V. cholera* Fur, and *H. pylori* Fur. Furthermore, unlike the [2Fe–2S] clusters in ferredoxin and other proteins, the [2Fe–2S] clusters in the Fur proteins are not stable and quickly release iron when the clusters are reduced, suggesting that under the reducing conditions of the cytosol, binding of the [2Fe–2S] cluster in Fur is transient and its status may be responsive to levels of intracellular free iron content. The results led us to propose that binding of a [2Fe–2S] cluster is highly conserved among the Fur proteins containing Cys-93 and Cys-96, and that elevation of intracellular free iron content promotes the [2Fe–2S] cluster binding in Fur which in turn will regulate expression of target genes in bacteria.

Materials and methods

Protein purification

The genes encoding Fur proteins from *V. cholera*, *H. pylori*, and *M. gryphiswaldense* MSR-1 (Supplemental Materials) were synthesized (GenScript Co.) and cloned into expression plasmid pBAD/His-A (Invitrogen Co.). The codon usage was optimized for the protein expression in *E. coli* cells. Cloned plasmids were introduced into wild-type *E. coli* (MC4100) and the *iscA*/*sufA* mutant cells (Lu et al. 2008; Tan et al. 2009). Fur proteins were purified as described previously (Fontenot et al. 2020). *E. coli* ferredoxin was also purified from the *E. coli iscA*/*sufA* mutant cells as described previously (Rogers and Ding 2001). The concentration of purified Fur proteins

was measured at 280 nm after iron–sulfur clusters were removed by adding HCl (10 mM). The extinction coefficients of 5.6 mM^{-1} cm^{-1}, 6.8 mM^{-1} cm^{-1}, 7.0 mM^{-1} cm^{-1}, 11.3 mM^{-1} cm^{-1}, 5.1 mM^{-1} cm^{-1}, and 7.8 mM^{-1} cm^{-1} at 280 nm were used for calculating the protein concentrations of *E. coli* Fur, *H. influenzae* Fur, *V. cholerae* Fur, *H. pylori* Fur, *M. gryphiswaldense* MSR-1 Fur, and *E. coli* ferredoxin, respectively.

Redox titration of the *E. coli* Fur [2Fe–2S] clusters

A specially designed anaerobic cuvette was used for redox titrations as described by Dutton (1978). Briefly, purified *E. coli* Fur (containing 10 µM [2Fe–2S] clusters) was mixed with a redox mediator safranine O (0.5 µM) in buffer containing NaCl (500 mM), and Tris (20 mM, pH 8.0). The solution was purged with ultra-pure argon gas in a sealed cuvette with a micro magnetic stirrer for 50 min at room temperature. The redox potential was adjusted by adding a small amount of freshly prepared sodium dithionite using a gas-tight Hamilton syringe (Hamilton Co., Reno, NV). The redox potential was monitored using a redox microelectrode (Microelectrodes Inc., Bedford, NH). At each redox potential, a UV–Vis absorption spectrum of the solution was taken to determine the amount of the oxidized *E. coli* Fur [2Fe–2S] cluster. A freshly prepared ZoBell's solution containing $K_3Fe(CN)_6$ (5 mM) and $K_4Fe(CN)_6$ (5 mM) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) was used as a reference (E_h = 238 mV) for calibration of the microelectrode. The amounts of the oxidized Fur [2Fe–2S] clusters in the solution were plotted as a function of redox potentials in the solutions. The data were fitted to a Nernst equation using the KaleidaGraph software.

Iron release kinetics from the Fur [2Fe–2S] clusters

Iron release from Fur proteins was monitored by an iron indicator Ferrozine. Ferrozine binds Fe(II) to form a Ferrozine–Fe complex which has an absorption peak at 564 nm with an extinction coefficient of 27.9 mM^{-1} cm^{-1} (Cowart et al. 1993). For the experiments, Fur proteins (50 µM) was incubated with Ferrozine (500 µM) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0) for 5 min before freshly prepared sodium dithionite (a final concentration of 2 mM) was added. The amplitude of the absorption peak at 564 nm of the reaction solution was continuously monitored using Jasco V-750 spectrophotometer for 15 min after addition of sodium dithionite.

Total iron content analyses

Total iron content in Fur proteins was analyzed by incubating the proteins (50 µM) with Ferrozine (500 µM) and L-cysteine (4 mM) at 90 °C for 10 min. The samples were then centrifuged to remove the precipitated proteins. The amplitude of the absorbance peak at 564 nm of the Ferrozine–Fe(II) complex was used to calculate the total iron content of the protein samples using an extinction coefficient of 27.9 mM^{-1} cm^{-1} (Cowart et al. 1993).

Results and discussion

Fur homologs from *V. cholerae* and *H. pylori* bind a [2Fe–2S] cluster when expressed in the *E. coli iscA*/*sufA* mutant cells

Previous studies have shown that *E. coli* Fur and *H. influenzae* Fur bind a [2Fe–2S] cluster via the conserved Cys-93 and Cys-96 when expressed in the *E. coli iscA*/*sufA* mutant cells in which intracellular free iron content is elevated (Fontenot et al. 2020). To test whether binding of a [2Fe–2S] cluster is conserved among Fur proteins, we chose Fur homologs from two pathogenic bacteria: *V. cholerae* (Sheikh and Taylor 2009) and *H. pylori* (Dian et al. 2011), and a magnetotactic bacterium: *M. gryphiswaldense* MSR-1 (Deng et al. 2015). The sequence alignment of the Fur proteins from *E. coli*, *H. influenzae*, *V. cholerae*, and *H. pylori*, and *M. gryphiswaldense* MSR-1 is shown in Supplemental Materials using the alignment software (Madeira et al. 2019). While Fur proteins from *E. coli*, *H. influenzae*, *V. cholerae*, and *H. pylori* have the conserved Cys-93 and Cys-94, Fur protein from *M. gryphiswaldense* MSR-1 does not have any cysteine residues.

The genes encoding Fur proteins from *V. cholerae*, *H. pylori*, and *M. gryphiswaldense* MSR-1 were synthesized and cloned into plasmid pBAD/His-A. Each plasmid was introduced into the *E. coli iscA*/*sufA* mutant cells, and Fur proteins were

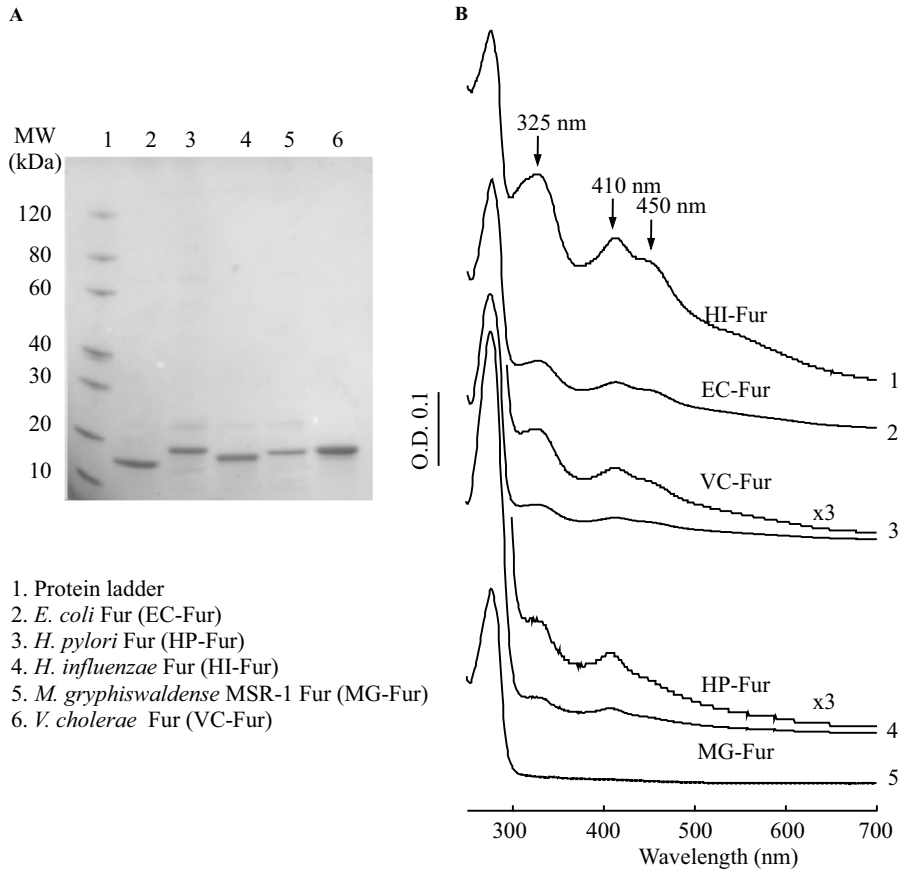

Fig. 1 Binding of a [2Fe–2S] cluster in the Fur proteins expressed in the *E. coli iscA*/*sufA* mutant cells. Each of genes encoding *V. cholerae* Fur, *H. pylori* Fur, *H. influenzae* Fur, and *M. gryphiswaldense* MSR-1 Fur was cloned into pBAD/His-A plasmid. The cloned plasmid was introduced into the *E. coli iscA*/*sufA* mutant cells. Fur proteins were purified from the cells. **A** SDS PAGE gel of purified Fur proteins. Lane 1, protein ladder [PAGE-Master protein standard (GenScript co.)]. Lane 2, *E. coli* Fur; Lane 3, *H. pylori* Fur; Lane 4, *H. influenzae* Fur; Lane 5, *M. gryphiswaldense* MSR-1 Fur; Lane 6, *V. cholerae* Fur. About 5 µM of Fur proteins (100 µM) mixed with 4× loading buffer were loaded for SDS PAGE gel. **B** UV–Visible absorption spectra of purified Fur proteins. Purified Fur proteins (50 µM) were diluted in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). Spectrum 1, *H. influenzae* Fur; spectrum 2, *E. coli* Fur; spectrum 3; *V. cholerae* Fur (a spectrum with 3× absorption amplitude was also shown); spectrum 4, *H. pylori* Fur (a spectrum with 3× absorption amplitude was also shown); spectrum 5, *M. gryphiswaldense* MSR-1 Fur. The absorption peaks at 325 nm, 410 nm, and 450 nm indicate the binding of a [2Fe–2S] cluster in the Fur proteins

purified (Fig. 1A) as described previously for *E. coli* Fur and *H. influenzae* Fur (Fontenot et al. 2020). Figure 1B shows that *V. cholerae* Fur (spectrum 3) and *H. pylori* Fur (spectrum 4), like *H. influenzae* Fur (spectrum 1) and *E. coli* Fur (spectrum 2), had three absorption peaks at 325 nm, 410 nm, and 450 nm, indicating that both *V. cholerae* Fur and *H. pylori* Fur bind a [2Fe–2S] cluster. In contrast, *M. gryphiswaldense* MSR-1 Fur (spectrum 5), which does not have any cysteine residues, had no absorption peaks at 325 nm, 410 nm, and 450 nm. Thus, Fur proteins that contain the conserved Cys-93 and Cys-96 can bind a [2Fe–2S] cluster in the *E. coli iscA*/*sufA* mutant cells.

Interestingly, different Fur proteins with the conserved Cys-93 and Cys-96 appear to have distinct binding activity for the [2Fe–2S] cluster (Fig. 1B). Using an extinction coefficient of 10 mM^{-1} cm^{-1} at 410 nm for the [2Fe–2S] cluster in Fur proteins (Fontenot et al. 2020), we estimated that the occupancy of the [2Fe–2S] cluster was 56 ± 12% for *H. influenzae* Fur, 25 ± 6% for *E. coli* Fur, ~ 10 ± 4% for *V.*

cholerae Fur, and ~ 8 ± 3% for *H. pylori* Fur ($n = 3$). The variation of the [2Fe–2S] cluster binding activity of the Fur proteins suggests that different bacteria may have different optimal intracellular free iron levels for the iron homeostasis. It is also possible that the [2Fe–2S] clusters in different Fur proteins may have unique interactions with the iron–sulfur cluster assembly machinery or have different stabilities in their native cells. Regardless, the results demonstrate that *V. cholerae* Fur and *H. pylori* Fur, like *E. coli* Fur and *H. influenzae* Fur, can bind a [2Fe–2S] cluster, and that binding of a [2Fe–2S] cluster is highly conserved among the Fur proteins that contain the conserved Cys-93 and Cys-96.

Binding of a [2Fe–2S] cluster in *V. cholerae* Fur and *H. pylori* Fur is diminished when expressed in wild-type *E. coli* cells

The Fur proteins from *V. cholerae* and *H. pylori* were also expressed in wild-type *E. coli* cells which have a relatively lower intracellular free iron content than the *E. coli iscA*/*sufA* mutant cells (Fontenot et al. 2020). Figure 2 shows that *V. cholerae* Fur (spectrum 3) and *H. pylori* Fur (spectrum 4) failed to bind any [2Fe–2S] clusters when expressed in wild-type *E. coli* cells. On the other hand, *H. influenzae* Fur (Fig. 2, spectrum 1) and *E. coli* Fur (Fig. 3, spectrum 2) still contained a [2Fe–2S] cluster when expressed in wild-type *E. coli* cells. However, the occupancies of [2Fe–2S] clusters (the amplitudes of the absorption peaks) in *H. influenzae* Fur and *E. coli* Fur purified from wild-type *E. coli* cells (Fig. 2) were significantly lower than those purified from the *E. coli iscA*/*sufA* mutant cells (Fig. 1B), suggesting that binding of a [2Fe–2S] cluster in Fur proteins is regulated by the levels of intracellular free iron content, and that different Fur proteins have a distinct binding activity for the [2Fe-2S] cluster.

It has been proposed that Fur proteins from *V. cholerae*, *H. pylori*, and many other bacteria bind ferrous iron and form an iron-bound Fur to regulate expression of target genes (Bagg and Neilands 1987; Hantke 2001; Pinochet-Barros and Helmann 2018). However, the iron-bound Fur has only been reconstituted *in vitro* (Mills and Marletta 2005), and has never been isolated from any bacteria. Here, we have found that Fur proteins from *E. coli*, *H. influenzae*, *V. cholerae*, and *H. pylori* can bind a [2Fe–2S] cluster (but not a mononuclear iron), and that elevated intracellular free iron promotes binding of the [2Fe–2S] cluster in Fur proteins (Figs. 1, 2). It must be pointed out that binding of the [2Fe–2S] cluster in Fur proteins does not require the deletion of IscA and SufA, because *H. influenzae* Fur and *E. coli* Fur can also bind a [2Fe–2S] cluster in wild-type *E. coli* cells (Fig. 2B). Instead, deletion of IscA and SufA results in elevation of intracellular free iron content (Jensen and Culotta 2000; Fontenot et al. 2020) and binding of a [2Fe–2S] cluster in Fur proteins in the *E. coli* cells. Indeed, preliminary studies have indicated that deletion of Fur can also increase intracellular free iron content and promote binding of the [2Fe–2S] cluster in Fur proteins in *E. coli* cells (Fontenot and Ding, unpublished results). Thus, Fur proteins may sense intracellular

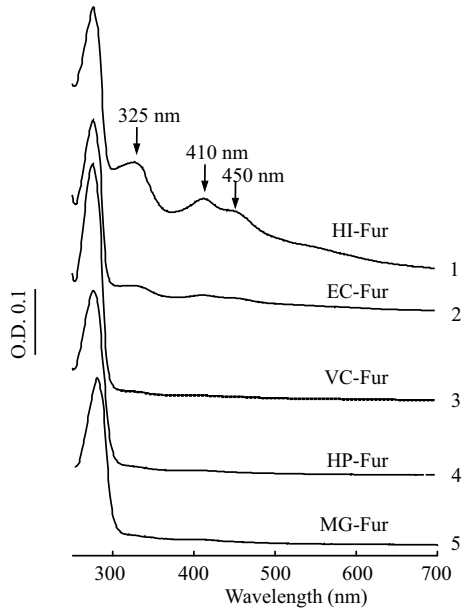

Fig. 2 Binding of a [2Fe–2S] cluster in Fur proteins expressed in wild-type *E. coli* cells. The plasmid expressing *E. coli* Fur, *V. cholerae* Fur, *H. pylori* Fur, *H. influenzae* Fur, or *M. gryphiswaldense* MSR-1 Fur was introduced into *E. coli* MC4100 cells. Each Fur protein was purified from the cells. Purified Fur proteins were diluted in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). Spectrum 1, *H. influenzae* Fur (50 μM); spectrum 2, *E. coli* Fur (50 μM); spectrum 3; *V. cholerae* Fur (50 μM); spectrum 4, *H. pylori* Fur (25 μM); spectrum 5, *M. gryphiswaldense* MSR-1 Fur (50 μM). The absorption peaks at 325 nm, 410 nm, and 450 nm indicate the binding of a [2Fe–2S] cluster in the Fur proteins

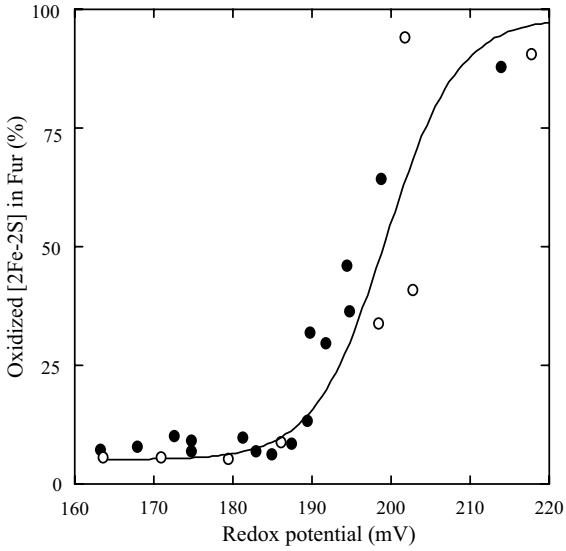

Fig. 3 Redox titration of the *E. coli* Fur [2Fe–2S] cluster. *E. coli* Fur was purified from the *E. coli iscA*/*sufA* mutant cells. *E. coli* Fur protein (containing 10 µM [2Fe–2S] clusters) was incubated with Safranin O (0.5 µM) in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0). Oxygen in the solution was purged using pure argon gas for 50 min. The redox potential was monitored using a micro redox electrode and adjusted by adding a small aliquot of freshly prepared sodium dithionite. The amplitudes of the absorption peak at 410 nm of the oxidized [2Fe–2S] cluster in Fur were plotted as a function of redox potentials in the solution. The data were fitted to a Nernst Equation with $N = 1$

free iron content via reversibly binding a [2Fe–2S] cluster in bacteria.

The *E. coli* [2Fe–2S] clusters release iron when the clusters are reduced

If Fur senses intracellular free iron content via reversibly binding a [2Fe–2S] cluster, the cluster in Fur proteins must be in a dynamic equilibrium with intracellular free iron pool in bacteria. However, purified Fur [2Fe–2S] clusters are quite stable in solution under aerobic conditions. Thus, we postulated that redox state of the [2Fe–2S] clusters in Fur may change the stability of the clusters.

To compare the stability of the [2Fe–2S] clusters in Fur when the clusters were reduced or oxidized, we first determined the redox midpoint potential of the *E. coli* Fur [2Fe–2S] clusters using the redox potentiometry as described in Dutton (1978). The data were fitted to the Nernst Equation with a redox midpoint potential (E_m) of 198 mV ± 20 mV (pH 8.0) (Fig. 3). Because the E_m value (198 ± 20 mV) of the *E. coli* Fur [2Fe–2S] clusters is much higher than the intracellular redox potential (~ − 200 mV) in *E. coli* (Ding and Demple 1997), the Fur [2Fe–2S] clusters are most likely in a reduced state in *E. coli* cells under normal growth conditions.

The purified *E. coli* Fur (containing 10 µM [2Fe–2S] clusters) was then incubated with an iron indicator Ferrozine (Cowart et al. 1993). Figure 4A shows that the *E. coli* Fur [2Fe–2S] clusters released very little or no Fe(II) after 20 min incubation with Ferrozine, confirming that the oxidized Fur [2Fe–2S] clusters are stable. However, when the purified *E. coli* Fur (containing 10 µM [2Fe–2S] clusters) was incubated with Ferrozine, followed by addition of freshly prepared sodium dithionite to reduce the [2Fe–2S] clusters, about 75% of total iron in the *E. coli* Fur [2Fe–2S] clusters were released within 2 min after addition of sodium dithionite (Fig. 4A). The Fur protein was also re-purified from the incubation solutions after the Fur [2Fe–2S] clusters were reduced with sodium dithionite. UV–Vis absorption measurements confirmed that the [2Fe–2S] clusters were removed from Fur upon reduction, while the oxidized [2Fe–2S] clusters in Fur remained unchanged (Fig. 4B).

To test whether other iron–sulfur proteins could also release iron upon reduction, we purified *E. coli* ferredoxin, a protein containing a [2Fe–2S] cluster (Rogers and Ding 2001), from the *E. coli iscA*/*sufA* mutant cells. Purified ferredoxin was pre-incubated with Ferrozine, followed by addition of freshly prepared sodium dithionite. Figure 4C shows that the ferredoxin [2Fe–2S] clusters released very little or no Fe(II) whether the clusters were oxidized or reduced. Re-purification of ferredoxin from the incubation solutions further showed that the ferredoxin [2Fe–2S] clusters remained intact after reduction with sodium dithionite (Fig. 4D). Similarly, the [2Fe–2S] clusters of the *E. coli* redox transcription factor SoxR (Ding et al. 1996) did not release any iron after reduction with sodium dithionite (data not shown). Thus, the *E. coli* Fur [2Fe–2S] clusters are unique in releasing Fe(II) upon reduction of the clusters.

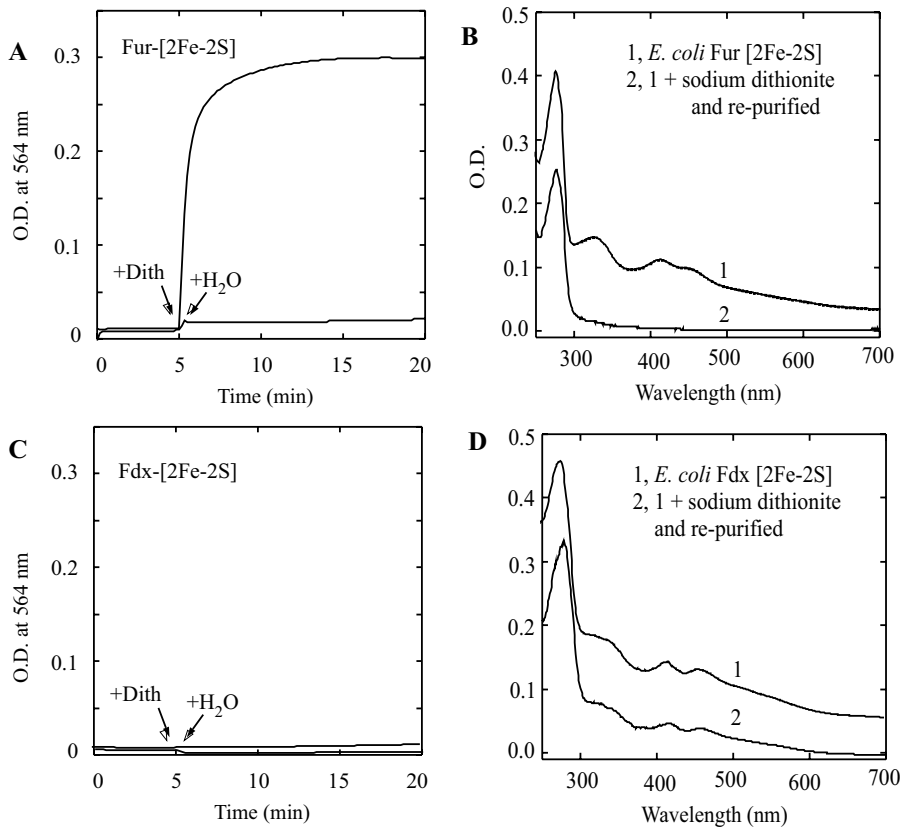

Fig. 4 The *E. coli* Fur [2Fe–2S] cluster releases iron upon reduction. **A** Purified *E. coli* Fur (containing 10 μM [2Fe–2S] cluster) was incubated with Ferrozine (500 μM) in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM) at room temperature. After 5 min incubation, either H_2O or freshly prepared sodium dithionite (2 mM) was added to the incubation solutions. The absorption at 564 nm of the Fe-Ferrozine complex was monitored as a function of time. **B** UV–Visible absorption spectra of re-purified Fur proteins. After incubation of the *E. coli* Fur with or without sodium dithionite in the presence of Ferrozine (500 μM) for 20 min at room temperature, the protein was re-purified. Spectrum 1, Fur incubated with Ferrozine only; spectrum 2, Fur incubated with Ferrozine and sodium dithionite. **C** Purified *E. coli* ferredoxin (containing 10 μM [2Fe–2S] cluster) was incubated with Ferrozine (500 μM) in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). After 5 min incubation at room temperature, either H_2O or freshly prepared sodium dithionite (2 mM) was added to the incubation solutions. The absorption at 564 nm of the Ferrozine–Fe complex was monitored as a function of time. **D** UV–Visible absorption spectra of re-purified ferredoxin proteins. After incubation of the *E. coli* ferredoxin with or without sodium dithionite in the presence of Ferrozine at room temperature for 20 min, ferredoxin was re-purified from the incubation solutions. Spectrum 1, ferredoxin incubated with Ferrozine only; spectrum 2, ferredoxin incubated with Ferrozine and sodium dithionite. The results are representatives from three independent experiments

The unstable nature of the reduced [2Fe–2S] clusters in Fur proteins is also conserved

The Fur proteins of *H. influenzae*, *V. cholera*, and *H. pylori* purified from the *E. coli iscA*/*sufA* mutant cells were also subjected to the [2Fe–2S] cluster stability analyses. Figure 5 shows that while the oxidized [2Fe–2S] clusters of the Fur proteins were stable, the Fur [2Fe–2S] clusters quickly released iron after addition of sodium dithionite. About 75–80% of total iron in the [2Fe–2S] clusters of each Fur protein were released within 2 min upon reduction of the cluster, indicating that the unstable nature of the reduced [2Fe–2S] clusters are conserved among the Fur proteins. Thus, Fur proteins may transiently bind a reduced [2Fe–2S] cluster when intracellular free iron content is elevated and quickly release iron when intracellular free iron content is decreased in cells.

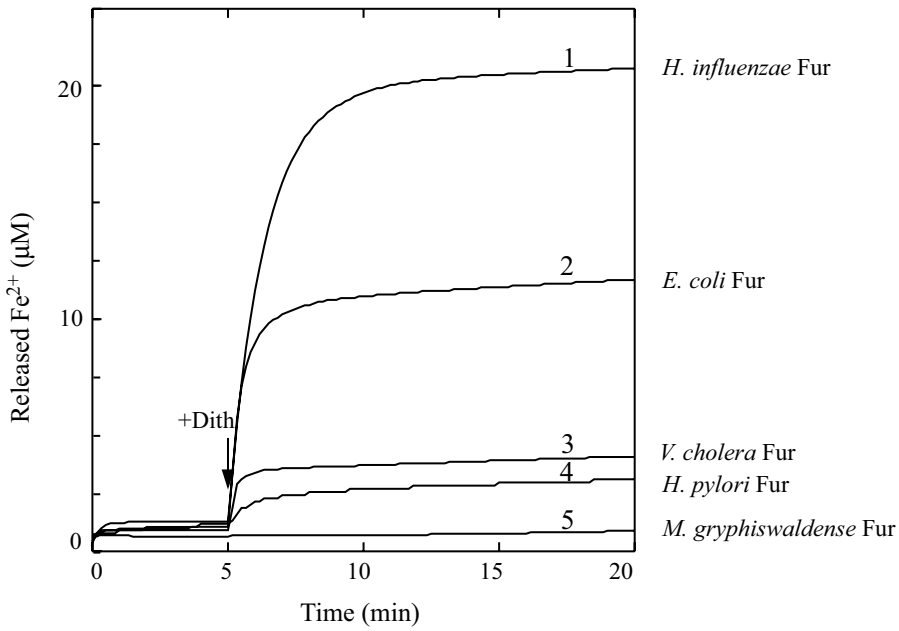

Fig. 5 Iron release from the [2Fe–2S] clusters of other Fur proteins upon reduction. *H. influenzae* Fur (trace 1), *E. coli* Fur (trace 2), *V. cholerae* Fur (trace 3), *H. pylori* Fur (trace 4), or *M. gryphiswaldense* MSR-1 Fur (50 μM) purified from the *E. coli iscA/sufA* mutant cells was pre-incubated with Tris (20 mM, pH 8.0), NaCl (500 mM) and Ferrozine (500 μM) for 5 min at room temperature, followed by adding freshly prepared sodium dithionite (2 mM). The iron release from the Fur [2Fe–2S] clusters was monitored at 564 nm as a function of time. The released iron concentration (Ferrozine–Fe complex) was calculated using an extinction coefficient of 27.9 mM^{-1} cm^{-1}. The data are representatives of three independent experiments

Use of an iron–sulfur cluster to sense intracellular free iron content is not unprecedented. For example, in mammalian cells, the iron regulatory protein-1 (IRP-1) binds a [4Fe-4S] cluster when intracellular free iron content is elevated (Schalinske et al. 1997). In yeast cells, the intracellular free iron sensors Yap5 of *S. cerevisiae* (Li et al. 2012; Rietzschel et al. 2015) and Fep1 of *Pichia pastoris* (Cutone et al. 2016) also bind a [2Fe–2S] cluster in response to an elevated intracellular free iron content. Evidently, assembly of a [2Fe–2S] cluster in Fur proteins not only requires intracellular free iron, but also sulfide. The molecular mechanisms by which the [2Fe–2S] clusters are assembled and disassembled in Fur proteins in response to intracellular free iron content remain to be further investigated. Nevertheless, our results suggest that Fur can reversibly bind a transient [2Fe–2S] cluster in response to elevation of intracellular free iron content and release iron from the clusters when intracellular free iron content is decreased in bacteria.

Conclusion

In summary, we find that binding of a [2Fe–2S] cluster is highly conserved among Fur proteins that contain Cys-93 and Cys-96 and that elevation of intracellular free iron content promotes binding of a [2Fe–2S] cluster in Fur proteins in *E. coli* cells. Among the Fur proteins studied, *H. influenzae* Fur has the strongest binding activity for the [2Fe–2S] cluster, followed by *E. coli* Fur, *V. cholerae* Fur, and *H. pylori* Fur, indicating that Fur proteins from various bacteria have a distinct binding activity for the [2Fe–2S] cluster. We conclude that the global regulator Fur may represent a new member of the transcription factor family that senses intracellular free iron content to regulate the expression of target genes via reversible binding of a [2Fe–2S] cluster in bacteria. Research is in progress to illustrate the molecular mechanisms on how Fur binds

a [2Fe–2S] cluster and how the [2Fe–2S] cluster-bound Fur represses expression of target genes in bacteria.

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