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

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Abstract

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 due to deletion of the iron-sulfur cluster assembly proteins IscA and SufA. The acid-labile iron and sulfide content analyses in conjunction with the electron paramagnetic resonance 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. The occupancy of the [2Fe-2S] cluster in Fur protein is about 31% in the *E. coli iscA/sufA* mutant cells and is decreased to about 4% in wild-type *E. coli* cells. Depletion of the intracellular "free" iron content using the membrane-permeable iron chelator 2,2'-dipyridyl 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, as the Fur homolog from *Haemophilus influenzae* expressed in *E. coli* cells also reversibly binds a [2Fe-2S] cluster to sense intracellular iron homeostasis.

Introduction

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), while 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 in *E. coli* (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 electron paramagnetic resonance (EPR) and Mössbauer spectroscopy measurements show that the red Fur protein binds a novel [2Fe-2S] cluster. The site-directed mutagenesis studies further indicate that the conserved cysteine residues (Cys-93, Cys-96, and Cys-133) in the *E. coli* Fur are required for the binding of the [2Fe-

2S] cluster. The occupancy of the [2Fe-2S] cluster in Fur protein is about 31% when expressed in the *E. coli iscA/sufA* mutant cells and is decreased to about 4% in wild-type *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 wild-type and *iscA/sufA* mutant *E. coli* cells. Because 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 homolog from *Haemophilus influenzae* can also bind a [2Fe-2S] cluster via the conserved cysteine residues when expressed in *E. coli* cells.

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 electron paramagnetic resonance (EPR) measurements following the procedures described in (28). Briefly, exponentially growing *E. coli* cells were treated with a membrane-permeable iron chelator desferrioxamine. The cells were then washed with a membrane-impermeable iron chelator diethylenetriaminepentaacetic acid to remove the extracellular "free" iron. As the desferrioxamine-ferric 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. glutathione, citrate, and phosphorylated sugar intermediates) (6,28-30). As shown in Figure 1, the intracellular "chelatable" iron content in the *iscA/sufA* mutant cells is about two times as that of wild-type cells grown in LB (Luria-Bertani) medium under aerobic conditions. Because this assay is of limited utility as it cannot observe ferrous iron and ferric-nanoparticles (both of which have been identified in cells (31,32)), it would be difficult to determine the exact concentration of the intracellular "free" iron in *E. coli* cells. Nevertheless, the results in Figure 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 (Figure 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* wild-type 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 wild-type *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 (insert in Figure 2A). The UV-visible absorption measurements showed that the red Fur protein has three major absorption peaks at 325 nm, 410 nm, and 450 nm, in addition to the protein peak at 280 nm (Figure 2A), suggesting that the red Fur protein may bind a mononuclear iron or iron-sulfur cluster.

Careful examination of the UV-visible spectra in Figure 2A revealed that the Fur protein purified from wild-type *E. coli* cells also has the absorption peaks at 325 nm, 410 nm, and 450 nm, although their amplitudes are only about one-eighth of those of the red Fur protein purified from the *E. coli iscA/sufA* mutant cells (Figure 2B). The small amplitudes of the absorption peaks at 325 nm, 410 nm, and 450 nm of the Fur protein purified from

wild-type *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 wild-type *E. coli* cells, and the relative concentration of the red Fur protein in the *iscA/sufA* mutant cells is about eight times as that in wild-type 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±0.2 iron and 0.4±0.2 sulfide atoms per each Fur monomer in the purified red Fur protein, suggesting that the red Fur protein likely contains an iron-sulfur cluster. As the *E. coli* Fur has previously been characterized as a zinc binding protein (33), we also analyzed the zinc content of purified Fur, and found that the zinc content is decreased from 1.8±0.3 atoms per Fur monomer when purified from wild-type *E. coli* cells to 1.4±0.2 atoms per Fur monomer when purified from the *iscA/sufA* mutant cells (n=3), indicating that binding of iron-sulfur cluster affects the zinc binding in Fur.

The iron-sulfur cluster in the red Fur protein was further investigated by the EPR spectroscopy. While as-purified red Fur protein has no EPR signals, the dithionite-reduced red Fur protein has an EPR signal at $g_x = 1.91$, $g_y = 1.94$ and $g_z = 2.00$ (Figure 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 temperature and in applied magnetic fields (38,39). As shown in Figure 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, $\Delta E_Q = 0.53(1)$ mm/s and linewidths of 0.33 mm/s. These parameters are typical for $[2Fe\text{-}2S]^{2+}$ 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 δ and ΔE_Q which are similar to values seen for the protein-bound [2Fe-2S] clusters (39,40), the diamagnetism of the sample provides unambiguous evidence for the presence of an oxidized $[2Fe\text{-}2S]^{2+}$ cluster, which is also consistent with the UV-visible absorption spectrum (Figure 2A). The spectra in low applied magnetic fields show a minor spectral component, indicated by the small arrow in Figure 3 (top spectrum). Collecting spectra at 180 K, at which the fast spin fluctuation limit is a reasonable assumption, affords the quantification of all iron in the sample in terms of two components: the major [2Fe-2S] cluster component and the minor ferrous iron component. Based on these spectra, the ferrous iron component contributes about 8% of the total iron in the sample, and the rest of the iron (about 92% of the total iron) exists as a [2Fe-2S] cluster in the Fur protein. At low temperature (6 K or below), the ferrous component can further be simulated with two distinct doublets with equal contributions ($\delta = 1.23(1)/\Delta E_Q = 3.50$ mm/s and $\delta = 1.36/\Delta E_Q = 2.80$ mm/s), which correspond to high-spin ferrous iron with pseudo-octahedral N/O coordination (42). The ferrous components appear as two small quadrupole doublets in the 0.07 T spectrum and they are in the background, broadened by hyperfine in interactions in the high-field spectrum. Thus, the Mössbauer spectroscopy studies demonstrated that the red Fur protein

primarily binds a [2Fe-2S] cluster likely with cysteine coordination.

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). Site 1 and site 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 the selected amino acid residues in site 1 or site 2 were replaced with alanine. The Fur mutants were then expressed in the *E. coli iscA/sufA* mutant cells and purified. Figure 4A shows the results of two *E. coli* Fur mutants (Glu-108 to Ala of site 1 and His-90 to Ala of site 2). Both mutations have very little or no effect on the [2Fe-2S] cluster binding in the Fur protein when expressed in the *E. coli iscA/sufA* mutant cells. Similar results were also observed when His-87 (site 1) or His-88 (site 2) was replaced with Ala (data not shown). Thus, site 1 and site 2 of the *E. coli* Fur protein are likely not involved in binding the [2Fe-2S] cluster.

The third metal binding site in the *E. coli* Fur protein consists of three conserved 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. Figure 4B show that when Cys-93, Cys-96, or Cys-133 was replaced with Ala, the *E. coli* Fur mutant protein failed to bind the [2Fe-2S] cluster when expressed in the *E. coli iscA/sufA* mutant cells, suggesting that these cysteine residues are required for the *E. coli* Fur to bind the [2Fe-2S] cluster. The fourth cysteine residue (Cys-138) in the *E. coli* Fur is not conserved. Attempts to replace Cys-138 with Ala in the *E. coli* Fur protein were not successful. We instead replaced 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 (Supplementary Figure 1), indicating that Cys-138 could provide the fourth ligand for 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 (Figure 4B), which is consistent with the Mössbauer spectroscopy data (Figure 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 wild-type *E. coli* cells expressing recombinant Fur protein with 2,2'-dipyridyl. Fur protein was then purified from the cells. Figure 5A shows that addition of 200 μM of 2,2'-dipyridyl effectively removes the [2Fe-2S] cluster from the Fur protein in wild-type *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 (Figure 5B). Furthermore, the binding of the [2Fe-2S] cluster in Fur is reversible in *E. coli* cells, as 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). Since 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 de-activation 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 *Haemophilus 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 *Haemophilus influenzae*, a Gram-negative facultatively anaerobic pathogenic bacterium. The *H. influenzae* Fur has 65% identity and 77% similarity with the *E. coli* Fur (Figure 6A). The *H. influenzae* Fur protein was expressed in the *E. coli iscA/sufA* mutant cells and purified from the cells. As shown in Figure 6B, the purified *H. influenzae* Fur has the same absorption peaks at 325 nm, 410 nm, 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, while 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 nm, 410 nm, and 450 nm of the purified *H. influenzae* Fur were much higher than those of the red *E. coli* Fur (Figure 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 about 68%, while that of the *E. coli* Fur protein is about 31% (Figure 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 wild-type *E. coli* cells and purified both proteins. As shown in Figure 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 (~ 4%) when expressed in wild-type *E. coli* cells. We also carried out the *in vitro* reconstitution experiments. When the apo-form *E. coli* Fur was incubated with four-fold excess of ferrous iron and sulfide in the presence of dithiothreitol, only about 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 four-fold excess of ferrous iron and sulfide under the same experimental conditions, about 50% of the apo-form *H. influenzae* Fur was reconstituted with a [2Fe-2S] cluster (Supplementary Figure 2). Thus, while 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 ferric uptake regulator (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 that the intracellular "free" iron content is mainly regulated by Fur (5-9) and to substantially increase the intracellular "free" iron concentration could 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 due to deletion of IscA and its homologs increases the intracellular "free" iron content (22,24,26) (Figure 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 Fe-coordination (about 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 wild-type *E. coli* cells

(Figure 2). The occupancy of the [2Fe-2S] cluster in the Fur protein is about 31% in the *E. coli iscA/sufA* mutant cells and is decreased to about 4% in wild-type *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 wild-type and *iscA/sufA* mutant cells (Figure 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 $[2Fe\text{-}2S]^{2+}$ and ~ 0.45 mm/s for the $[4Fe\text{-}4S]^{2+}$ (38) (Supplementary Table 1). The Mössbauer parameters observed in the *E. coli* red Fur protein ($\delta = 0.29(2)$ mm/s and $\Delta E_Q = 0.53(1)$) (Figure 3) represent a typical [2Fe-2S] cluster and are virtually identical to the [2Fe-2S] cluster associated with the oxygen-exposed FNR protein ($\delta = 0.28(1)$ mm/s and $\Delta E_Q = 0.58(2)$ mm/s) (39) and the [2Fe-2S] cluster of the human mitochondrial glutaredoxin 2 (Grx2) ($\delta = 0.27$ mm/s and $\Delta E_Q = 0.60$ mm/s) (40). It should be pointed out that the Mössbauer spectrum of the *E. coli* red Fur protein (Figure 3) is 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 (41,44), representing the binding of ferrous iron at site 2 via His-33, Glu-81, His-88, and His-90 in Fur protein (44). While a small fraction (about 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), about 92% of the total iron content in the red Fur protein is assigned to the [2Fe-2S] cluster in the protein (Figure 3). Thus, 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 sulfur cluster biogenesis. Use of an iron-sulfur cluster to sense the intracellular "free" iron content is not unprecedented. In mammalian cells, the iron regulatory protein 1 (IRP-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 wild-type and *iscA/sufA* mutant *E. coli* cells contain the [2Fe-2S] cluster (Figure 6); 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), since 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 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 wild-type *E. coli* cells, only about 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 about 31% (Figure 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 as $CX_2CX_{37}C$ motif. The CX_2C 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 did not completely eliminate 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, while 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 (Figure 6B 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. As only crystal structure of a truncated *E. coli* Fur (residues 1-82) is available (10), we have modeled a full-length *E. coli* Fur protein (Figure 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, Cys-113) in the *E. coli* Fur are closely positioned for hosting a [2Fe-2S] cluster (Figure 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 wild-type *E. coli* strain (MC4100) as described in (22). With exception of the Mössbauer sample preparation, *E. coli* wild-type and the *iscA/sufA* mutant strains were grown in LB (Luria-Bertani) 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* wild-type (MC4100) or the *iscA/sufA* mutant cells. Fur protein was overproduced in the *E. coli* cells by adding 0.2% L-arabinose for four hours, and purified following the procedure described in (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, as 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 $mM^{-1}cm^{-1}$ 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 wild-type 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 (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 (56).

EPR measurements of purified Fur

The X-band Electron Paramagnetic Resonance (EPR) spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions were: microwave frequency, 9.47 GHz; microwave power, 1.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 20 K; receiver gain, $2x10^5$.

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 (28). Overnight bacterial cultures were diluted 100-fold into 500 ml of fresh LB media at 37°C with agitation. Cultures were grown to an O.D. at 600 nm of 0.2. Cells were harvested by centrifugation at 8000 rpm. for 5 min, and pellets were re-suspended in LB containing 20 mM desferrioxamine to O.D. at 600 nm of 5.0. The cells were then incubated at 37°C for 15 min and washed with 10 mM diethylenetriaminepentaacetic acid (DTPA) once. The suspension was further washed twice with 20 mM cold Tris-Cl (pH 7.4) and re-suspended 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 ^{57}Fe-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 ^{57}Fe (10 μM) under aerobic growth conditions. The ^{57}Fe-labeled Fur purified from the *E. coli iscA/sufA* mutant cells had the same UV-visible absorption spectrum with the absorption peaks at 325 nm, 410 nm, and 450 nm. Mössbauer spectra were recorded on a closed-cycle refrigerator spectrometer, model CCR4K (SeeCo, Edina, MN) 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. Mössbauer spectra were analyzed using the software SpinCount (Michael Hendrich, Ph.D., Carnegie Mellon University and WMOSS4 (Ion Prisecaru, www.wmoss.org).

Data availability

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

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Competing financial interests

The authors declare no competing financial interests.

Author Contributions

All authors participated in designing and performing the experiments and reviewed the results and approved the final version of the manuscript.

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Figure legends

Figure 1. Deletion of IscA and SufA results in accumulation of the intracellular "free" iron content in *E. coli* cells. A), EPR spectra of *E. coli* wild-type cells treated with or without desferrioxamine (desf). **B)**, EPR spectra of the *E. coli iscA/sufA* mutant cells treated with or without desferrioxamine (desf). Experimental conditions were described in Experimental Procedures. The data are representatives of three independent experiments.

Figure 2. The *E. coli* Fur has a bright red color when expressed in the *E. coli iscA/sufA* mutant cells. Recombinant *E. coli* Fur was expressed in the *E. coli* wild-type and the *E. coli iscA/sufA* mutant cells grown in LB medium under aerobic conditions. **A)**, UV-visible absorption spectra of purified Fur proteins. Spectrum 1, Fur protein purified from wild-type *E. coli* cells. Spectrum 2, Fur protein purified from the *iscA/sufA* mutant cells. The protein (100 μM) was dissolved in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl. Insert was a photograph of purified Fur proteins from wild-type (1) and the *iscA/sufA* mutant (2) cells. **B)**, UV-visible absorption spectrum of the Fur protein purified from wild-type *E. coli* cells. Same as in A), except the Y-axis is expanded by 8 folds. **C)**, SDS-PAGE gel of purified Fur proteins from wild-type *E. coli* cells (lane 1) and from the *iscA/sufA* mutant cells (lane 2). Lane M, molecular weight ladder. **D)**, EPR spectra of the *E. coli* Fur protein purified from the *iscA/sufA* mutant cells. The Fur protein (500 μM) was reduced with freshly prepared sodium dithionite (10 mM), and immediately frozen in liquid nitrogen until the EPR measurement.

Figure 3. Variable-field Mössbauer spectra of the ^{57}Fe-enriched Fur protein purified from the *E. coli iscA/sufA* mutant cells. The ^{57}Fe-labeld *E. coli* Fur protein was purified from the *E. coli iscA/sufA* mutant cells growing in M9 minimum medium supplemented with ^{57}Fe (10 μM). The protein concentration of ^{57}Fe-labeld Fur was about 1.0 mM. Mössbauer spectra were collected at cryogenic temperatures at 5.5 K and 70 mT (top) and 4.2 K and 7.0 T (bottom). The magnetic field was applied parallel to the observed gamma-radiation. Hash marks are raw data, lines are spectral simulations with the parameters shown and discussed in the text. The linewidths (full width at half-maximum) are 0.33 mm/s. The arrow marks the high-energy line of the ferrous components discussed in the text.

Figure 4. The conserved cysteine residues in the *E. coli* Fur are the ligands for the [2Fe-2S] cluster. The Fur mutant proteins were constructed by the site-directed mutagenesis and purified from the *E. coli iscA/sufA* mutant cells. **A)**, UV-visible absorption spectra of the wild-type Fur (spectrum 1), the mutant H190A (spectrum 2), and the mutant E108A (spectrum 3). **B)**, UV-visible absorption spectra of the wild-type Fur (spectrum 1), the mutant C93A (spectrum 2), the mutant C96A (spectrum 3), and the mutant C133A (spectrum 4). Each spectrum was offset for clarity. The protein concentrations were about 100 μM. The results are representatives of three independent experiments.

Figure 5. Depletion of intracellular "free" iron content removes the [2Fe-2S] cluster from Fur protein in *E. coli* cells. A), UV-visible absorption spectra of the *E. coli* Fur protein. Fur proteins were purified from the wild-type *E. coli* cells treated with 0 μM (spectrum 1) or 200 μM (spectrum 2) of 2,2'-dipyridyl, respectively. **B),** UV-visible absorption spectra of the *E. coli* Fur proteins. Fur proteins were purified from the *E. coli iscA/sufA* mutant cells treated with 0 μM (spectrum 1) or 200 μM (spectrum 2) of 2,2'-dipyridyl, respectively. The concentrations of purified proteins were about 60 μM. The results are representatives of three independent experiments.

Figure 6. The *Haemophilus 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* wild-type cells. The protein concentrations were 100 μM. The results are representatives of three independent experiments.

Figure 7. A structure model of the full-length *E. coli* Fur. Full length sequence of the *E. coli* Fur was modeled as described in (53). Site 1 is coordinated by His-87, Asp-89, Glu-108, and His-125. Site 2 is coordinated by His-33, Glu-81, His-88, and His-90. Site 3 is formed by Cys-93, Cys-96, and Cys-133. The structure model of the *E. coli* Fur was visualized using RasMol (57). The zinc binding sites and the [2Fe-2S] cluster binding site are indicated.

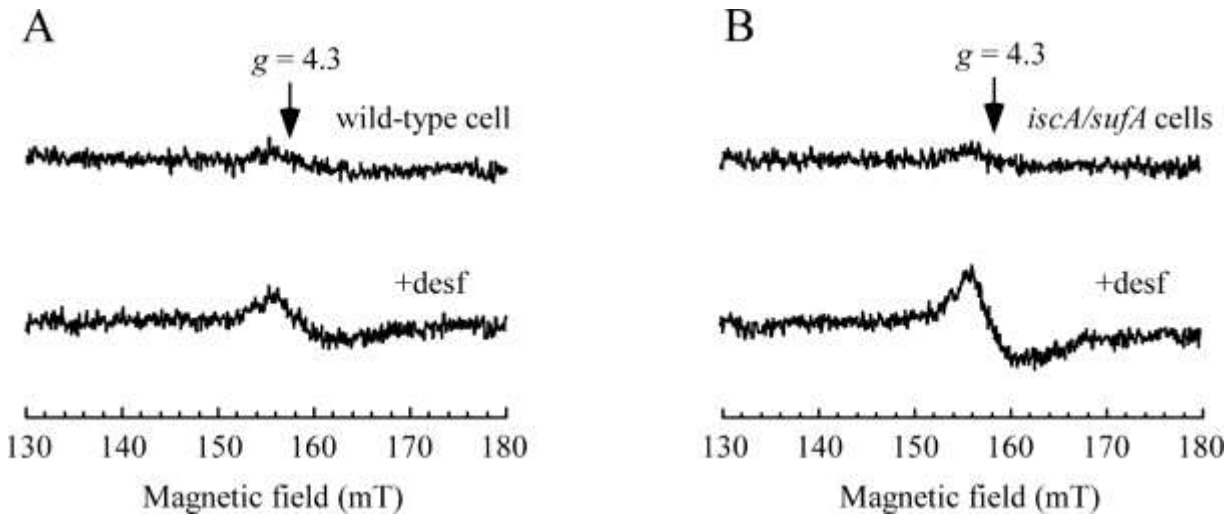
A
g = 4.3
wild-type cell
+desf
130
140
150
160
170
180
Magnetic field (mT)
B
g = 4.3
iscA/sufA cells
+desf
130
140
150
160
170
180
Magnetic field (mT)

Figure 1

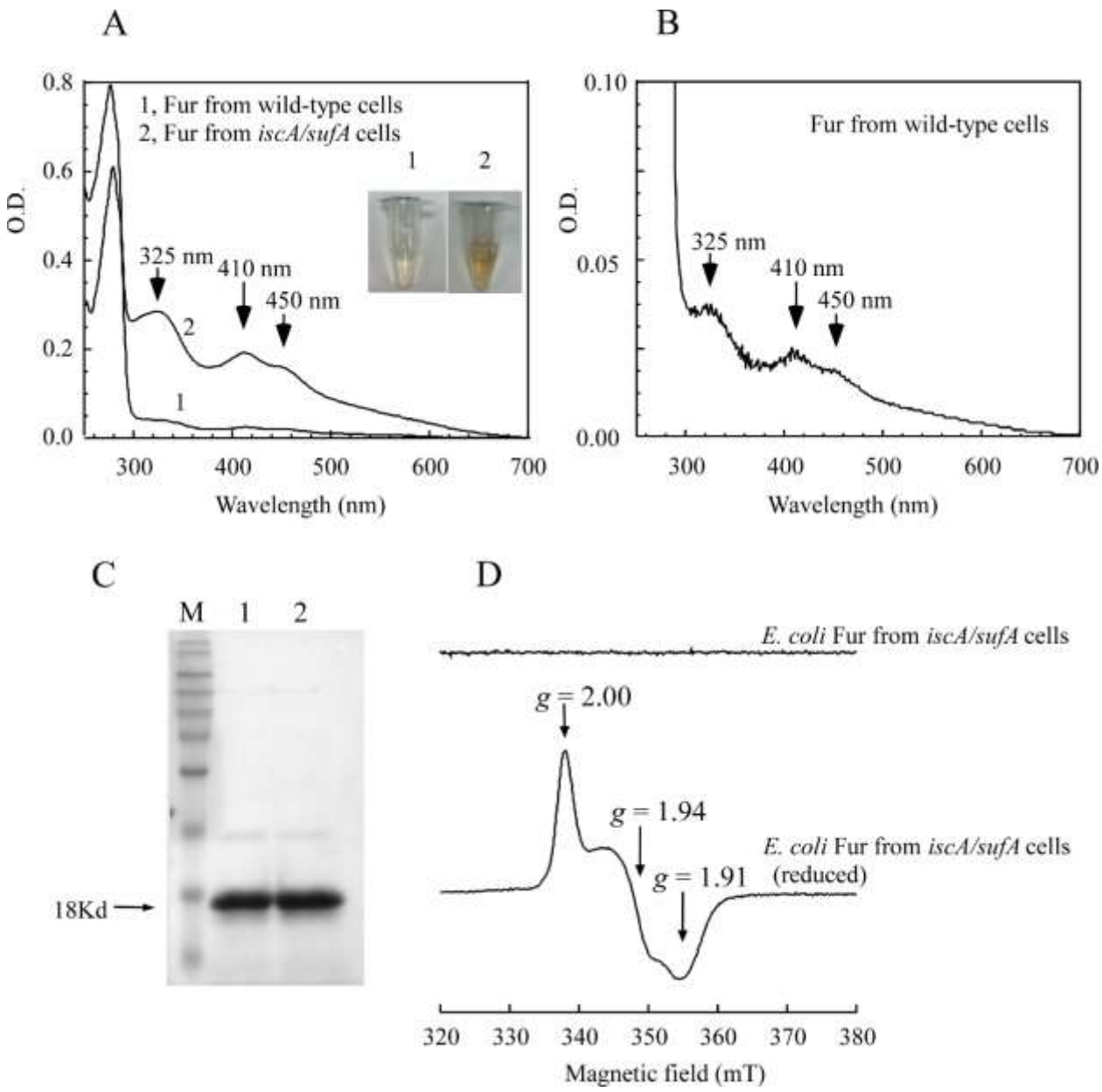
A
B
1, Fur from wild-type cells
2, Fur from iscA/sufA cells
O.D.
Wavelength (nm)
325 nm
410 nm
450 nm
Fur from wild-type cells
C
M
1
2
18Kd
D
E. coli Fur from iscA/sufA cells
g = 2.00
g = 1.94
g = 1.91
E. coli Fur from iscA/sufA cells (reduced)
Magnetic field (mT)

Figure 2

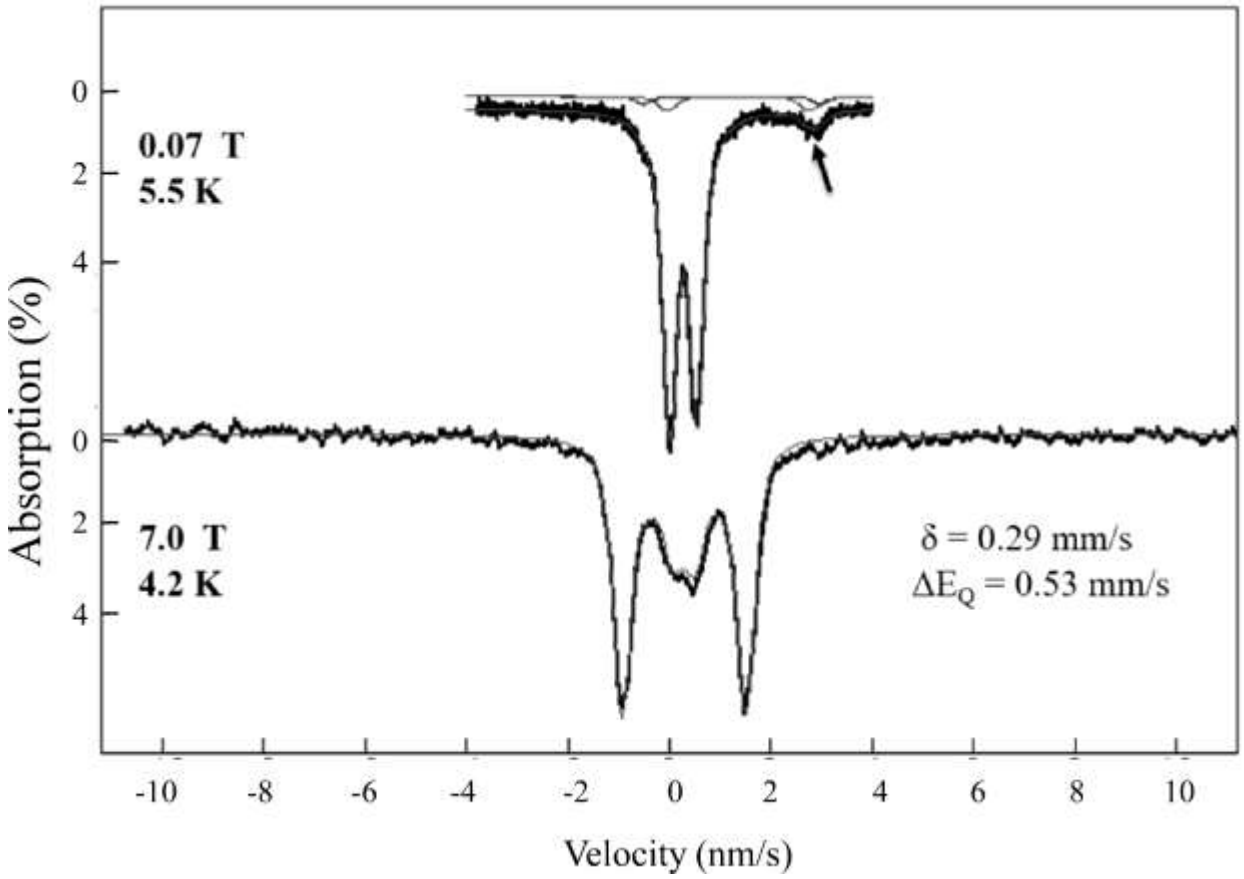
0.07 T
5.5 K
7.0 T
4.2 K
δ = 0.29 mm/s
ΔE_Q = 0.53 mm/s
Absorption (%)
Velocity (nm/s)
0
2
4
0
2
4
-10
-8
-6
-4
-2
0
2
4
6
8
10

Figure 3

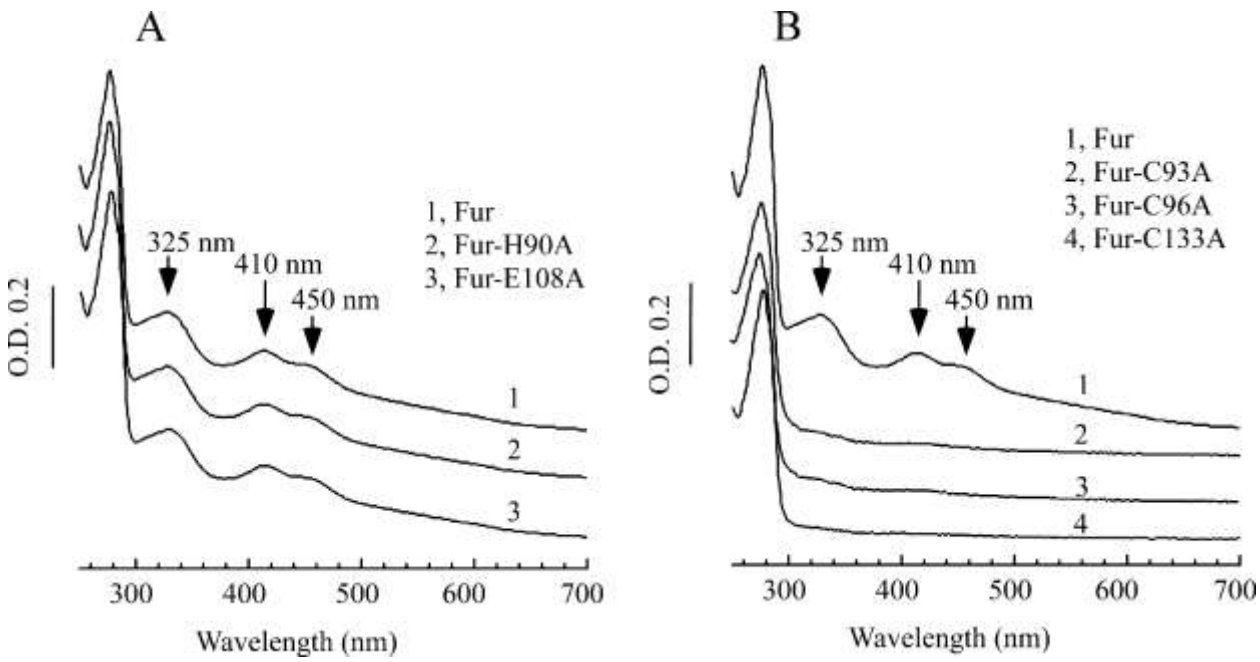
A
B
1, Fur
2, Fur-H90A
3, Fur-E108A
1, Fur
2, Fur-C93A
3, Fur-C96A
4, Fur-C133A
325 nm
410 nm
450 nm
O.D. 0.2
Wavelength (nm)
300
400
500
600
700

Figure 4

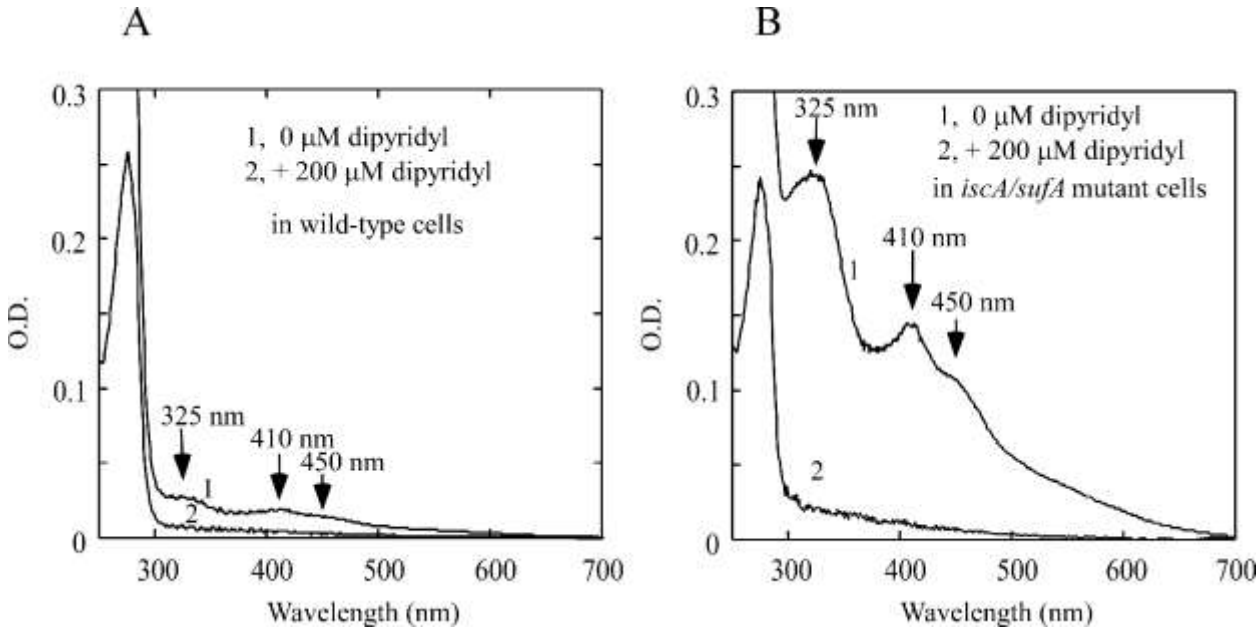
A
B
0.3
0.2
0.1
0
O.D.
1, 0 μM dipyridyl
2, + 200 μM dipyridyl
in wild-type cells
325 nm
410 nm
450 nm
1
2
300
400
500
600
700
Wavelength (nm)
1, 0 μM dipyridyl
2, + 200 μM dipyridyl
in iscA/sufA mutant cells

Figure 5

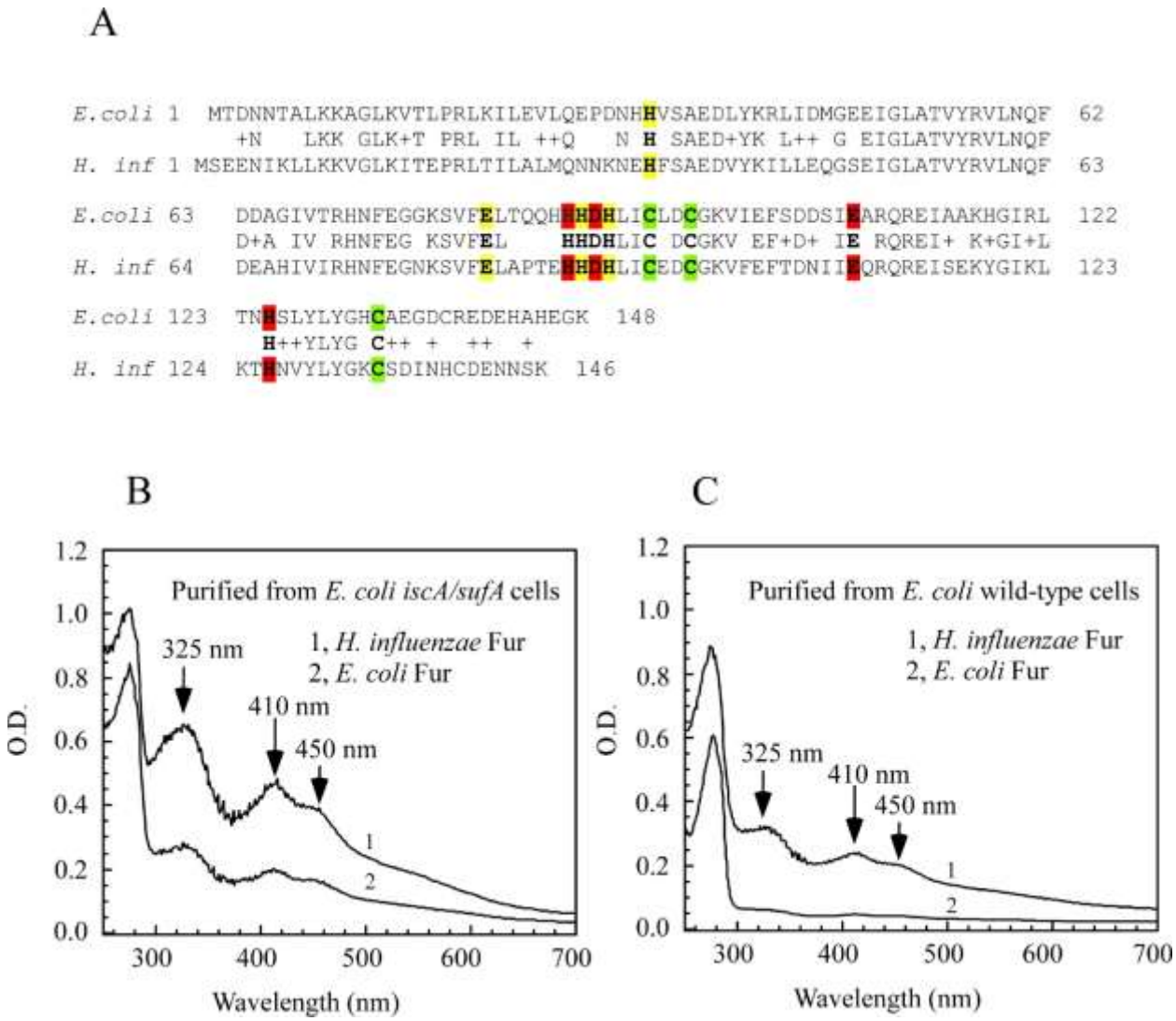
A
E.coli 1 MTDNNTALKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQF 62
+N LKK GLK+T PRL IL ++Q N H SAED+YK L++ G EIGLATVYRVLNQF
H. inf 1 MSEENIKLLKKVGLKITEPRLTILALMQNNKNEHFSAEDVYKILLEQGSEIGLATVYRVLNQF 63
E.coli 63 DDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRL 122
D+A IV RHNFEG KSVFEL HHDHLIC DCGKV EF+D+ IE RQREI+ K+GI+L
H. inf 64 DEAHIVIRHNFEGNKSVFELAPTEHHDHLICEDCGKVFEFTDNIIEQRQREISEKYGIKL 123
E.coli 123 TNHSLYLYGHCAEGDCREDEHAHEGK 148
H++YLYG C++ + ++ +
H. inf 124 KTHNVYLYGKCSDINHCDENNSK 146
B
Purified from E. coli iscA/sufA cells
325 nm
410 nm
450 nm
1, H. influenzae Fur
2, E. coli Fur
O.D.
Wavelength (nm)
C
Purified from E. coli wild-type cells
325 nm
410 nm
450 nm
1, H. influenzae Fur
2, E. coli Fur
O.D.
Wavelength (nm)

Figure 6

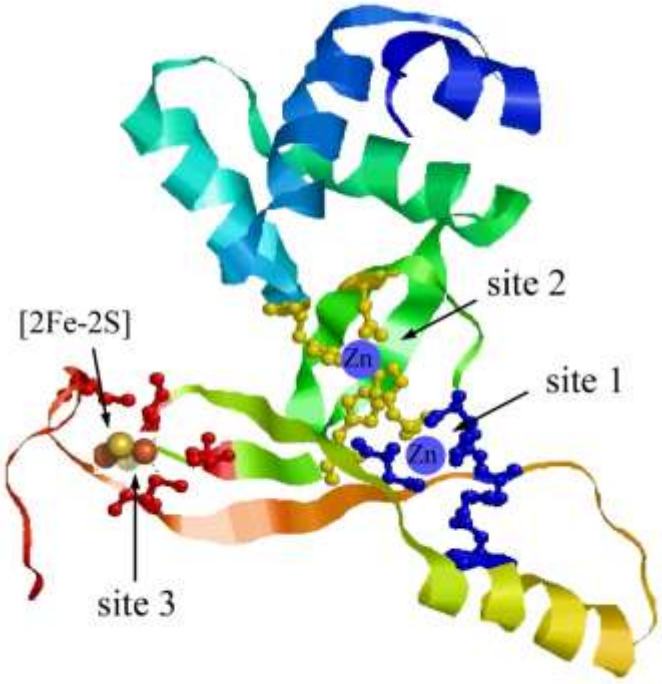

Figure 7