Ferric uptake regulator (Fur) reversibly binds a [2Fe-2S] cluster to sense intracellular iron homeostasis in escherichia coli

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The ferric uptake regulator (Fur) is a global transcription factor that regulates intracellular iron homeostasis in bacteria. The current hypothesis states that when the intracellular “free” iron concentration is elevated, Fur binds ferrous iron, and the iron-bound Fur represses the genes encoding for iron uptake systems and stimulates the genes encoding for iron storage proteins. However, the “iron-bound” Fur has never been isolated from any bacteria. Here we report that the Escherichia coli Fur has a bright red color when expressed in E. coli mutant cells containing an elevated intracellular free iron content because of deletion of the iron–sulfur cluster assembly proteins IscA and SufA. The acid-labile iron and sulfide content analyses in conjunction with the EPR and Mössbauer spectroscopy measurements and the site-directed mutagenesis studies show that the red Fur protein binds a [2Fe-2S] cluster via conserved cysteine residues (Cys-93, Cys-96, and Cys-133) in the Fur protein is ~31% in the E. coli iscA/sufA mutant cells and is decreased to ~4% in WT E. coli cells. Depletion of the intracellular free iron content using the membrane-permeable iron chelator 2,2´-dipyridyl (200 μM) effectively removes the [2Fe-2S] cluster from Fur in E. coli cells, suggesting that Fur senses the intracellular free iron content via reversible binding of a [2Fe-2S] cluster. The binding of the [2Fe-2S] cluster in Fur appears to be highly conserved, because the Fur homolog from Hemophilus influenzae expressed in E. coli cells also reversibly binds a [2Fe-2S] cluster to sense intracellular iron homeostasis.

The bacterial intracellular “free” iron concentration is primarily regulated by a global transcription factor, Ferric uptake regulator (Fur) (1–4). It has been generally assumed that when the intracellular free iron concentration is elevated, Fur binds free ferrous iron, and the iron-bound Fur represses the genes encoding for iron uptake systems and stimulates the genes encoding for iron storage proteins (5–9). The crystallographic studies of the Fur proteins from Escherichia coli (10), Mycobacterium tuberculosis (11), Vibrio cholerae (12), Helicobacter pylori (13), Campylobacter jejuni (14), and Francisella tularensis (15) have revealed that Fur protein exists as a homodimer or tetramer (8) with each monomer containing three putative metal-binding sites. The first metal-binding site (site 1) is coordinated by His-87, Asp-89, Glu-108, and His-125 (the residue numbers are based on the E. coli Fur), whereas the second site (site 2) is coordinated by His-33, Glu-81, His-88, and His-90 (12). The third metal-binding site (site 3) is formed by three conserved cysteine residues (Cys-93, Cys-96, and Cys-133) (11–15). However, the metal-binding sites in purified Fur proteins are often occupied by zinc or other metal ions, and the “iron-bound” Fur has never been isolated from any bacteria. Iron–sulfur proteins are the major iron-containing proteins in cells (16). Recent studies have demonstrated that iron–sulfur clusters in proteins are assembled by a group of dedicated proteins (17, 18). Among the iron–sulfur cluster assembly proteins in E. coli, IscA has been characterized as an alternative scaffold (19) or iron chaperone to recruit the intracellular free iron for iron–sulfur cluster assembly (20, 21). Depletion of IscA and its homologs inhibits the [4Fe-4S] cluster assembly without affecting the [2Fe-2S] cluster assembly (22). Saccharomyces cerevisiae (23), and human (24) cells, indicating that the [2Fe-2S] clusters and [4Fe-4S] clusters have distinct biogenesis pathways (25). Furthermore, deletion of IscA and its homologs significantly increases the intracellular free iron content in E. coli (22), S. cerevisiae (26), and human (24) cells. Inspired by these observations, we reasoned that the global iron regulator Fur may become iron-bound in the E. coli mutant cells in which IscA and its paralog SufA are deleted. Here, we find that recombinant E. coli Fur protein indeed has a bright red color when expressed in the E. coli iscA/sufA mutant cells under aerobic growth conditions. The iron and sulfide content analyses and the EPR and Mössbauer spectroscopy measurements show that the red Fur protein binds a novel [2Fe-2S] cluster. Site-directed mutagenesis studies further indicate that the conserved cysteine residues (Cys-93, Cys-96, and Cys-133) in E. coli Fur are required for the binding of the [2Fe-2S] cluster. The occupancy of the [2Fe-2S] cluster in Fur protein is ~31% when expressed in the E. coli iscA/sufA mutant cells and is decreased to ~4% in WT E. coli cells. Moreover, when the intracellular free iron content is depleted using the membrane-permeable iron chelator 2,2´-dipyridyl (200 μM), the [2Fe-2S] cluster in Fur is effectively removed in both WT and iscA/sufA mutant E. coli cells. Because the addition of 2,2´-dipyridyl (200 μM) to E. coli cells switches on the expression of the Fur-repressed targeted genes in E. coli cells (27), we propose that the E. coli Fur may sense the intracellular free iron content via reversible binding of a [2Fe-2S] cluster. Importantly, binding of the [2Fe-2S] cluster in Fur appears to be highly conserved as the Fur
Results

Deletion of the iron–sulfur cluster assembly protein IscA and its paralog SufA leads to accumulation of the intracellular free iron content in E. coli cells

In S. cerevisiae (26) and human (24) cells, depletion of the iron–sulfur cluster assembly protein IscA homologs results in substantial iron accumulation in mitochondria. To evaluate the intracellular free iron content in the E. coli mutant cells in which IscA and its paralog SufA were deleted (22), we used the whole-cell EPR measurements following the procedures described in Ref. 28. Briefly, exponentially growing E. coli cells were treated with the membrane-permeable iron chelator desferrioxamine. The cells were then washed with the membrane-impermeable iron chelator diethylenetriaminepentaacetic acid to remove the extracellular free iron. Because the desferrioxamine-ferrous iron complex has an EPR signal at $g = 4.3$, the amplitude of the EPR signal represents the relative concentration of the intracellular “chelatable” iron (28). The detected intracellular chelatable iron pool has been defined as the free iron associated with metabolites (e.g. GSH, citrate, and phosphorylated sugar intermediates) (6, 28–30). As shown in Fig. 1, the intracellular chelatable iron content in the iscA/sufA mutant cells is approximately two times that of WT cells grown in LB medium under aerobic conditions. This assay is of limited utility because it cannot observe ferrous iron and ferric nanoparticles (both of which have been identified in cells (31, 32)); therefore, it would be difficult to determine the exact concentration of the intracellular free iron in E. coli cells. Nevertheless, the results in Fig. 1 clearly suggested that deletion of IscA and its homolog SufA in E. coli cells increases the intracellular chelatable iron content, consistent with the previous observations made in S. cerevisiae (26) and human (24) cells.

The ferric uptake regulator (Fur) has a bright red color when expressed in the E. coli iscA/sufA mutant cells

Here, we took advantage of the E. coli iscA/sufA mutant, which has an elevated intracellular free iron content (Fig. 1), to explore the possible iron binding of the global iron regulator Fur in vivo. In the experiments, we introduced a plasmid (pBAD) expressing the E. coli Fur into E. coli WT and the iscA/sufA mutant cells grown in LB medium under aerobic conditions. Recombinant Fur protein was purified from both cells. As reported previously by other research groups (33–35), the Fur protein purified from WT E. coli cells is essentially colorless. In contrast, the Fur protein purified from the E. coli iscA/sufA mutant cells has a bright red color (inset in Fig. 2A). The UV-visible absorption measurements showed that the red Fur protein has three major absorption peaks at 325, 410, and 450 nm, in addition to the protein peak at 280 nm (Fig. 2A), suggesting that the red Fur protein may bind a mononuclear iron or iron–sulfur cluster.

Careful examination of the UV-visible spectra in Fig. 2A revealed that the Fur protein purified from WT E. coli cells also has the absorption peaks at 325, 410, and 450 nm, although their amplitudes are only approximately one-eighth of those of the red Fur protein purified from the E. coli iscA/sufA mutant cells (Fig. 2B). The small amplitudes of the absorption peaks at 325, 410, and 450 nm of the Fur protein purified from WT E. coli cells might have been overlooked previously, especially when the concentration of purified Fur protein was low. Thus, the red Fur protein is present not only in the iscA/sufA mutant cells but also in WT E. coli cells, and the relative concentration of the red Fur protein in the iscA/sufA mutant cells is approximately eight times that in WT cells.

The red Fur protein contains a [2Fe-2S] cluster

To determine whether the red Fur protein binds a mononuclear iron or iron–sulfur cluster, we first analyzed the acid-labile iron and sulfide contents of the protein. From three independent experiments, we found $0.6 \pm 0.2$ iron and $0.4 \pm 0.2$ sulfide atoms per each Fur monomer purified from WT E. coli cells also has the absorption peaks at 325, 410, and 450 nm, although their amplitudes are only approximately one-eighth of those of the red Fur protein purified from the E. coli iscA/sufA mutant cells (Fig. 2B). The small amplitudes of the absorption peaks at 325, 410, and 450 nm of the Fur protein purified from WT E. coli cells might have been overlooked previously, especially when the concentration of purified Fur protein was low. Thus, the red Fur protein is present not only in the iscA/sufA mutant cells but also in WT E. coli cells, and the relative concentration of the red Fur protein in the iscA/sufA mutant cells is approximately eight times that in WT cells.

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The red Fur protein contains a [2Fe-2S] cluster

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The iron–sulfur cluster in the red Fur protein was further investigated by the EPR spectroscopy. Although as-purified red Fur protein has no EPR signals, the dithionite-reduced red Fur protein has an EPR signal at $g_z = 1.91$, $g_y = 1.94$, and $g_x = 2.00$ (Fig. 2D), which is similar to that of other iron–sulfur proteins (36, 37), thus confirming that the red Fur protein contains an iron–sulfur cluster. Interestingly, unlike other iron–sulfur proteins, the reduced [2Fe-2S] cluster in Fur is not stable and quickly decomposes, thus preventing spin quantification of the [2Fe-2S] cluster in Fur using the EPR spectroscopy.

To explore the nature of the iron–sulfur cluster in the red Fur protein, we have utilized Mössbauer spectroscopy (38). The $^{57}$Fe-enriched Fur protein was prepared from the E. coli iscA/sufA mutant cells grown in $^{57}$Fe-enriched M9 minimum medium. The Mössbauer spectroscopy was conducted at
variable temperatures and in applied magnetic fields (38, 39). As shown in Fig. 3, the 5.5 K Mössbauer spectrum in a 70-mT applied field exhibits a well-defined quadrupole doublet with isomer shift $\delta = 0.29(2)$ mm/s, $\Delta E_Q$ of 0.53(1) mm/s and line widths of 0.33 mm/s. These parameters are typical for $[2\text{Fe}-2\text{S}]^{2+}$ clusters with distorted tetrahedral thiolate coordination (39, 40) and are very different from the Mössbauer spectrum of the in vitro ferrous iron-reconstituted $E. coli$ Fur which has an isomer shift of $\delta = 1.19$ (1) mm/s and a quadrupole splitting of $\Delta E_Q = 3.47$ (2) mm/s (41). The spectrum in 7.0-T applied magnetic field exhibits magnetic splitting that could be attributed solely to the applied field. The absence of internal fields in the 7.0-T spectrum indicates that the cluster in the sample is diamagnetic, consistent with two iron Fe$^{3+}$ ions, which are antiferromagnetically coupled. Together with the observed values of the $\delta$ and $\Delta E_Q$, which are similar to values seen for the protein-bound $[2\text{Fe}-2\text{S}]^{2+}$ clusters (39, 40), the diamagnetism of the sample provides unambiguous evidence for the presence of an oxidized $[2\text{Fe}-2\text{S}]^{2+}$ cluster, which is also consistent with the UV-visible absorption spectrum (Fig. 2A). The spectra in low applied magnetic fields show a minor spectral component, indicated by the small arrow in Fig. 3.
The cysteine residues in Fur are the likely ligands for the [2Fe-2S] cluster

The E. coli Fur protein has three distinct metal-binding sites (10). Sites 1 and 2 are coordinated by His, Asp, and Glu, and binding of zinc in these sites has been attributed to stabilization of the Fur structure (10). To test whether site 1 or site 2 is involved in binding the [2Fe-2S] cluster, we constructed the Fur mutants in which each cysteine residues (Cys-93, Cys-96, and Cys-133) (10). We thus constructed Fur mutants in which each of the cysteine residues was replaced with alanine. The fourth cysteine residue (Cys-138) in the E. coli Fur protein is not conserved. Attempts to replace Cys-138 with Ser and found that mutation of Cys-138 to Ser did not abolish the [2Fe-2S] cluster binding in the E. coli Fur but significantly changed the UV-visible absorption spectrum of the [2Fe-2S] cluster in Fur. Regardless, the results clearly suggested that the E. coli Fur protein likely binds the [2Fe-2S] cluster via the conserved cysteine residues (Fig. 4B), which is consistent with the Mössbauer spectroscopy data (Fig. 3).

Depletion of the intracellular free iron content removes the [2Fe-2S] cluster from the Fur in E. coli cells

If the [2Fe-2S] cluster-bound Fur protein represents an active form of the Fur repressor in E. coli cells when the intracellular free iron content is elevated, it is expected that depletion of the intracellular free iron content will remove the [2Fe-2S] cluster from Fur. Because the membrane-permeable iron chelator 2,2′-dipyridyl has often been used to deplete the intracellular free iron content in E. coli cells (27), we treated WT E. coli cells expressing recombinant Fur protein with 2,2′-dipyridyl. Fur protein was then purified from the cells. Figure 5 shows that addition of 200 μM of 2,2′-dipyridyl effectively removes the [2Fe-2S] cluster from the Fur protein in WT E. coli cells. We also treated the E. coli isca/sufA mutant cells expressing recombinant Fur with 200 μM of 2,2′-dipyridyl and found that the [2Fe-2S] cluster is also removed from the Fur protein in the E. coli isca/sufA mutant cells by 2,2′-dipyridyl (Fig. 5B).
Furthermore, the binding of the [2Fe-2S] cluster in Fur is reversible in *E. coli* cells, because addition of ferrous ammonium sulfate (300 μM) to the 2,2´-dipyridyl–treated *E. coli* cells restores the [2Fe-S] cluster binding in Fur (data not shown). Because addition of 2,2´-dipyridyl to *E. coli* cells switches on the expression of the Fur-repressed targeted genes in *E. coli* cells (27), we propose that removal of the [2Fe-2S] cluster from Fur in *E. coli* cells by 2,2´-dipyridyl may represent deactivation of Fur in response to depletion of the intracellular free iron content and that Fur senses the intracellular free iron content via reversible binding of the [2Fe-2S] cluster in *E. coli* cells.

The *H. influenzae* Fur also binds a [2Fe-2S] cluster

The Fur protein is highly conserved among bacteria (10–15). To test whether other Fur proteins can also bind a [2Fe-2S] cluster, we synthesized a gene encoding the Fur homolog from *H. influenzae*, a Gram-negative facultatively anaerobic pathogenic bacterium. The *H. influenzae* Fur has 65% identity and 77% similarity with the *E. coli* Fur (Fig. 6A). The *H. influenzae* Fur protein was expressed in the *E. coli* *iscA*/*sufA* mutant cells and purified from the cells. As shown in Fig. 6B, the purified *H. influenzae* Fur has the same absorption peaks at 325, 410, and 450 nm as the *E. coli* red Fur protein. Furthermore, mutations of the conserved cysteine residues (Cys-94, Cys-97, and Cys-134) to Ala in the *H. influenzae* Fur eliminated the [2Fe-2S] cluster binding (data not shown), suggesting that like the *E. coli* Fur, the *H. influenzae* Fur binds the [2Fe-2S] cluster via the conserved cysteine residues.

Interestingly, although the protein yield of the *H. influenzae* Fur was similar to that of the *E. coli* Fur when expressed in the *E. coli* *iscA*/*sufA* mutant cells, the amplitudes of the absorption peaks at 325, 410, and 450 nm of the purified *H. influenzae* Fur were much higher than those of the red *E. coli* Fur (Fig. 6B). Assuming that the extinction coefficient of the [2Fe-2S] cluster in Fur proteins at 450 nm is 10 mM$^{-1}$ cm$^{-1}$ (36, 37), we estimated that the occupancy of the [2Fe-2S] cluster in the *H. influenzae* Fur expressed in the *E. coli* *iscA*/*sufA* mutant cells is ~68%, whereas that of the *E. coli* Fur protein is ~31% (Fig. 6B). This result implies that the *H. influenzae* Fur has a higher binding affinity for the [2Fe-2S] cluster than the *E. coli* Fur in the *E. coli* *iscA*/*sufA* mutant cells. To test this idea further, we expressed the *H. influenzae* Fur and the *E. coli* Fur in WT *E. coli* cells and purified both proteins. As shown in Fig. 6C, the occupancy of the [2Fe-2S] cluster in the *H. influenzae* Fur (~30%) is again much higher than that of the *E. coli* Fur (~3%) when expressed in WT *E. coli* cells. We also carried out the *in vitro* reconstitution experiments. When the apo-form *E. coli* Fur was incubated with 4-fold excess of ferrous iron and sulfide in the presence of DTT, only ~5% of the apo-form *E. coli* Fur was reconstituted with a [2Fe-2S] cluster. In contrast, when the apo-form *H. influenzae* Fur was incubated with 4-fold excess of ferrous iron and sulfide under the same experimental

**Figure 6.** The *H. influenzae* Fur also binds a [2Fe-2S] cluster when expressed in *E. coli* cells. A, the sequence alignment of the *H. influenzae* Fur and the *E. coli* Fur. The three metal-binding sites in Fur proteins are highlighted in red (site 1), yellow (site 2), and green (site 3), respectively. B, UV-visible absorption spectra of the *H. influenzae* Fur (spectrum 1) and the *E. coli* Fur (spectrum 2) purified from the *E. coli* *iscA*/*sufA* mutant cells. C, UV-visible absorption spectra of the *H. influenzae* Fur (spectrum 1) and the *E. coli* Fur (spectrum 2) purified from the *E. coli* WT cells. The protein concentrations were 100 μM. The results are representative of three independent experiments.
conditions, ~50% of the apo-form *H. influenzae* Fur was reconstituted with a [2Fe-2S] cluster (Fig. S2). Thus, whereas both the *E. coli* Fur and the *H. influenzae* Fur can bind a [2Fe-2S] cluster via conserved cysteine residues, the *H. influenzae* Fur has a much higher binding affinity for the [2Fe-2S] cluster than the *E. coli* Fur, suggesting that *E. coli* and *H. influenzae* may have distinct genetic responses to intracellular iron homeostasis via Fur.

**Discussion**

In the past decades, it has been well-established that when the intracellular free iron content is elevated in bacteria, the global transcription factor Fur binds free ferrous iron to repress the genes encoding for iron uptake systems and to stimulate the genes encoding for iron storage proteins in bacteria (5–9). Although the purified *E. coli* Fur has been reconstituted with ferrous iron in vitro (41, 43), the iron-bound Fur has never been isolated from *E. coli* or any other bacteria. This could be because the intracellular free iron content is mainly regulated by Fur (5–9), and substantially increasing the intracellular free iron concentration would be challenging without deleting Fur in bacteria. The *E. coli* *iscA/sufA* mutant (22) provides a unique opportunity to explore the possible iron binding of Fur in vivo, because deficiency of iron–sulfur cluster biogenesis caused by deletion of *iscA* and its homologs increases the intracellular free iron content (22, 24, 26) (Fig. 1). Furthermore, deletion of *iscA* and its homologs only inhibits [4Fe-4S] cluster assembly without affecting [2Fe-2S] cluster assembly in *E. coli* (22), *S. cerevisiae* (23), and human (24) cells. Here, we took advantage of the *E. coli isca/sufA* mutant cells (22) and found that the Fur protein expressed in the *E. coli isca/sufA* mutant cells has a bright red color. The iron and sulfide content analyses in conjunction with the UV-visible absorption, EPR, and Mössbauer measurements suggest that the red Fur protein primarily binds a [2Fe-2S] cluster, and only a minor fraction of the mononuclear iron coordination (~8% of total iron content) is associated with Fur (possibly in site 1 or 2). Additional studies reveal that the [2Fe-2S] cluster-bound Fur is present not only in the *iscA/sufA* mutant cells but also in WT *E. coli* cells (Fig. 2). The occupancy of the [2Fe-2S] cluster in the Fur protein is ~31% in the *E. coli isca/sufA* mutant cells and is decreased to ~4% in WT *E. coli* cells. Furthermore, depletion of the intracellular free iron content using the membrane-permeable iron chelator 2,2'-dipyridyl (200 μM) effectively removes the [2Fe-2S] cluster from the Fur protein in both WT and *iscA/sufA* mutant cells (Fig. 5), suggesting that the *E. coli* Fur senses the intracellular free iron content via reversible binding of a [2Fe-2S] cluster in cells.

The UV-visible absorption and EPR spectra of the *E. coli* red Fur protein are reminiscent of [2Fe-2S] cluster-containing proteins (36, 37). The unambiguous evidence for the presence of a [2Fe-2S] cluster in the *E. coli* red Fur protein comes from the Mössbauer spectroscopic studies. In the literature, the Mössbauer isomer shifts (δ) for diamagnetic iron–sulfur clusters with tetrahedral cysteine ligation have been documented to be in the range of ~0.27 mm/s for the [2Fe-2S]2+ and ~0.45 mm/s for the [4Fe-4S]3+ (38) (Table S1). The Mössbauer parameters observed in the *E. coli* red Fur protein (δ = 0.29(2) mm/s and ΔEQ = 0.53(1)) (Fig. 3) represent a typical [2Fe-2S] cluster and are virtually identical to the [2Fe-2S] cluster associated with the oxygen-exposed FNR protein (δ = 0.28(1) mm/s and ΔE = 0.58(2) mm/s) (39) and the [2Fe-2S] cluster of the human mitochondrial glutaredoxin 2 (Grx2) (δ = 0.27 mm/s and ΔE = 0.60 mm/s) (40). It should be pointed out that the Mössbauer spectrum of the *E. coli* red Fur protein (Fig. 3) is very different from the Mössbauer spectrum of the *in vitro* ferrous iron-reconstituted *E. coli* Fur, which has an isomer shift of δ = 1.19 (1) mm/s and a quadrupole splitting of ΔE = 3.47(2) mm/s (41, 44), representing the binding of ferrous iron at site 2 via His-33, Glu-81, His-88, and His-90 in Fur protein (44). Although a small fraction (~8%) of the total iron content in the red Fur protein is found to be the mononuclear iron component (some of the iron component could be generated during protein purification), ~92% of the total iron content in the red Fur protein is assigned to the [2Fe-2S] cluster in the protein (Fig. 3). Thus, again against all previous ideas, the *E. coli* Fur is a novel [2Fe-2S] cluster-binding protein.

Iron–sulfur clusters are the major group of iron-containing co-factors in cells. It has been reported that biogenesis of iron–sulfur clusters is regulated not only by the iron–sulfur cluster assembly transcription factor IscR (45) but also by the global iron regulator Fur (3, 46). Our finding that Fur senses the intracellular free iron content via binding of a [2Fe-2S] cluster provides a new aspect for the physiological link between intracellular free iron homeostasis and iron–sulfur cluster biogenesis. Use of an iron–sulfur cluster to sense the intracellular free iron content is not unprecedented. In mammalian cells, IRP-1 (iron regulatory protein 1) regulates the intracellular free iron content by reversible binding of a [4Fe-4S] cluster in response to an elevated intracellular free iron content (47). It is appealing to suggest that the *E. coli* Fur, like IRP-1, may also bind a [4Fe-4S] cluster in response to elevation of the intracellular free iron content. However, this is not likely, because (a) the *E. coli isca/sufA* mutant cells cannot assemble [4Fe-4S] clusters in proteins under aerobic growth conditions (22), eliminating the possibility of the [4Fe-4S] cluster binding in Fur protein in the *iscA/sufA* mutant cells; (b) purification of recombinant Fur protein from the *E. coli isca/sufA* cells under argon atmosphere does not significantly change the content of the [2Fe-2S] cluster in Fur protein (data not shown); (c) the *H. influenzae* Fur proteins expressed in both WT and *iscA/sufA* mutant *E. coli* cells contain the [2Fe-2S] cluster (Fig. 6); and (d) in yeast cells, the cellular iron sensors Yap5 of *S. cerevisiae* (48, 49) and Fep1 of *Pichia pastoris* (50) also bind a [2Fe-2S] cluster in response to an elevated intracellular free iron content. Thus, it is most likely that Fur binds a [2Fe-2S] cluster (not a [4Fe-4S] cluster or a mononuclear iron) when the intracellular free iron content is elevated in the *E. coli isca/sufA* mutant cells. Use of a [2Fe-2S] cluster in Fur to sense the intracellular free iron content may represent physiological connections between intracellular iron homeostasis and regulation of acid tolerance, oxidative stress response, and bacterial virulence (8), because assembly of the [2Fe-2S] cluster in Fur requires not only the intracellular free iron but also sulfide that is derived from L-cysteine by cysteine desulfurase IscS (17). In this context, we propose

**EDITORS' PICK:** Fur senses intracellular iron via binding a [2Fe-2S] cluster
that while elevation of the intracellular free iron content together with available sulfide leads to assembly of a [2Fe-2S] cluster in Fur and formation of an active Fur repressor in cells, depletion of the intracellular free iron content results in disassembly of the [2Fe-2S] cluster in Fur and inactivates Fur as a repressor. In WT E. coli cells, only ~4% of Fur protein binds a [2Fe-2S] cluster, indicating that majority of Fur will be in an inactive form under normal growth conditions.

In the E. coli iscA/sufA mutant cells, an elevated intracellular free iron content increases the occupancy of the [2Fe-2S] cluster in Fur protein to ~31% (Fig. 2), which would shift a significant amount of inactive Fur to an active Fur repressor. Thus, reversible binding of the [2Fe-2S] cluster in Fur may reflect the intracellular free iron content and define the activity of Fur as a global transcription regulator in E. coli cells.

Fur is highly conserved among bacteria (11–15). With a few exceptions, the conserved three cysteine residues in Fur proteins from both Gram-negative and Gram-positive bacteria are arranged in a CX3-CX3-C motif. The CX3-C sequence has often been associated with proteins that bind iron–sulfur clusters (51). Using the site-directed mutagenesis, we have identified three conserved cysteine residues in the E. coli Fur as the likely ligands for the [2Fe-2S] cluster. The fourth cysteine residue (Cys-138) is not conserved, and mutation of Cys-138 to Ser seemed to change the [2Fe-2S] cluster binding in the E. coli Fur. Thus, the fourth ligand for the [2Fe-2S] cluster could be Cys-138 in the E. coli Fur. It is worth mentioning that among 16 mutations of the metal-binding sites in the E. coli Fur protein, only Cys-93 and Cys-96 were found to be important for the repressor activity of Fur in E. coli cells (52), indicating that the [2Fe-2S] cluster binding could be crucial for the physiological function of Fur in E. coli cells. Interestingly, although both the E. coli Fur and the H. influenzae Fur can bind a [2Fe-2S] cluster via the conserved cysteine residues, the binding affinity of the H. influenzae Fur for the [2Fe-2S] cluster is significantly higher than that of the E. coli Fur in E. coli cells (Fig. 6, B and C). The higher binding affinity for the [2Fe-2S] cluster implies that the H. influenzae Fur will become an active repressor at a lower intracellular free iron content than the E. coli Fur. Thus, H. influenzae and E. coli likely have distinct genetic responses to intracellular iron homeostasis via Fur, and the higher binding affinity of the H. influenzae Fur for the [2Fe-2S] cluster could be vital for regulating intracellular iron homeostasis in H. influenzae. The specific amino acid residues that contribute to the higher binding affinity of the H. influenzae Fur for the [2Fe-2S] cluster and their physiological significance remain to be further investigated.

In summary, the E. coli Fur is a novel [2Fe-2S] protein, and occupancy of the [2Fe-2S] cluster in Fur is regulated by the intracellular free iron content. When the intracellular free iron content is elevated, Fur reversibly binds a [2Fe-2S] cluster via the conserved cysteine residues in E. coli cells. Because only the crystal structure of a truncated E. coli Fur (residues 1-82) is available (10), we have modeled a full-length E. coli Fur protein (Fig. 7) using the RaptorX structure prediction software (53). Overall, the predicted structure of the full-length E. coli Fur is similar to the crystal structures of Fur proteins from other bacteria (11–15). In the predicted model, the conserved cysteine residues (Cys-93, Cys-96, and Cys-113) in the E. coli Fur are closely positioned for hosting a [2Fe-2S] cluster (Fig. 7). We envision that binding of the [2Fe-2S] cluster will change the protein conformation of Fur in response to an elevated intracellular free iron content and switch an inactive Fur to an active [2Fe-2S]-bound Fur repressor in bacteria.

**Experimental procedures**

**E. coli strains**

The E. coli iscA/sufA mutant was previously constructed from WT E. coli strain (MC4100) as described in Ref. 22. With exception of the Mössbauer sample preparation, E. coli WT and the iscA/sufA mutant strains were grown in LB medium at 37 °C under aerobic conditions.

**Protein purification**

Genes encoding the E. coli Fur and the H. influenzae Fur were synthesized (Genscript Co.) and cloned to plasmid pBAD for protein expression in E. coli cells. The plasmid with the cloned gene was introduced into the E. coli WT (MC4100) or the iscA/sufA mutant cells. Fur protein was overproduced in the E. coli cells by adding 0.2% L-arabinose for 4 h and purified following the procedure described in Ref. 33. In some experiments, an N-terminal His tag was used for quick purification of Fur protein from E. coli cells. The N-terminal His tag has no contribution to the [2Fe-2S] cluster binding in Fur protein, because the Fur protein with or without His tag purified from the E. coli iscA/sufA mutant cells have the same red color and same UV-visible absorption spectrum. The purity of all purified proteins was greater than 95% as judged by electrophoresis.
analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The UV-visible absorption spectra of purified proteins were recorded in a Beckman DU640 UV-visible absorption spectrometer. The extinction coefficients for the E. coli apo-Fur and the H. influenzae apo-Fur at 280 nm are 6.2 and 6.9 mM⁻¹ cm⁻¹, respectively.

Site-directed mutagenesis studies
Site-directed mutagenesis was carried out using the QuikChange kit (Agilent Co.). The mutations were confirmed by direct sequencing (Eurofins Genomics Co.). The mutated Fur proteins were expressed in the E. coli iscA/sufA mutant cells and purified as described for the WT Fur protein.

Iron, sulfide, and zinc content analyses
Total iron content in protein samples was determined using the iron indicator ferrozine following the procedures described in Ref. 54. The absorption peak at 562 nm of the Fe(II)–ferrozine complex was used for quantifying the iron content using an extinction coefficient of 27.9 mM⁻¹ cm⁻¹. The sulfide content in protein samples was determined following the procedures described by Siegel (55). The zinc content in protein samples was determined using 4-(2-pyridylazo)-resorcinol as described in Ref. 56.

EPR measurements of purified fur
The X-band EPR spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions were as follows: microwave frequency, 9.47 GHz; microwave power, 1.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; and receiver gain, 2 × 10⁵.

Intracellular chelatable iron content analyses using the whole-cell EPR
The intracellular free iron content in E. coli cells were measured following the procedures described in Ref. 28. Overnight bacterial cultures were diluted 100-fold into 500 ml of fresh LB medium at 37 °C with agitation. The cultures were grown to an A₆₀₀ of 0.2. The cells were harvested by centrifugation at 8000 rpm for 5 min, and the pellets were resuspended in LB containing 20 mM desferrioxamine to an A₆₀₀ of 5.0. The cells were then incubated at 37 °C for 15 min and washed with 10 mM diethylenetriaminepentaacetic acid once. The suspension was further washed twice with 20 mM cold Tris-Cl (pH 7.4) and resuspended in 20 mM cold Tris-Cl (pH 7.4) containing 10% glycerol. The cell suspension was transferred to an EPR tube and frozen in liquid nitrogen until EPR measurements.

Mössbauer spectroscopy
For the Mössbauer experiments, the ⁵⁷Fe-labeled Fur was prepared by expressing the protein in the E. coli iscA/sufA mutant cells grown in M9 minimum medium supplemented with 20 amino acids (each amino acid at 40 μg/ml), thiamine (0.5 μg/ml), glycerol (0.2%), and ⁵⁷Fe (10 μM) under aerobic growth conditions. The ⁵⁷Fe-labeled Fur purified from the E. coli iscA/sufA mutant cells had the same UV-visible absorption spectrum with the absorption peaks at 325, 410, and 450 nm. The Mössbauer spectra were recorded on a closed-cycle refrigerator spectrometer (model CCR4K; SeeCo, Edina, MN, USA) equipped with a 0.07-T permanent magnet, maintaining temperatures between 5 and 300 K. High-field (7.0 T) spectra were collected in Dr. Yisong (Alex) Guo’s laboratory (Carnegie Mellon University) on a constant-acceleration spectrometer housed in a cryostat equipped with a superconducting magnet at 4.2 K. The samples consisted of buffered solutions of protein in Delrin 1.0-ml cups, frozen in liquid nitrogen. The isomer shifts are quoted at 5 K with respect to iron metal standard at 298 K. The Mössbauer spectra were analyzed using the software SpinCount (Michael Hendrich, Ph.D., Carnegie Mellon University and WM0SS4 (Ion Prisecaru).

Data availability
All data generated during this study are included in this published article and its supporting information files.

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