Published in final edited form as:
Biochem J. 2010 December 15; 432(3): 429–436. doi:10.1042/BJ20101507.

In vivo Evidence for the Iron Binding Activity of an Iron-Sulfur Cluster Assembly Protein IscA in *Escherichia coli*

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SYNOPSIS

IscA is a key member of the iron-sulfur cluster assembly machinery in prokaryotic and eukaryotic organisms; however, the physiological function of IscA still remains elusive. Here we report the *in vivo* evidence demonstrating the iron binding activity of IscA in *Escherichia coli* cells. Supplement of exogenous iron (1μM) in the M9 minimal medium is sufficient to maximize the iron binding in IscA expressed in *E. coli* cells under aerobic growth conditions. In contrast, IscU, an iron-sulfur cluster assembly scaffold protein, or CyaY, a bacterial frataxin homologue, fails to bind any iron in *E. coli* cells under the same experimental conditions. Interestingly, the strong iron binding activity of IscA is greatly diminished in *E. coli* cells under anaerobic growth conditions. Additional studies reveal that oxygen in medium promotes the iron binding in IscA and that the iron binding in IscA in turn prevents formation of biologically inaccessible ferric hydroxide under aerobic conditions. Consistent with the differential iron binding activity of IscA under aerobic and anaerobic conditions, we find that IscA and its paralog SufA are essential for the iron-sulfur cluster assembly in *E. coli* cells under aerobic growth conditions but not under anaerobic growth conditions. The results provide the *in vivo* evidence that IscA may act as an iron chaperone for the biogenesis of iron-sulfur clusters in *E. coli* cells under aerobic conditions.

Keywords

Iron-sulfur cluster biogenesis; human IscA homologue; intracellular iron content

INTRODUCTION

In *Escherichia coli*, IscA is a key member of the iron-sulfur cluster assembly machinery [1–4], and is highly conserved from bacteria to humans [5]. Deletion of IscA and its paralog SufA in *E. coli* cells results in deficiency of the iron-sulfur cluster assembly in multiple proteins [6,7] and a null-growth phenotype in the M9 minimal medium under aerobic conditions [8]. Depletion of IscA in *Azotobacter vinelandii* also causes a null-growth phenotype in the modified Burks minimal medium under elevated oxygen conditions [9]. In *Saccharomyces cerevisiae*, depletion of IscA homologues leads to iron accumulation in mitochondria and dependency on lysine and glutamate for the cell growth under aerobic conditions [10,11]. In *Schizosaccharomyces pombe*, deletion of IscA homologues is lethal

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Author Contributions: All authors contributed to the experimental design and data collections. H.D. wrote the paper.

[12]. In cultured human HeLa cells, RNAi (RNA interference) knockdown of the IscA homologue (hIscA1) dramatically decreases the enzyme activity of iron-sulfur proteins in both mitochondrion and cytosol [13]. However, the specific function of IscA in the biogenesis of iron-sulfur clusters still remains controversial. One hypothesis suggests that IscA and SufA may act as regulatory proteins that control the iron homeostasis and redox stress responses in cyanobacterium *Synechococcus* sp. strain PCC 7002 [14], although the underlying molecular mechanism is not fully understood. The second hypothesis states that IscA and SufA are the alternative scaffold/carrier proteins that bind transient iron-sulfur clusters and transfer the assembled clusters to target proteins [15–22]. However, unlike other proposed iron-sulfur cluster assembly scaffold proteins such as IscU [23], purified *E. coli* IscA [24–28] and human IscA [29] have a strong iron binding activity with an iron association constant of 1.0×10^{19} M^{-1} in the presence of the thioredoxin/thioredoxin reductase system under aerobic conditions. Furthermore, the iron center in IscA can be readily mobilized by L-cysteine [30] and transferred for the iron-sulfur cluster assembly in a proposed scaffold IscU *in vitro* [27], suggesting that IscA/SufA may also act as iron chaperones to recruit intracellular iron [28] and deliver the iron for the iron-sulfur cluster assembly in proteins [27]. Nevertheless, the physiological relevance of iron binding in IscA for the biogenesis of iron-sulfur clusters has not been addressed.

In this study, we present the *in vivo* evidence demonstrating the strong iron binding activity of IscA in *E. coli* cells. Supplement of exogenous iron (1μM) in the M9 minimal medium is sufficient to maximize the iron binding occupancy of IscA expressed in *E. coli* cells under aerobic growth conditions. In contrast, the iron-sulfur cluster assembly scaffold protein IscU [23] or the bacterial frataxin homologue CyaY [31–34] fails to bind any iron in *E. coli* cells under the same experimental conditions. Importantly, the strong iron binding in IscA is greatly diminished in *E. coli* cells under anaerobic growth conditions. Additional studies indicate that oxygen in the medium promotes the iron binding in IscA by oxidizing ferrous iron to ferric iron in the iron binding site of IscA and that the iron binding in IscA in turn prevents formation of biologically inaccessible ferric hydroxide under aerobic conditions. Consistent with these observations, we find that IscA and SufA are largely dispensable under anaerobic growth conditions but are essential for the iron-sulfur cluster assembly in *E. coli* cells under aerobic growth conditions. The physiological roles of IscA/SufA in the biogenesis of iron-sulfur clusters will be discussed.

EXPERIMENTAL

Cell growth and protein expression

Overnight culture of *E. coli* (BL21(DE3) strain containing the expression vector pTISCA [24], pTSUFA [8], pTISCU [27], or pTCYAY [33] for expressing recombinant *E. coli* IscA, SufA, IscU, or CyaY, respectively) was diluted (1:100) in the M9 minimal medium containing glucose (0.2%), thiamin (5 μg/ml), and 20 amino acids (each at 10 μg/ml). After four hours of incubation at 37°C with aeration (250 rpm), the M9 minimal media were supplemented with or without ferric citrate 10 min before the protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (200 μM). If the protein was expressed under anaerobic conditions, the *E. coli* cells were purged with pure argon gas for 20 min before isopropyl β-D-1-thiogalactopyranoside was added to the sealed flask. The cells were grown for additional one hour before being harvested and washed once with the protein purification buffer (20 mM Tris (pH 8.0), 500 mM NaCl). Protein was purified as described previously [27], and the purity of purified proteins was over 95% judging from the SDS polyacrylamide gel electrophoresis. The amounts of the acid-labile iron and sulfide in purified proteins were analyzed according to the Fischer's method [35] and the Siegel's method [36], respectively, using purified *E. coli* ferredoxin [2Fe-2S] cluster [37] as a standard.

Intracellular iron content measurements in *E. coli* cells

The intracellular iron content of *E. coli* cells was measured using the *in vivo* EPR (electron paramagnetic resonance) following the procedures described in [38,39]. Briefly, overnight *E. coli* cells were diluted in the M9 minimal medium supplemented with different amounts of exogenous iron under aerobic conditions. The exponentially growing *E. coli* cells (O.D. at 600 nm = 0.5) were harvested, washed once, and re-suspended in the M9 minimal medium to O.D. at 600 nm = 20.0. After additional 30 min incubation, the cells were treated with a membrane permeable iron chelator desferrioxamine (20 mM) and a non-permeable iron chelator diethylenetriaminepentaacetic acid (10 mM) for 15 min. The cells were then chilled at 4°C, harvested, and washed twice with ice-cold buffer containing Tris (20 mM, pH 8.0) and NaCl (0.5 M), and re-suspended in the same ice-cold buffer. Iron standards for the EPR measurements were prepared by making serial dilutions of $FeCl_3$ and desferrioxamine (1 mM) in Tris (20 mM, pH 8.0). The EPR spectra were recorded at X-band on a Bruker ESR-300 spectrometer using an Oxford Instruments ESR-9 flow cryostat. The routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 10 K; receive gain, 1.0×10^5.

Iron-sulfur cluster assembly in IscU *in vitro*

The *E. coli* IscU and cysteine desulfurase IscS were prepared as described previously [27]. Typically, purified IscU (50 μM) was incubated with IscS (1 μM), NaCl (200 mM), Tris (20 mM, pH 8.0) with various iron source in the presence of dithiothreitol (2 mM). L-cysteine (1 mM) was added to initiate the iron-sulfur cluster assembly reaction. The amount of the iron-sulfur clusters assembled in IscU was monitored at 456 nm [23] in a Beckman DU-640 UV-Visible spectrometer equipped with a temperature controller.

Aconitase activity assay in the cell extracts—The cell extracts were prepared from the *E. coli* cells containing recombinant aconitase B [40] by passing the cells through French press once. Aliquots were transferred to pre-incubation solutions containing Tris (50 mM, pH 8.0), $MgCl_2$ (10 mM), and D,L-isocitrate (10 mM) at 30°C. The aconitase activity was monitored following the formation of *cis*-aconitate at 240 nm using an extinction coefficient of 3.6 $mM^{-1}cm^{-1}$ [40].

RESULTS

IscA is an iron binding protein in *E. coli* cells under aerobic growth conditions

To explore the *in vivo* iron binding activity of IscA, we expressed recombinant IscA in *E. coli* cells grown in the M9 minimal medium under aerobic growth conditions. Figure 1A shows that IscA purified from *E. coli* cells grown in the M9 minimal medium without any exogenous iron had very little or no absorption peaks of the iron or iron-sulfur cluster binding in the protein. The acid-labile iron and sulfide content analyses also showed that purified IscA contained less than 0.02 iron atoms and 0.01 sulfide atoms per IscA dimer (n = 3). However, when the M9 minimal medium was supplemented with ferric citrate (5.0 μM), IscA purified from *E. coli* cells had a major absorption peak at 315 nm, indicative of iron binding in IscA [24] (Figure 1A). The acid-labile iron and sulfide content analysis further revealed that purified IscA contained 0.60±0.11 iron atoms and 0.03 sulfide atoms per IscA dimer (n = 3), suggesting that IscA binds iron, but not iron-sulfur clusters, in *E. coli* cells under the experimental conditions used.

In parallel experiments, the proposed iron-sulfur cluster assembly scaffold protein IscU [23] was also expressed in *E. coil* cells grown in the M9 minimal medium supplemented with or without exogenous iron (5.0 μM ferric citrate). Figure 1B shows that supplement of

exogenous iron in the M9 minimal medium did not increase the iron binding in *E. coli* IscU, consistent with the notion that IscU has a weak iron binding activity [23,27]. We also expressed the bacterial frataxin homologue CyaY [33], a putative iron donor for the iron-sulfur cluster assembly [41], in *E. coli* cells under the same experimental conditions. As shown in Figure 1C, *E. coli* CyaY, like IscU, failed to bind iron whether or not the M9 minimal medium was supplemented with exogenous iron (5.0 μM ferric citrate) (Figure 1C). Thus, IscA has its unique iron binding activity in *E. coli* cells under aerobic growth conditions.

To further determine the iron binding activity of IscA *in vivo*, the *E. coli* cells expressing IscA were grown in the M9 minimal medium supplemented with increasing concentrations of exogenous iron under aerobic growth conditions. Figure 2A shows that the iron binding in IscA was almost linearly increased as the exogenous iron concentration in the M9 minimal medium was increased from 0 to 1.0μM, and appeared to be saturated (at approx.. 60% occupancy) above 1.0 μM iron (Figure 2B). Further increase of exogenous iron (up to 50 μM) in the M9 minimal medium failed to fully saturate the iron binding occupancy in IscA expressed in *E. coli* cells under aerobic growth conditions (data not shown).

Since elevated intracellular iron content will promote production of deleterious hydroxyl free radicals via the Fenton reaction [42], we reasoned that the intracellular iron concentration could be the limiting factor for the iron binding in IscA in *E. coli* cells. To test this hypothesis, we adopted the *in vivo* electron paramagnetic resonance (EPR) approach developed by the Imlay's group [38] to probe the intracellular iron content of *E. coli* cells grown in the M9 minimal medium supplemented with exogenous iron under aerobic growth conditions. The amplitude of the EPR signal at $g = 4.0$ reflects the relative amount of the intracellular iron content in *E. coli* cells [38,39]. Figure 2C shows that the intracellular iron content of *E. coli* cells grown in the M9 minimal medium without exogenous iron was very low, indicating that *E. coli* cells are under iron starvation in the M9 minimal medium. As the concentration of exogenous iron in the M9 minimal medium was increased, the amplitude of the EPR signal at $g = 4.0$ was progressively increased and plateaued at approx. 1 μM exogenous iron (Figure 2D). The saturation curve of the intracellular iron content in *E. coli* cells (Figure 2D) generally correlates with that of the iron binding of IscA in *E. coli* cells (Figure 2B). Thus, the partial occupancy (~60%) of the iron binding in IscA in *E. coli* cells could be due to the limited intracellular iron content under the experimental conditions.

The iron binding activity of IscA is greatly diminished in *E. coli* cells under anaerobic conditions

Because IscA has also been characterized as an alternative iron-sulfur cluster assembly scaffold/carrier protein [15–22], we decided to express IscA in *E. coli* cells grown in the M9 minimal medium under anaerobic growth conditions, hoping to purify an iron-sulfur cluster-bound IscA from the cells. To our surprise, IscA purified from the *E. coli* cells grown in the M9 minimal medium supplemented with 2 μM exogenous iron under anaerobic conditions had only a small absorption peak at 315 nm (Figure 3A). The acid-labile iron and sulfide content analyses further showed that purified IscA contained 0.12±0.03 iron atoms and 0.03 sulfide atoms per IscA dimer (n = 3), suggesting that the iron binding activity of IscA is greatly diminished in *E. coli* cells under anaerobic growth conditions.

One of the likely explanations for the diminished iron binding in IscA in *E. coli* cells could be that the intracellular iron content is severely limited under anaerobic conditions. Using the whole cell EPR measurements as described above, we found that the intracellular iron content of *E. coli* cells grown in the M9 minimal medium supplemented with 2 μM exogenous iron under anaerobic conditions was similar to, if not higher than, that of *E. coli* cells under aerobic growth conditions (data not shown). We then asked whether oxygen

could directly promote the iron binding in IscA in *E. coli* cells. To test this hypothesis, we prepared apo-IscA as described in [24] and incubated apo-IscA with ferrous iron and dithiothreitol under aerobic and anaerobic conditions. Figure 3B shows that while IscA had a strong iron binding activity *in vitro* under aerobic conditions as reported previously [24,26], IscA had very little or no iron binding under anaerobic conditions, indicating that oxygen does have a crucial role for the strong iron binding in IscA. To directly test the binding affinity of apo-IscA for ferric iron, we incubated apo-IscA pre-reduced with dithiothreitol with an equal amount of ferric iron ($FeCl_3$) under anaerobic conditions and found that apo-IscA was indeed converted into the iron-bound IscA after incubation (data not shown). Thus, oxygen is able to promote the iron binding in IscA probably by oxidizing ferrous iron to ferric iron in the iron binding site of IscA.

Role of IscA in the iron-sulfur cluster assembly under aerobic and anaerobic conditions

Our previous studies indicated that the iron-loaded IscA can readily provide iron for iron-sulfur cluster assembly in IscU *in vitro* under aerobic conditions [27]. However, since IscA has very little or no iron binding activity under anaerobic conditions (Figure 3), it is possible that IscA may be dispensable for iron-sulfur cluster assembly under anaerobic conditions. To test this hypothesis, we re-evaluated the role of IscA in the biogenesis of iron-sulfur clusters under aerobic and anaerobic conditions.

Figure 4 shows that under anaerobic conditions, “free” ferrous iron was able to provide iron for iron-sulfur cluster assembly in IscU *in vitro* (Figure 4A) as the absorption peaks at 402 nm and 456 nm reflecting the iron-sulfur cluster assembly in IscU [23] quickly appeared. Under the same anaerobic conditions, the iron-bound IscA had similar activity in providing iron for the iron-sulfur cluster assembly in IscU (Figure 4C). However, under aerobic conditions, pre-incubation of “free” ferrous iron resulted in deficiency of the iron-sulfur cluster assembly in IscU (Figure 4B), whereas the iron-bound IscA could still efficiently provide the iron for the iron-sulfur cluster assembly in IscU after the same pre-incubation under aerobic conditions (Figure 4D). If an equal amount of apo-IscA was mixed with “free” ferrous iron before pre-incubation under aerobic conditions, the iron also remained available for the iron-sulfur cluster assembly in IscU (data not shown). Thus IscA may have an essential role in preventing formation of biologically inaccessible iron and providing the iron for iron-sulfur cluster assembly under aerobic conditions, but not under anaerobic conditions.

To re-evaluate physiological role of IscA in the biogenesis of iron-sulfur clusters in *E. coli* cells under aerobic and anaerobic conditions, we used an *E. coli* mutant in which both IscA and its paralog SufA were in-frame deleted [8]. A plasmid expressing recombinant iron-sulfur protein aconitase B [43] was introduced into the $iscA^{-1}/sufA^{-1}$ mutant and its parental wild-type cells as described in [8]. Recombinant aconitase B was expressed in the *E. coli* cells in rich LB medium under aerobic or anaerobic conditions and purified. Figure 5A shows that iron-sulfur clusters were assembled in recombinant aconitase B in the wild-type *E. coli* cells under both aerobic and anaerobic conditions. However, in the $iscA^{-1}/sufA^{-1}$ mutant cells, the iron-sulfur cluster assembly in aconitase B occurred only under anaerobic conditions, but not under aerobic conditions. Because the aconitase B activity requires an intact iron-sulfur cluster, we also analyzed the total aconitase activity in the cell extracts prepared from the wild-type and the $iscA^{-1}/sufA^{-1}$ mutant cells. As shown in Figure 5B, deletion of IscA/SufA in *E. coli* cells resulted in inactivation of aconitase B under aerobic conditions, but not under anaerobic conditions, further indicating that the $iscA^{-1}/sufA^{-1}$ mutant fails to assemble iron-sulfur clusters in aconitase B under aerobic conditions. Similar results were obtained when the iron-sulfur protein dihydroxyacid dehydratase [44] was expressed in the wild-type and the $iscA^{-1}/sufA^{-1}$ mutant cells (data not shown). Collectively, the results suggest that IscA and its paralog SufA are essential for the

biogenesis of iron-sulfur clusters in *E. coli* cells under aerobic conditions but not under anaerobic conditions.

If IscA and SufA were dispensable for the iron-sulfur cluster assembly in *E. coli* cells under anaerobic conditions, deletion of both IscA and SufA should have minimal effects on cell growth of *E. coli* in the M9 minimal medium under anaerobic conditions. Indeed, while deletion of IscA/SufA resulted in a null-growth phenotype of *E. coli* cells on the M9 minimal medium plate under aerobic conditions (Figure 5C) as reported previously [8], deletion of IscA/SufA did not prevent cell growth of *E. coli* under anaerobic conditions (Figure 5D).

DISCUSSION

In this study, we present the *in vivo* evidence demonstrating the iron binding activity of IscA in *E. coli* cells under aerobic conditions. Supplement of exogenous iron (1μM) in the M9 minimal medium is sufficient to maximize the iron binding occupancy of IscA in *E. coli* cells under aerobic growth conditions (Figures 1 and 2). IscU, a proposed iron-sulfur cluster assembly scaffold protein [23], and CyaY, a bacterial frataxin homolog that has been postulated as an iron donor for the iron-sulfur cluster assembly [41], fail to bind any iron in *E. coli* cells under the same experimental conditions (Figure 1). Importantly, the iron binding in IscA is greatly diminished *in vivo* and *in vitro* under anaerobic conditions (Figure 3). Additional studies reveal that oxygen promotes the iron binding in IscA likely by oxidizing ferrous iron to ferric iron in the binding site of the protein, and that the iron binding in IscA in turn prevents formation of biologically inaccessible ferric hydroxide and facilitates the iron-sulfur cluster assembly under aerobic conditions (Figure 4). Consistent with the differential iron binding activity of IscA under aerobic and anaerobic conditions, we find that IscA and its paralog SufA are essential for the iron-sulfur cluster assembly in *E. coli* cells under aerobic conditions but are dispensable under anaerobic conditions (Figure 5). Taken together, the results suggest that IscA/SufA may act as iron chaperones for the iron-sulfur cluster biogenesis under aerobic conditions.

We wish to emphasize that the results described in this study do not exclude the possibility that IscA/SufA may also act as alternative scaffold/carrier proteins for the biogenesis of iron-sulfur clusters as proposed by others [15–22]. Instead, we propose that two models for the function of IscA/SufA may be reconciled by suggesting that IscA/SufA could be bifunctional. When both the intracellular sulfide/L-cysteine and iron are abundant, iron-sulfur clusters may be assembled in IscA/SufA, and IscA/SufA act as alternative scaffold/carrier proteins for the biogenesis of iron-sulfur clusters as reported [15–22]. On the other hand, when sulfide/L-cysteine is limited in cells, IscA/SufA may act as iron chaperones to recruit intracellular iron for the iron-sulfur cluster assembly. The observation that supplement of exogenous iron (1 μM) in the M9 minimal medium is sufficient to maximize the iron binding occupancy of IscA expressing in *E. coli* cells (Figure 2) strongly suggests that IscA has a high iron binding affinity not only *in vitro* [24,26] but also *in vivo*. Moreover, the bifunctional model for IscA/SufA is entirely consistent with the crystal structures of IscA [3,4] and SufA [45] in which the conserved “cysteine pocket” could readily accommodate a mononuclear iron center or an iron-sulfur cluster without significant re-arrangements of protein structure.

The salient finding of this study is that IscA and its paralog SufA are essential for the iron-sulfur cluster assembly under aerobic conditions but not under anaerobic conditions (Figure 5). One interpretation could be that the biogenesis of iron-sulfur clusters is under high demand under aerobic conditions in such that deletion of IscA/SufA may result in severe deficiency of the iron-sulfur cluster assembly activity [7]. Alternatively, another IscA

homologue ErpA [19] may substitute the function of IscA and SufA to support the iron-sulfur cluster assembly under anaerobic growth conditions. ErpA is involved in isoprenoid biosynthesis, and is required for cell growth of *E. coli* by either aerobic and anaerobic respiration but not fermentation [19]. Therefore, it cannot be ascertained as how ErpA could substitute IscA/SufA for the biogenesis of iron-sulfur clusters under anaerobic conditions but not under aerobic conditions [7]. In this context, we would like to offer new interpretation for the differential requirement of IscA/SufA for the biogenesis of iron-sulfur clusters in *E. coli* cells under aerobic and anaerobic conditions. We propose that under anaerobic conditions intracellular ferrous iron is readily available, and that IscA and SufA are not required to recruit intracellular iron for the biogenesis of iron-sulfur clusters (Figure 4). Consequently, deletion of both IscA and SufA has very little or no effect on the iron-sulfur cluster assembly in *E. coli* cells (Figure 5). Under aerobic conditions, however, the intracellular iron concentration is limited [38,39] as elevated intracellular iron contents are highly toxic to cells [42], and IscA/SufA become essential to recruit intracellular iron and deliver iron for the biogenesis of iron-sulfur clusters (Figure 4B and D). Consistent with this notion, deletion of IscA/SufA results in deficiency of the iron-sulfur cluster assembly in aconitase B, dihydroxyacid dehydratase and several other iron-sulfur proteins in *E. coli* cells under aerobic conditions [6] (Figure 5). Nevertheless, additional experiments are needed to further illustrate the dynamic iron binding and physiological roles of IscA/SufA in cells under aerobic and anaerobic conditions.

Acknowledgments

This work was supported in part by the US Public Health Service Grant (CA107494) from the National Institutes of Health, the Chinese National Natural Science Foundation Grant (30770448), the Science and Technology Key Program of Zhejiang Province Grant (2006C14025), and the Natural Science Foundation of Zhejiang Province Grant (Y2081075 and 84107002).

Abbreviations

AcnB	aconitase B
CyaY	bacterial frataxin homologue
EPR	electron paramagnetic resonance

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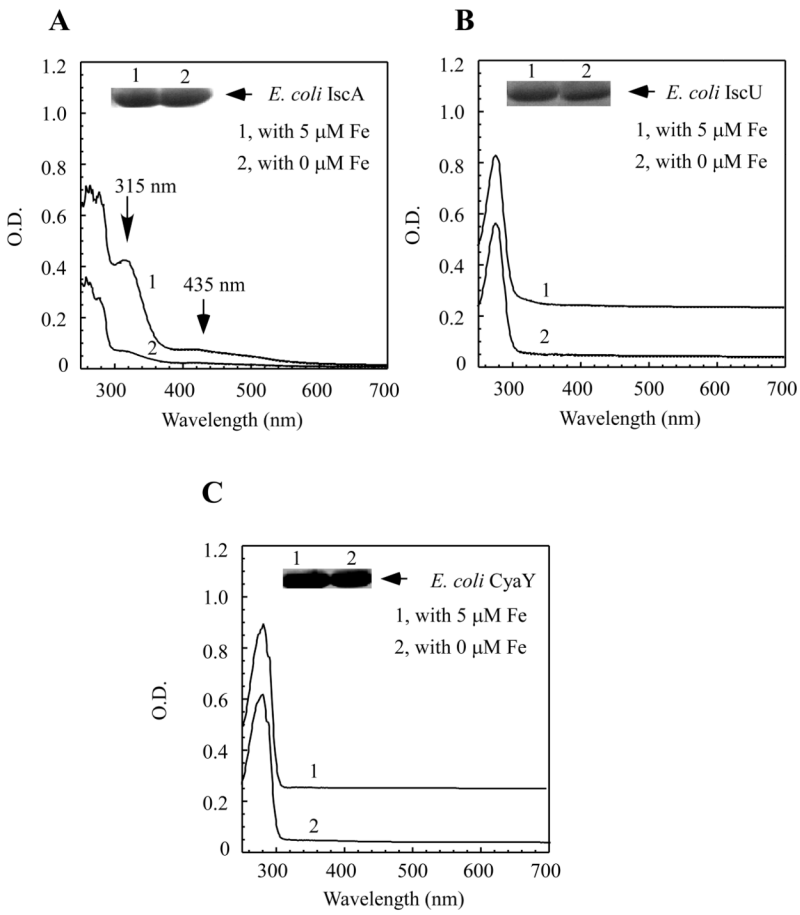

Figure 1. IscA is an iron binding protein in *E. coli* cells under aerobic conditions

A) Recombinant IscA was expressed in *E. coli* cells grown aerobically in the M9 minimal medium supplemented with (spectrum 1) or without (spectrum 2) 5.0μM ferric citrate and purified as described in the Experimental section. The protein concentrations were about 120 μM. **B)** Recombinant IscU was expressed in *E. coli* cells grown aerobically in the M9 minimal medium supplemented with (spectrum 1) or without (spectrum 2) 5.0μM ferric citrate and purified. The protein concentrations were about 60 μM. **C)** Recombinant CyaY was expressed in *E. coli* cells grown aerobically in the M9 minimal medium supplemented with (spectrum 1) or without (spectrum 2) 5μM ferric citrate and purified. The protein concentrations were about 20 μM. The insert in each panel is a photograph of the SDS/ PAGE gel of the proteins purified from the *E. coli* cells grown in the M9 minimal medium supplemented with (lane 1) or without (lane 2) 5.0μM ferric citrate. The results are representatives from three independent experiments.

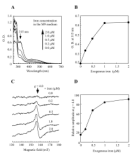

Figure 2. Relative iron binding activity of IscA in *E. coli* cells under aerobic conditions

A) UV-visible absorption spectra of recombinant IscA purified from *E. coli* cells grown aerobically in the M9 minimal medium supplemented with ferric citrate (0.0, 0.2, 0.5, 1.0, and 2.0μM). The protein concentration of purified IscA was about 250 μM. **B)** The apparent iron binding activity of IscA in *E. coli* cells. The amplitude of the absorption peak at 315 nm of purified IscA in A) was plotted as a function of the exogenous iron concentration supplemented in the M9 minimal medium. **C)** The EPR spectra of the *E. coli* cells treated with an iron indicator desferrioxamine. The *E. coli* cells grown in the M9 minimal medium supplemented with ferric citrate (0.0, 0.2, 0.5, 1.0, and 2.0 μM) were subject to the intracellular iron content measurements using the iron indicator desferrioxamine as described in the Experimental section. The amplitude of the EPR signal at $g = 4.0$ reflects the relative intracellular iron concentration in *E. coli* cells. **D)** The amplitudes of the EPR signal at $g = 4.0$ in C) were plotted as a function of the exogenous iron concentration supplemented in the M9 minimal medium. The data are representatives from three independent experiments.

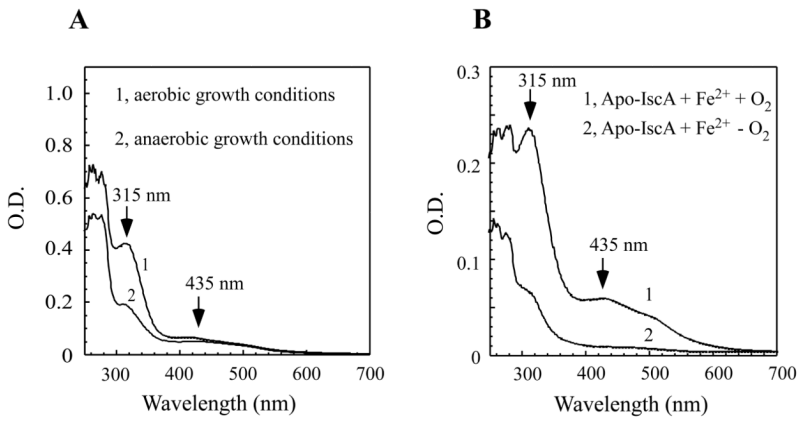

Figure 3. The iron binding activity of IscA *in vivo* and *in vitro* under aerobic or anaerobic conditions

A) The iron binding activity of IscA *in vivo* under aerobic and anaerobic conditions. UV-visible absorption spectra of IscA purified from the *E. coli* cells grown in the M9 minimal medium supplemented with 2.0μM ferric citrate under aerobic (spectrum 1) or anaerobic (spectrum 2) growth conditions. The protein concentration was about 150 μM. **B)** The iron binding activity of IscA *in vitro* under aerobic and anaerobic conditions. Apo-IscA (100 μM) was incubated with ferrous ammonium sulfite (50μM) and dithiothreitol (2 mM) under aerobic (spectrum 1) or anaerobic (spectrum 2) conditions at 25°C for 10 min. IscA was re-purified from the incubation solutions and subject to the UV-visible absorption measurements.

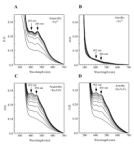

Figure 4. IscA promotes *in vitro* iron-sulfur cluster assembly in IscU under aerobic conditions

The iron-sulfur cluster assembly in IscU (50 μM) was carried out *in vitro* by incubating with cysteine desulfurase (IscS) (1 μM), dithiothreitol (2 mM), L-cysteine (1 mM), and different iron sources. The absorption peaks at 402 nm and 456 nm indicate the iron-sulfur cluster assembly in IscU. **A)** Ferrous ammonium sulfate (50 μM) was used for the iron-sulfur cluster assembly in IscU under anaerobic conditions. **B)** Ferrous ammonium sulfate (50 μM) was pre-incubated at 37°C for 3 hours under aerobic conditions before being used for the iron-sulfur cluster assembly in IscU. **C)** The iron-bound IscA (containing 50 μM iron) was used for the iron-sulfur cluster assembly in IscU under anaerobic conditions. **D)** The iron-bound IscA (containing 50 μM iron) was pre-incubated at 37°C for 3 hours under aerobic conditions before being used for the iron-sulfur cluster assembly in IscU. The experiments were repeated three times, and similar results were obtained.

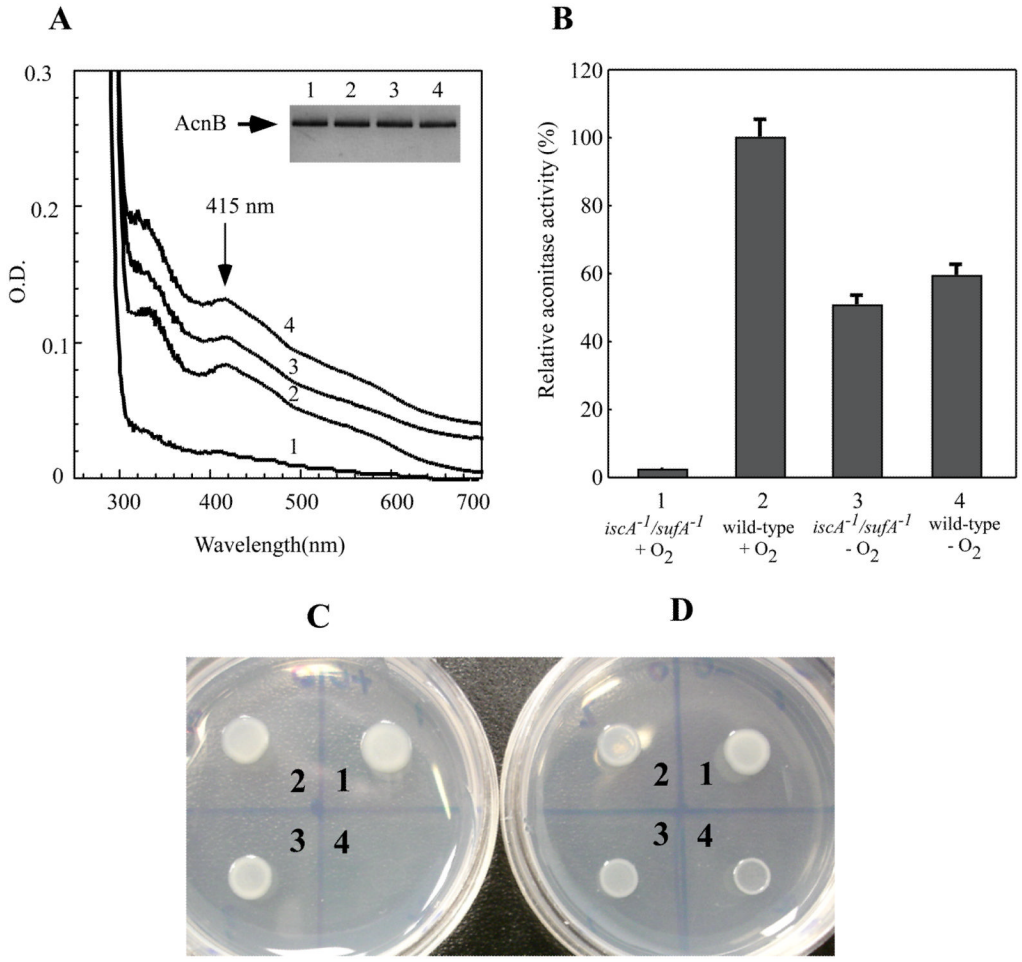

Figure 5. IscA and its paralog SufA are required for the iron-sulfur cluster assembly in aconitase B in *E. coli* cells under aerobic conditions

A) Recombinant aconitase B was purified from the *iscA*$^{-1}$/*sufA*$^{-1}$ mutant *E. coli* cells grown in LB medium under aerobic (spectrum 1) and anaerobic (spectrum 3) conditions or from the wild-type *E. coli* cells grown under aerobic (spectrum 2) and anaerobic (spectrum 4) conditions. The absorption peaks at 415 nm of purified aconitase B indicates the iron-sulfur cluster in the protein. The insert is a photograph of the SDS/PAGE gel of purified aconitase B. **B)** Recombinant aconitase B was expressed in the *iscA*$^{-1}$/*sufA*$^{-1}$ mutant *E. coli* cells grown in LB medium under aerobic (sample 1) and anaerobic (sample 3) conditions or in the wild-type *E. coli* cells grown under aerobic (sample 2) and anaerobic (sample 4) conditions. The aconitase activity in the cell extracts was measured as described in the Experimental section. Values are the means ± S.D. for three independent experiments. **C)** About 2×10^5 cells of the wild-type (1), the *iscA*$^{-1}$ mutant (2), the *sufA*$^{-1}$ mutant (3), and the *iscA*$^{-1}$/*sufA*$^{-1}$ mutant (4) were spotted on the M9 minimal medium plates containing 0.2% glucose but without any amino acids and thiamin. The plate was incubated at 37°C overnight under aerobic conditions. **D)** Same as in C) except that the plate was incubated at 37°C overnight under anaerobic conditions.