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IscA/SufA Paralogs Are Required for the [4Fe-4S] Cluster Assembly in Enzymes of Multiple Physiological Pathways in *Escherichia coli* under Aerobic Growth Conditions

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Synopsis

IscA/SufA paralogs are the members of the iron-sulfur cluster assembly machinery in *Escherichia coli*. While deletion of either IscA or SufA has only a mild effect on cell growth, deletion of both IscA and SufA results in a null-growth phenotype in minimal medium under aerobic growth conditions. Here we report that cell growth of the *iscA/sufA* double mutant (*E. coli* strain in which both *iscA* and *sufA* had been in-frame-deleted) can be partially restored by supplementing with BCAAs (branched-chain amino acids) and thiamin. We further demonstrate that deletion of IscA/SufA paralogs blocks the [4Fe-4S] cluster assembly in IlvD (dihydroxyacid dehydratase) of the BCAA biosynthesis pathway in *E. coli* cells under aerobic conditions and that addition of the iron-bound IscA/SufA efficiently promotes the [4Fe-4S] cluster assembly in IlvD and restores the enzyme activity in vitro, suggesting that IscA/SufA may act as an iron donor for the [4Fe-4S] cluster assembly under aerobic conditions. Additional studies reveal that IscA/SufA are also required for the [4Fe-4S] cluster assembly in protein ThiC of the thiamin biosynthesis pathway, aconitase B of the citrate acid cycle, and endonuclease III of the DNA base excision repair pathway in *E. coli* under aerobic conditions. Nevertheless, deletion of IscA/SufA does not significantly affect the [2Fe-2S] cluster assembly in the redox transcription factor SoxR, ferredoxin, and the siderophore-iron reductase FhuF. The results suggest that the biogenesis of the [4Fe-4S] clusters and the [2Fe-2S] clusters may have distinct pathways and that IscA/SufA paralogs are essential for the [4Fe-4S] cluster assembly, but are dispensable for the [2Fe-2S] cluster assembly in *E. coli* under aerobic conditions.

Keywords

aconitase; branched-chain amino acids; dihydroxyacid dehydratase; iron-sulfur clusters; IscA/SufA paralogs; thiamin

Introduction

Iron-sulfur clusters are one of the most ancient and ubiquitous redox centers in biology. They are involved in diverse physiological processes including respiratory electron transfer, nitrogen fixation, photosynthesis, biosynthesis of amino acids, thiamin, heme, biotin, and lipoic acid, DNA synthesis and repair, RNA modification, and the regulation of gene expression [1,2]. However, the mechanism underlying the iron-sulfur cluster assembly is still not fully understood [3]. The discovery of cysteine desulfurase (NifS) in *Azotobacter vinelandii* by

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Dean's group in 1990s [4] led to identification of a highly conserved **i**ron-**s**ulfur **c**luster assembly gene cluster *iscRSUA-hscBA-fdx* in *Escherichia coli* and many other bacteria [5,6] (*hsc* refers to heat-shock cognate proteins A and B and *fdx* refers to ferredoxin). IscR is a repressor that contains a [2Fe-2S] cluster [7]. Disassembly of the [2Fe-2S] cluster in IscR de-activates its function as a repressor and switches on expression of the gene cluster *iscRSUA-hscBA-fdx* [8,9]. IscS is a cysteine desulfurase that catalyses desulfurization of L-cysteine and transfers sulfane sulfur to a proposed iron-sulfur cluster assembly scaffold IscU [10-15]. HscB and HscA are two heat shock cognate proteins that specifically interact with IscU [16] and promote transfer of assembled clusters from IscU to apo-ferredoxin in an ATP dependent reaction [17,18]. Ferredoxin encoded by gene *fdx* contains a stable [2Fe-2S] cluster. Recent studies indicated that ferredoxin could be involved in the [4Fe-4S] cluster formation in IscU [19]. For the function of IscA, at least three hypotheses have been proposed. The first hypothesis stated that IscA may act as an alternative iron-sulfur cluster assembly scaffold since IscA, like IscU, can host an iron-sulfur cluster and transfer the cluster to target proteins in vitro [20-23]. The second hypothesis suggested that IscA may act as a regulatory protein for iron homeostasis and redox stress responses in cyanobacterium *Synechococcus* sp. strain PCC 7002, as deletion of IscA and its paralog promotes cell growth in A+ medium under the iron limitation condition [24]. The third hypothesis stated that IscA may act as an iron donor for the iron-sulfur cluster assembly [25-29]. Purified IscA from *E. coli* binds ferrous iron (ferrous ammonium sulfate) in the presence of dithiothreitol or the thioredoxin/thioredoxin reductase system with an iron association constant of $2.0\times10^{19}M^{-1}$, and that the iron center in IscA can be mobilized specifically by L-cysteine [29] for the iron-sulfur cluster assembly in IscU in vitro [27,29]. Furthermore, IscA is able to recruit iron from the iron storage protein ferritin A and transfer the iron for the iron-sulfur cluster assembly in IscU [30]. These results led us to propose that the primary function of IscA is to recruit intracellular iron and deliver iron for the biogenesis of iron-sulfur clusters [27].

IscA is highly conserved from bacteria to yeast [25], plants [31], and humans [32]. It has been reported that depletion of IscA in *A. vinelandii* resulted in a null-growth phenotype under elevated oxygen conditions [33]. In *Saccharomyces cerevisiae*, deletion of the IscA homologs led to accumulation of iron in mitochondria and a dependency on lysine and glutamate for cell growth under aerobic conditions [25]. However, the mechanism underlying the IscA-deletion phenotypes has not been fully explored. In *E. coli*, IscA has two additional paralogs: ErpA and SufA. ErpA, which maps at a distance from any iron-sulfur cluster assembly-related genes, has been characterized as a dedicated scaffold for maturation of the key iron-sulfur enzymes in the isoprenoids biosynthesis pathway [34]. SufA, on the other hand, is a member of the second iron-sulfur cluster assembly gene cluster *sufABCDSE* [35]. Purified SufA, like IscA, can bind ferrous iron (ferrous ammonium sulfate) and subsequently provide iron for the iron-sulfur cluster assembly in IscU in vitro [28]. While deletion of either IscA or SufA only has a mild effect on cell growth of *E. coli*, deletion of both IscA and SufA results in a null-growth phenotype in minimal medium under aerobic conditions [28]. Cell growth of the *iscA/sufA* double mutant is fully restored when either IscA or SufA is re-introduced into the *iscA/sufA* double mutant, further indicating that IscA and SufA have complementary roles for the biogenesis of iron-sulfur clusters [28].

Here we report that cell growth of the *E. coli iscA/sufA* double mutant can be partially restored by supplementing with the branched-chain amino acids (BCAA) and thiamin in growth medium under aerobic conditions. We further demonstrate that IscA/SufA paralogs are required for the [4Fe-4S] cluster assembly in dihydroxyacid dehydratase (IlvD) of the BCAA biosynthesis pathway [36], ThiC of the thiamin biosynthesis pathway [37], aconitase B of the citrate acid cycle [38], and endonuclease III of the DNA base excision repair pathway [39] in *E. coli* cells under aerobic growth conditions. In contrast, deletion of IscA/SufA paralogs does not significantly affect the [2Fe-2S] cluster assembly in the redox transcription factor SoxR

[40], ferredoxin [41], and the siderophore-iron reductase FhuF [42]. The results suggest that IscA/SufA paralogs are required for the [4Fe-4S] cluster assembly but are dispensable for the [2Fe-2S] cluster assembly in *E. coli* cells under aerobic conditions, and that the biogenesis of the [4Fe-4S] clusters and the [2Fe-2S] clusters may have very distinct pathways in cells.

Experimental

Mutant strains and cell growth

The *E. coli* deletion mutants in which *iscA* and *sufA* were in-frame deleted were previously constructed [28]. Each deletion in *E. coli* cells was confirmed by PCR as described in [28]. For cell growth analysis, overnight cell cultures grown in Luria-Bertani (LB) medium were washed twice with minimal medium containing glucose (0.2%) before inoculated on minimal medium plates or in liquid minimal medium at 37°C with aeration (250 rpm). Cell growth was recorded by measuring the optical density of cell culture at 600 nm. When indicated, minimal medium was supplemented with the three branched-chain amino acids (at final concentrations of 90 μg/mL) and/or thiamin (at a final concentration of 1.0 μg/mL).

Protein expression and purification

The DNA fragments encoding dihydroxyacid dehydratase (IlvD) [36], ThiC [37], aconitase B [38], endonuclease III [39], SoxR [40], ferredoxin [41], and siderophore-iron reductase FhuF [42] were amplified from the wild-type *E. coli* genomic DNA using PCR. The primers used for PCR amplifications are listed in Table 1. The PCR products were digested with restriction enzymes and ligated to pBAD (Invitrogen co). The cloned plasmid was introduced into the *iscA/sufA* double mutant and its parental wild-type (MC4100) using electroporation. The DNA sequence of each cloned gene was confirmed by direct sequencing (Genomic Facility at Louisiana State University). Typically, *E. coli* cells hosting the expression plasmid were grown to O.D. at 600 nm of 0.6 before the protein expression was induced by adding L-arabinose (to a final concentration of 0.02%) for additional 3 hour under aerobic growth conditions. For expression of ThiC, the final concentration of L-arabinose used was 0.005% to avoid aggregation of the expressed ThiC. The His-tagged proteins were purified using the Ni-nitrilotriacetate-agarose column (Qiagen co.) followed by passing through a High-Trap desalting column as described previously [43]. All solutions used for protein purification were purged with pure argon gas. The concentration of purified proteins was calculated from the absorption peak at 280 nm using an extinction coefficient of 35.2 $mM^{-1}cm^{-1}$ for IlvD, 77.9 $mM^{-1}cm^{-1}$ for ThiC, 73.6 $mM^{-1}cm^{-1}$ for aconitase B, 18.6 $mM^{-1}cm^{-1}$ for endonuclease III, 15.7 $mM^{-1}cm^{-1}$ for SoxR, 7.8 $mM^{-1}cm^{-1}$ for ferredoxin, and 56.5 $mM^{-1}cm^{-1}$ for FhuF. The amounts of the acid-labile iron and sulfide contents in protein samples were analyzed according to the Fischer's method [44] and Siegel's method [45], respectively.

Iron-sulfur cluster assembly in dihydroxyacid dehydratase (IlvD) in vitro

For the iron-sulfur cluster assembly in IlvD, apo-IlvD (10 μM) was incubated with the iron-bound IscA or SufA (containing 75 μM iron) and IscS (0.5 μM) in the presence of dithiothreitol (2 mM), NaCl (200 mM), and Tris (20 mM, pH 8.0) in an open-to-air micro-centrifuge tube. The iron-sulfur cluster assembly reaction was initiated by adding L-cysteine (0.5 mM) to the incubation solution at 37°C. The [4Fe-4S] cluster assembly in IlvD was followed by the UV-visible absorption measurements and by the enzyme activity assay (see below). IlvD was then re-purified from the incubation solutions using a Mono-Q column attached the FPLC system.

Activity assay for dihydroxyacid dehydratase (IlvD)

The enzyme activity of IlvD was measured using substrate D,L-2,3-dihydroxy-isovalerate which was synthesized according to the method of Cioffi et al. [46]. All chemical reagents used

for the D,L-2,3-dihydroxy-isovalerate synthesis were obtained from Sigma-Aldrich (St. Louis, MO). Aliquots of the cell extracts prepared from *E. coli* cells containing recombinant IlvD were immediately transferred to pre-incubated solutions containing Tris (50 mM, pH 8.0), $MgCl_2$ (10 mM), and D,L-2,3-dihydroxyisovalerate (10 mM) at 37°C as described in [47]. The reaction product (oxo acids) was monitored at 240 nm using an extinction coefficient of 0.19 $mM^{-1}cm^{-1}$ [36].

Activity assay for the redox transcription factor SoxR in vivo

A plasmid (pTN1530) containing a reporter gene *soxS::lacZ* [48] was introduced into the *iscA/sufA* double mutant and its parental wild-type (MC4100) cells using electroporation. Overnight *E. coli* cells containing pTN1530 were diluted to OD at 600 nm of 0.01 in fresh LB medium. After incubation at 37°C with aeration (250 rpm) to OD at 600 nm of 0.1, paraquat (100 μM) was added to the cell cultures. Aliquots (25 μL) were taken from cell cultures after continuous incubation with aeration (250 rpm) at 37°C for 0, 20, 40 and 60 min. The β-galactosidase activity in cells was measured following the procedure described in [48].

Results

Cell growth of the *iscA/sufA* double mutant can be partially restored by supplementing with the branched-chain amino acids (BCAA) and thiamin in growth medium

In previous studies [28], we reported that in-frame deletion of either IscA or SufA in *E. coli* had only a mild effect on cell growth, and that deletion of both IscA and SufA paralogs resulted in a null-growth phenotype in minimal medium under aerobic growth conditions (Figure 1A). Cell growth of the *iscA/sufA* double mutant was fully restored when either IscA or SufA was re-introduced into the *iscA/sufA* double mutant [28], demonstrating that IscA and SufA have complementary roles for the biogenesis of iron-sulfur clusters in *E. coli* cells.

While the *iscA/sufA* double mutant had no growth on minimal medium plates under aerobic growth conditions (Figure 1A), it grew slowly on rich medium LB plates (Figure 1B), indicating that deletion of IscA/SufA paralogs may result in paucity of some essential metabolites for cell growth. Since deficiency of the branched-chain amino acids (BCAA) [49] and thiamin [50] has been considered a hallmark of oxidative inactivation of iron-sulfur enzymes in bacteria, we postulated that deletion of both IscA and SufA may prevent the iron-sulfur cluster assembly in key enzymes, thus blocking the biosyntheses of BCAA and thiamin in *E. coli* cells. Figure 1C shows that addition of the three branched-chain amino acids (leucine, isoleucine and valine) indeed partially restored cell growth of the *E. coli iscA/sufA* double mutant in minimal medium under aerobic growth conditions. While thiamin alone did not recover any noticeable cell growth of the *iscA/sufA* double mutant, addition of both thiamin and BCAA significantly increased cell growth under aerobic growth conditions. Thus, deletion of IscA/SufA paralogs has resulted in the BCAA and thiamin auxotrophy in *E. coli* cells under aerobic growth conditions.

The *iscA/sufA* double mutant fails to assemble the [4Fe-4S] cluster in dihydroxyacid dehydratase (IlvD) under aerobic growth conditions

The BCAA biosynthesis pathway in *E. coli* contains two iron-sulfur enzymes, namely IlvD [36] and LeuCD [49]. IlvD converts 2,3-dihydroxy-isovalerate to 2-ketoisovalerate [36], whereas LeuCD converts 2-isopropylmalate to 3-isopropylmalate [49]. Both IlvD and LeuCD require an intact [4Fe-4S] cluster for their catalytic activity. Because LeuCD is a two-subunit enzyme that dissociates during purification [49], we chose IlvD for further investigation.

To explore the function of IscA/SufA paralogs in the [4Fe-4S] cluster assembly in IlvD, we expressed IlvD in the wild-type, the *iscA* mutant, the *sufA* mutant, and the *iscA/sufA* double

mutant cells grown in LB medium under aerobic growth conditions. The SDS polyacrylamide gel electrophoresis analysis showed that the amounts of IlvD expressed in the wild-type and the IscA/SufA deletion mutants were very similar to each other (Figure 2B). However, unlike IlvD expressed in the wild-type or the *iscA* and *sufA* single mutant cells, IlvD expressed in the *iscA/sufA* double mutant cells had little or no enzyme activity (Figure 2A). Recombinant IlvD was then purified from the wild-type and the *iscA/sufA* double mutant cells following the procedures described in the Experimental. As shown in Figure 2C, while IlvD purified from the wild-type *E. coli* cells had a typical absorption peak at 415 nm of the [4Fe-4S] cluster and was active [36], IlvD purified from the *iscA/sufA* double mutant had no absorption features of any iron-sulfur clusters and no enzyme activity. The acid-labile iron and sulfide content analyses further revealed that IlvD purified from the wild-type cells contained about 1.6±0.4 mol of iron and 1.4±0.3 mol of sulfide per mol of protein (n=3), indicating that on average about 40% of purified IlvD contained an intact [4Fe-4S] cluster. In contrast, IlvD purified from the *iscA/sufA* double mutant cells had no detectable amounts of the acid-labile iron and sulfide.

The iron-bound IscA/SufA can efficiently provide iron for the [4Fe-4S] assembly in IlvD under aerobic conditions

As reported previously, both IscA and SufA purified from *E. coli* can bind ferrous iron and act as an iron donor for the iron-sulfur cluster assembly in IscU in vitro [27-29]. To test whether IscA/SufA paralogs can provide iron for the [4Fe-4S] cluster assembly in IlvD, we prepared the iron-bound IscA as described in [51], and incubated with apo-IlvD (purified from the *iscA/sufA* double mutant cells), cysteine desulfurase IscS, and L-cysteine in the presence of dithiothreitol at 37°C under aerobic conditions. Figure 3A shows that the enzyme activity of IlvD was quickly re-stored during the incubation with the iron-bound IscA, IscS and L-cysteine. In control where the iron-bound IscA was replaced with apo-IscA, no enzyme activity of IlvD was recovered after incubation. IlvD was then re-purified from the incubation solution using an anion-exchange Mono-Q column. The UV-visible absorption measurements of re-purified IlvD confirmed that the [4Fe-4S] cluster was assembled in IlvD after incubation with the iron-bound IscA, IscS and L-cysteine (Figure 3B). Similarly, when the iron-bound SufA was included in the incubation solution instead of IscA, the enzyme activity of IlvD was also restored and the [4Fe-4S] cluster assembled in the protein (data not shown). Thus, IscA/SufA paralogs may act as iron donors for the [4Fe-4S] cluster assembly in IlvD under aerobic conditions.

Because IscA/SufA paralogs have also been characterized as alternative scaffolds for the iron-sulfur cluster assembly [20-23], it would be imperative to examine whether IscA/SufA can directly transfer the assembled iron-sulfur clusters to apo-IlvD. In the experiments, IscA with pre-assembled iron-sulfur cluster was prepared as described in [27] and incubated with apo-IlvD in the presence of dithiothreitol at 37°C under aerobic conditions. While the enzyme activity of apo-IlvD could indeed be restored by incubation with the iron-sulfur cluster bound IscA, pre-incubation of the iron-sulfur cluster bound IscA rapidly diminished its ability to re-activate apo-IlvD under aerobic conditions (data not shown). This is likely because the iron-sulfur cluster pre-assembled in IscA is oxygen-labile [20-22], thus resulting in failure to re-activate apo-IlvD under aerobic conditions (see Discussion).

IscA/SufA paralogs are required for the [4Fe-4S] assembly in other enzymes in *E. coli* under aerobic growth conditions

Because supplement of thiamin to the growth medium containing BCAA can further increase cell growth of the *iscA/sufA* double mutant under aerobic growth conditions (Figure 1C), we speculated that deletion of IscA/SufA paralogs may also lead to deficiency of thiamin in *E. coli* cells. The thiamin biosynthesis pathway in *E. coli* contains at least two iron-sulfur enzymes: ThiC [37] and ThiH [52]. ThiH catalyzes the synthesis of the 4-methyl-5-beta

hydroxyethyl-thiazole monophosphate moiety of thiamin pyrophosphate [52]; whereas ThiC catalyzes the formation of 4-amino-5-hydroxymethyl-2-methylpyrimidine [37]. Both ThiC and ThiH require an intact [4Fe-4S] cluster for their catalytic activity [50]. To explore the role of IscA/SufA paralogs in the [4Fe-4S] cluster assembly in the enzymes of the thiamin biosynthesis pathway, we expressed recombinant ThiC in the wild-type and the *iscA/sufA* double mutant cells in LB medium under aerobic growth conditions. The SDS polyacrylamide gel electrophoresis analysis showed that deletion of IscA/SufA paralogs in *E. coli* cells did not significantly affect expression of ThiC and subsequent protein purification. Figure 4A shows that ThiC purified from the wild-type cells had a clear absorption peak at 410 nm of the [4Fe-4S] cluster as reported previously [37], and contained about 1.1±0.2 iron and 1.2 ±0.2 sulfide per protein (n=3). In contrast, ThiC purified from the *iscA/sufA* double mutant did not have any absorption peaks of iron-sulfur clusters and no detectable amounts of the acid-labile iron and sulfide. Since the [4Fe-4S] cluster is required for the activity of ThiC [37], lack of the [4Fe-4S] cluster in ThiC would block the thiamin biosynthesis in the *iscA/sufA* double mutant under aerobic growth conditions.

Figure 1C also indicated supplement of both BCAA and thiamin did not fully restore cell growth of the *iscA/sufA* double mutant, suggesting that deletion of IscA/SufA paralogs may have a much broader effect on the iron-sulfur cluster assembly in *E. coli* cells. Because the aconitase B [4Fe-4S] cluster represents the major aconitase activity in *E. coli* cells [38], we chose to examine the [4Fe-4S] cluster assembly in aconitase B in the wild-type and the *iscA/sufA* double mutant cells in LB medium under aerobic growth conditions. SDS polyacrylamide gel electrophoresis analysis showed that the amount of aconitase B expressed in the wild-type and the *iscA/sufA* double mutant cells was similar to each other. However, unlike the aconitase B expressed in the wild-type cells, the aconitase B expressed in the *iscA/sufA* double mutant cells was completely inactive (data not shown). Recombinant aconitase B was then purified from the wild-type and the *iscA/sufA* double mutant cells. As shown in Figure 4B, aconitase B purified from the wild-type cells had a typical absorption peak at 413 nm of the [4Fe-4S] cluster and contained about 1.4±0.2 iron and 1.0±0.2 sulfide per protein (n=3). In contrast, aconitase B purified from the *iscA/sufA* double mutant cells had no absorption peaks of any iron-sulfur clusters and no detectable amounts of the acid-labile iron and sulfide.

Finally, we explored the [4Fe-4S] cluster assembly in endonuclease III of the DNA base excision repair pathway [39] in the *iscA/sufA* double mutant cells grown in LB medium under aerobic growth conditions. Unlike IlvD, ThiC or aconitase B, endonuclease III contains an oxygen-resistant [4Fe-4S] cluster [39]. Figure 4C shows that while endonuclease III purified from the wild-type cells had an absorption peak at 419 nm of the [4Fe-4S] cluster and contained about 2.6±0.2 iron and 2.1±0.3 sulfide per protein (n=3), endonuclease III purified from the *iscA/sufA* double mutant had no absorption peaks of iron-sulfur clusters and no detectable amounts of the acid-labile iron and sulfide. Thus, IscA/SufA paralogs are rquired for the [4Fe-4S] cluster assembly in IlvD of the BCAA biosynthesis pathway, ThiC of the thiamin biosynthesis pathway, aconitase B of the citrate acid cycle, and endonuclease III of the DNA base excision repair pathway in *E. coli* cells under aerobic growth conditions.

Deletion of IscA/SufA paralogs does not significantly affect the [2Fe-2S] cluster assembly in the redox transcription factor SoxR in *E. coli* under aerobic growth conditions

Another type of iron-sulfur clusters found in cells is the [2Fe-2S] cluster [1-3]. It would be pertinent to examine the role of IscA/SufA paralogs in the [2Fe-2S] cluster assembly. We utilized the transcription factor SoxR as an example, as SoxR requires an intact [2Fe-2S] cluster for its redox activation in response to oxidative stress in *E. coli* cells [40]. Under normal physiological conditions, the SoxR [2Fe-2S] cluster is in reduced state and SoxR remains inactive [53]. When *E. coli* cells are subjected to oxidative stress, the reduced SoxR [2Fe-2S]

cluster becomes oxidized and SoxR is switched on to stimulate expression of its target gene *soxS* [53]. To monitor the SoxR activation, we introduced a plasmid containing a reporter gene *soxS::lacZ* [48] into the wild-type and the *iscA/sufA* double mutant *E. coli* cells.

Figure 5A shows that for the wild-type *E. coli* cells, SoxR quickly became activated upon addition of the redox cycling reagent paraquat as reported previously [48]. For the *iscA/sufA* double mutant, we noticed that the basal level of the SoxR activation was considerably higher than that of the wild-type cells (Figure 5B), indicating that deletion of IscA/SufA paralogs may cause an elevated basal oxidative stress. Nevertheless, when the *iscA/sufA* double mutant was exposed to paraquat, the SoxR activation was further increased to a level that is comparable to that of the paraquat-treated wild-type cells (Figure 5B). The results suggested that the SoxR [2Fe-2S] cluster is fully functional in response to oxidative stress in the *iscA/sufA* double mutant under aerobic growth conditions.

We then expressed recombinant SoxR in the wild-type and the *iscA/sufA* double mutant cells in LB medium under aerobic growth conditions. The UV-visible absorption measurements showed that SoxR purified from the wild-type (Figure 5C) and from the *iscA/sufA* double mutant cells (Figure 5D) had nearly identical spectra, further suggesting that IscA/SufA paralogs are not essential for the [2Fe-2S] cluster assembly in SoxR in *E. coli* cells under aerobic conditions.

IscA/SufA paralogs are dispensable for the [2Fe-2S] cluster assembly in other proteins in *E. coli* under aerobic growth conditions

To test whether IscA/SufA paralogs are required for the [2Fe-2S] cluster assembly in other proteins, we expressed the recombinant ferredoxin [2Fe-2S] cluster [41] in the wild-type and the *iscA/sufA* double mutant cells. Figure 6A shows that ferredoxin purified from the wild-type and the *iscA/sufA* double mutant cells also had a similar UV-visible absorption spectrum indicating the presence of the [2Fe-2S] cluster. The iron and sulfide content analyses showed that ferredoxin purified from the wild-type (0.6±0.1 iron and 0.5±0.2 sulfide per protein (n=3)) and from the *iscA/sufA* double mutant (0.5±0.1 iron and 0.4±0.2 sulfide per protein (n=3)) were very similar to each other.

We also examined the [2Fe-2S] cluster assembly in the siderophore-iron reductase FhuF [42] in the wild-type and the *iscA/sufA* double mutant cells in LB medium under aerobic growth conditions. Figure 6B shows that FhuF purified from the wild-type and the *iscA/sufA* double mutant cells had a typical UV-visible absorption spectrum indicating the presence of the [2Fe-2S] cluster. The iron and sulfide content analyses further revealed that FhuF purified from the wild-type (1.1±0.1 iron and 0.7±0.1 sulfide per protein (n=3)) and from the *iscA/sufA* double mutant (1.2±0.1 iron and 0.9±0.2 sulfide per protein (n=3)) were essentially the same. Thus, deletion of IscA/SufA paralogs does not significantly affect the [2Fe-2S] cluster assembly in the redox transcription factor SoxR, ferredoxin and siderophore-iron reductase FhuF in *E. coli* cells under aerobic growth conditions.

Discussion

Iron-sulfur cluster assembly requires a coordinated delivery of iron and sulfur in cells. While sulfide in iron-sulfur clusters is derived from L-cysteine via cysteine desulfurases [4], the iron donor for the iron-sulfur cluster assembly still remains elusive. It has been postulated that mitochondrial protein frataxin [54] and its bacterial homolog CyaY [55] may act as an iron donor for the iron-sulfur cluster assembly. However, deletion of frataxin/CyaY has little or no effect on iron-sulfur proteins in *E. coli* [56] and *S. cerevisiae* [57]. On the other hand, depletion of frataxin has been linked to the human neurodegenerative disease Friedreich's ataxia [58], indicating that frataxin/CyaY may have yet unknown and critical cellular functions. The other

proposed iron donors for the iron-sulfur cluster assembly are IscA/SufA paralogs [27]. In vitro studies have shown that IscA/SufA purified from *E. coli* can bind ferrous iron and deliver iron for the iron-sulfur cluster assembly in IscU [26-29]. While deletion of either IscA or SufA had only a mild effect on cell growth of *E. coli*, deletion of both IscA and SufA resulted in a null-growth phenotype in minimal medium under aerobic growth conditions [28]. These results led us to propose that IscA/SufA paralogs have an indispensable role in the biogenesis of iron-sulfur clusters in *E. coli* cells under aerobic conditions. Here we report that cell growth of the *iscA/sufA* double mutant can be partially restored by supplementing with the branched-chain amino acids (BCAA) and thiamin in growth medium under aerobic conditions. Furthermore, we find that the *iscA/sufA* double mutant fails to assemble the [4Fe-4S] cluster in dihydroxyacid dehydratase (IlvD) of the BCAA biosynthesis pathway, ThiC of the thiamin biosynthesis pathway, aconitase B of the citrate acid cycle, and endonuclease III of the DNA base excision repair pathway under aerobic growth conditions, suggesting that IscA/SufA paralogs are essential for the [4Fe-4S] cluster assembly in *E. coli* under aerobic growth conditions. On the other hand, deletion of IscA/SufA paralogs has little or no effect on the [2Fe-2S] cluster assembly in the redox transcription factor SoxR, ferredoxin and the siderophore-iron reductase FhuF in *E. coli* cells under aerobic growth conditions, indicating that IscA/SufA paralogs may be dispensable for the [2Fe-2S] cluster assembly in cells.

The specific function of IscA/SufA paralogs for the [4Fe-4S] cluster assembly in proteins could only be speculated. In the present study, we find that the iron-bound IscA/SufA can efficiently provide iron for the [4Fe-4S] cluster assembly in IlvD and restore the enzyme activity under aerobic conditions (Figure 3), suggesting that IscA/SufA paralogs may directly provide iron for the [4Fe-4S] cluster assembly in apo-IlvD. However, we could not exclude the possibility that IscA/SufA paralogs may also act as scaffolds for the iron-sulfur cluster assembly as proposed by others [20-23]. Indeed, IscA/SufA with pre-assembled iron-sulfur clusters can also restore the enzyme activity of IlvD in the presence of dithiothreitol (data not shown). Nevertheless, the iron-sulfur cluster pre-assembled in IscA/SufA paralogs is oxygen-labile [20-23], thus limiting its ability to re-activate apo-IlvD under aerobic conditions. On the other hand, the iron center in IscA is stable under aerobic conditions [51], and can be readily mobilized by L-cysteine [29] for the iron-sulfur cluster assembly in proteins. While the mechanism underlying the [4Fe-4S] cluster assembly could still not be ascertained, we propose that under aerobic conditions IscA/SufA paralogs may act as iron chaperons to make the iron accessible for the [4Fe-4S] cluster assembly in proteins such as IlvD, ThiC, aconitase B, and endonuclease III in *E. coli* cells.

A salient finding from the present study is that the *iscA/sufA* double mutant has an elevated level of basal oxidative stress (Figure 5B). Because IscA or SufA per se does not have anti-oxidative stress activity [26], it is possible that deficiency of the [4Fe-4S] cluster assembly in the *iscA/sufA* double mutant may result in accumulation of intracellular “free” iron and promote cellular oxidative stress. This would be consistent with the previous studies showing that deletion of the IscA homologs in *S. cerevisiae* led to accumulation of “free” iron in mitochondria [25]. Alternatively, the *iscA/sufA* double mutant may fail to assemble the [4Fe-4S] cluster in 6-phosphogluconate dehydratase [14], thus limiting cellular NADPH production and increasing basal oxidative stress. Additional experiments are required to illustrate the mechanism of the elevated basal oxidative stress in the *iscA/sufA* double mutant *E. coli* cells under aerobic growth conditions.

There are over 200 distinctive iron-sulfur proteins known so far [1-3]. The [2Fe-2S] clusters and the [4Fe-4S] clusters represent two major types of iron-sulfur clusters found in cells. Current model suggests that iron-sulfur cluster assembly scaffolds such as IscU [12] and NfuA [59-61] may host both the [2Fe-2S] clusters and the [4Fe-4S] clusters and transfer the “correct” clusters to target proteins. It has also been reported that the [2Fe-2S] clusters in IscU can be

reductively converted to the [4Fe-4S] clusters in vitro [19]. Nevertheless, conversion between the [2Fe-2S] clusters and the [4Fe-4S] clusters has yet to be demonstrated in vivo [12], and little is known for regulation of any scaffold proteins to bind the [4Fe-4S] clusters or the [2Fe-2S] clusters. Structurally, the [2Fe-2S] clusters and the [4Fe-4S] clusters are very different in their ligand arrangements within proteins. While there are two ligands from protein that bind each iron atom of the [2Fe-2S] cluster, there is only one ligand from protein that binds each iron atom of the [4Fe-4S] cluster. It is plausible that proteins that host the [2Fe-2S] cluster or the [4Fe-4S] cluster may have different binding affinity for "free" iron in cells because of their ligand arrangements for the iron-sulfur clusters. Preliminary studies indicated that at limited iron concentrations, the [2Fe-2S] clusters are preferably assembled in ferredoxin than the [4Fe-4S] cluster in IlvD under aerobic conditions (unpublished data). In summary, the results presented in this study led us to propose that under aerobic conditions IscA/SufA paralogs may act as iron chaperons to provide iron for the [4Fe-4S] cluster assembly but are dispensable for the [2Fe-2S] cluster assembly in *E. coli* cells, and that the [2Fe-2S] cluster and [4Fe-4S] cluster may have distinct assembly pathways.

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Abbreviations

BCAA the branched-chain amino acids

FhuF siderophore-iron reductase

IlvD dihydroxyacid dehydratase

iscA/sufA* double mutant** ***E. coli **strain** in which both *iscA* and *sufA* were in-frame deleted

IscS cysteine desulfurase

LeuCD isopropylmalate isomerase

References

1. Bandyopadhyay S, Chandramouli K, Johnson MK. Iron-sulfur cluster biosynthesis. Biochem Soc Trans 2008;36:1112–1119. [PubMed: 19021507]
2. Rouault TA, Tong WH. Iron-sulfur cluster biogenesis and human disease. Trends Genet 2008;24:398–407. [PubMed: 18606475]
3. Fontecave M, Ollagnier-de-Choudens S. Iron-sulfur cluster biosynthesis in bacteria: Mechanisms of cluster assembly and transfer. Arch Biochem Biophys 2008;474:226–237. [PubMed: 18191630]
4. Zheng L, White RH, Cash VL, Jack RF, Dean DR. Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci U S A 1993;90:2754–2758. [PubMed: 8464885]
5. Zheng L, Cash VL, Flint DH, Dean DR. Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii. J Biol Chem 1998;273:13264–13272. [PubMed: 9582371]
6. Takahashi Y, Nakamura M. Functional assignment of the ORF2-iscS-iscU-iscA-hscB-hscA-fdx-ORF3 gene cluster involved in the assembly of Fe-S clusters in Escherichia coli. J Biochem (Tokyo) 1999;126:917–926. [PubMed: 10544286]

7. Schwartz CJ, Giel JL, Patschkowski T, Luther C, Ruzicka FJ, Beinert H, Kiley PJ. IscR, an Fe-S cluster-containing transcription factor, represses expression of Escherichia coli genes encoding Fe-S cluster assembly proteins. Proc Natl Acad Sci U S A 2001;98:14895–14900. [PubMed: 11742080]
8. Giel JL, Rodionov D, Liu M, Blattner FR, Kiley PJ. IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O-regulated genes in Escherichia coli. Mol Microbiol 2006;60:1058–1075. [PubMed: 16677314]
9. Yeo WS, Lee JH, Lee KC, Roe JH. IscR acts as an activator in response to oxidative stress for the suf operon encoding Fe-S assembly proteins. Mol Microbiol 2006;61:206–218. [PubMed: 16824106]
10. Agar JN, Krebs C, Frazzon J, Huynh BH, Dean DR, Johnson MK. IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. Biochemistry 2000;39:7856–7862. [PubMed: 10891064]
11. Smith AD, Agar JN, Johnson KA, Frazzon J, Amster IJ, Dean DR, Johnson MK. Sulfur transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. J Am Chem Soc 2001;123:11103–11104. [PubMed: 11686732]
12. Raulfs EC, O'Carroll IP, Dos Santos PC, Unciuleac MC, Dean DR. In vivo iron-sulfur cluster formation. Proc Natl Acad Sci U S A 2008;105:8591–8596. [PubMed: 18562278]
13. Urbina HD, Silberg JJ, Hoff KG, Vickery LE. Transfer of Sulfur from IscS to IscU during Fe/S Cluster Assembly. J Biol Chem 2001;276:44521–44526. [PubMed: 11577100]
14. Schwartz CJ, Djaman O, Imlay JA, Kiley PJ. The cysteine desulfurase, IscS, has a major role in in vivo Fe-S cluster formation in Escherichia coli. Proc Natl Acad Sci U S A 2000;97:9009–9014. [PubMed: 10908675]
15. Cupp-Vickery JR, Urbina H, Vickery LE. Crystal structure of IscS, a cysteine desulfurase from Escherichia coli. J Mol Biol 2003;330:1049–1059. [PubMed: 12860127]
16. Silberg JJ, Hoff KG, Tapley TL, Vickery LE. The Fe/S assembly protein IscU behaves as a substrate for the molecular chaperone Hsc66 from Escherichia coli. J Biol Chem 2001;276:1696–1700. [PubMed: 11053447]
17. Chandramouli K, Johnson MK. HscA and HscB stimulate [2Fe-2S] cluster transfer from IscU to apoferredoxin in an ATP-dependent reaction. Biochemistry 2006;45:11087–11095. [PubMed: 16964969]
18. Bonomi F, Iametti S, Morleo A, Ta D, Vickery LE. Studies on the Mechanism of Catalysis of Iron-Sulfur Cluster Transfer from IscU[2Fe2S] by HscA/HscB Chaperones. Biochemistry. 2008
19. Chandramouli K, Unciuleac MC, Naik S, Dean DR, Huynh BH, Johnson MK. Formation and properties of [4Fe-4S] clusters on the IscU scaffold protein. Biochemistry 2007;46:6804–6811. [PubMed: 17506525]
20. Ollagnier-de-Choudens S, Mattioli T, Takahashi Y, Fontecave M. Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. J Biol Chem 2001;276:22604–22607. [PubMed: 11319236]
21. Krebs C, Agar JN, Smith AD, Frazzon J, Dean DR, Huynh BH, Johnson MK. IscA, an alternate scaffold for Fe-S cluster biosynthesis. Biochemistry 2001;40:14069–14080. [PubMed: 11705400]
22. Morimoto K, Yamashita E, Kondou Y, Lee SJ, Arisaka F, Tsukihara T, Nakai M. The asymmetric IscA homodimer with an exposed [2Fe-2S] cluster suggests the structural basis of the Fe-S cluster biosynthetic scaffold. J Mol Biol 2006;360:117–132. [PubMed: 16730357]
23. Ollagnier-De-Choudens S, Sanakis Y, Fontecave M. SufA/IscA: reactivity studies of a class of scaffold proteins involved in [Fe-S] cluster assembly. J Biol Inorg Chem 2004;9:828–838. [PubMed: 15278785]
24. Balasubramanian R, Shen G, Bryant DA, Golbeck JH. Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium Synechococcus sp. strain PCC 7002. J Bacteriol 2006;188:3182–3191. [PubMed: 16621810]
25. Jensen LT, Culotta VC. Role of Saccharomyces cerevisiae ISA1 and ISA2 in iron homeostasis. Mol Cell Biol 2000;20:3918–3927. [PubMed: 10805735]
26. Ding H, Yang J, Coleman LC, Yeung S. Distinct Iron Binding Property of Two Putative Iron Donors for the Iron-Sulfur Cluster Assembly: IscA and the Bacterial Frataxin Ortholog CyaY Under Physiological and Oxidative Stress Conditions. J Biol Chem 2007;282:7997–8004. [PubMed: 17244611]

27. Yang J, Bitoun JP, Ding H. Interplay of IscA and IscU in biogenesis of iron-sulfur clusters. J Biol Chem 2006;281:27956–27963. [PubMed: 16877383]
28. Lu J, Yang J, Tan G, Ding H. Complementary roles of SufA and IscA in the biogenesis of iron-sulfur clusters in Escherichia coli. Biochem J 2008;409:535–543. [PubMed: 17941825]
29. Ding B, Smith ES, Ding H. Mobilization of the iron centre in IscA for the iron-sulphur cluster assembly in IscU. Biochem J 2005;389:797–802. [PubMed: 15828873]
30. Bitoun JP, Wu G, Ding H. Escherichia coli FtnA acts as an iron buffer for reassembly of iron-sulfur clusters in response to hydrogen peroxide stress. Biometals 2008;21:693–703. [PubMed: 18618270]
31. Abdel-Ghany SE, Ye H, Garifullina GF, Zhang L, Pilon-Smits EA, Pilon M. Iron-sulfur cluster biogenesis in chloroplasts. Involvement of the scaffold protein CpIscA. Plant Physiol 2005;138:161–172. [PubMed: 15888686]
32. Cozar-Castellano I, Del Valle Machargo M, Trujillo E, Arteaga MF, Gonzalez T, Martin-Vasallo P, Avila J. hIscA: a protein implicated in the biogenesis of iron-sulfur clusters. Biochim Biophys Acta 2004;1700:179–188. [PubMed: 15262227]
33. Johnson DC, Unciuleac MC, Dean DR. Controlled expression and functional analysis of iron-sulfur cluster biosynthetic components within Azotobacter vinelandii. J Bacteriol 2006;188:7551–7561. [PubMed: 16936042]
34. Loiseau L, Gerez C, Bekker M, Ollagnier-de-Choudens S, Py B, Sanakis Y, Teixeira de Mattos J, Fontecave M, Barras F. ErpA, an iron sulfur (Fe S) protein of the A-type essential for respiratory metabolism in Escherichia coli. Proc Natl Acad Sci U S A. 2007
35. Takahashi Y, Tokumoto U. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem 2002;277:28380–28383. [PubMed: 12089140]
36. Flint DH, Emptage MH, Finnegan MG, Fu W, Johnson MK. The role and properties of the iron-sulfur cluster in Escherichia coli dihydroxy-acid dehydratase. J Biol Chem 1993;268:14732–14742. [PubMed: 8325851]
37. Martinez-Gomez NC, Downs DM. ThiC is an [Fe-S] cluster protein that requires AdoMet to generate the 4-amino-5-hydroxymethyl-2-methylpyrimidine moiety in thiamin synthesis. Biochemistry 2008;47:9054–9056. [PubMed: 18686975]
38. Varghese S, Tang Y, Imlay JA. Contrasting sensitivities of Escherichia coli aconitases A and B to oxidation and iron depletion. J Bacteriol 2003;185:221–230. [PubMed: 12486059]
39. Cunningham RP, Asahara H, Bank JF, Scholes CP, Salerno JC, Surerus K, Munck E, McCracken J, Peisach J, Emptage MH. Endonuclease III is an iron-sulfur protein. Biochemistry 1989;28:4450–4455. [PubMed: 2548577]
40. Ding H, Hidalgo E, Demple B. The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. J Biol Chem 1996;271:33173–33175. [PubMed: 8969171]
41. Ta DT, Vickery LE. Cloning, sequencing, and overexpression of a [2Fe-2S] ferredoxin gene from Escherichia coli. J Biol Chem 1992;267:11120–11125. [PubMed: 1317854]
42. Muller K, Matzanke BF, Schunemann V, Trautwein AX, Hantke K. FhuF, an iron-regulated protein of Escherichia coli with a new type of [2Fe-2S] center. Eur J Biochem 1998;258:1001–1008. [PubMed: 9990318]
43. Ding H, Harrison K, Lu J. Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. J Biol Chem 2005;280:30432–30437. [PubMed: 15985427]
44. Fischer DS. A method for the rapid detection of acute iron toxicity. Clin Chem 1967;13:6–11. [PubMed: 6016599]
45. Siegel LM. A Direct Microdetermination of Sulfide. Anal Biochem 1965;11:126–132. [PubMed: 14328633]
46. Cioffi EA, Shaw KJ, Bailey WF, Berg CM. Improved synthesis of the sodium salt of DL-alpha, beta-dihydroxyisovaleric acid. Anal Biochem 1980;104:485–488. [PubMed: 7004266]
47. Ren B, Zhang N, Yang J, Ding H. Nitric oxide-induced bacteriostasis and modification of iron-sulphur proteins in Escherichia coli. Mol Microbiol 2008;70:953–964. [PubMed: 18811727]
48. Nunoshiba T, Hidalgo E, Amabile Cuevas CF, Demple B. Two-stage control of an oxidative stress regulon: the Escherichia coli SoxR protein triggers redox-inducible expression of the soxS regulatory gene. J Bacteriol 1992;174:6054–6060. [PubMed: 1400156]

49. Jang S, Imlay JA. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J Biol Chem 2007;282:929–937. [PubMed: 17102132]
50. Dougherty MJ, Downs DM. A connection between iron-sulfur cluster metabolism and the biosynthesis of 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate in Salmonella enterica. Microbiology 2006;152:2345–2353. [PubMed: 16849799]
51. Ding H, Clark RJ. Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. Biochem J 2004;379:433–440. [PubMed: 14720122]
52. Martinez-Gomez NC, Robers M, Downs DM. Mutational analysis of ThiH, a member of the radical S-adenosylmethionine (AdoMet) protein superfamily. J Biol Chem 2004;279:40505–40510. [PubMed: 15271986]
53. Ding H, Demple B. In vivo kinetics of a redox-regulated transcriptional switch. Proc Natl Acad Sci U S A 1997;94:8445–8449. [PubMed: 9237996]
54. Yoon T, Cowan JA. Iron-sulfur cluster biosynthesis. Characterization of frataxin as an iron donor for assembly of [2Fe-2S] clusters in ISU-type proteins. J Am Chem Soc 2003;125:6078–6084. [PubMed: 12785837]
55. Layer G, Ollagnier-de Choudens S, Sanakis Y, Fontecave M. Iron-sulfur cluster biosynthesis: characterization of Escherichia coli CYaY as an iron donor for the assembly of [2Fe-2S] clusters in the scaffold IscU. J Biol Chem 2006;281:16256–16263. [PubMed: 16603772]
56. Li DS, Ohshima K, Jiralerspong S, Bojanowski MW, Pandolfo M. Knockout of the cyaY gene in Escherichia coli does not affect cellular iron content and sensitivity to oxidants. FEBS Lett 1999;456:13–16. [PubMed: 10452520]
57. Duby G, Foury F, Ramazzotti A, Herrmann J, Lutz T. A non-essential function for yeast frataxin in iron-sulfur cluster assembly. Hum Mol Genet 2002;11:2635–2643. [PubMed: 12354789]
58. Campuzano V, Montermini L, Lutz Y, Cova L, Hindelang C, Jiralerspong S, Trottier Y, Kish SJ, Faucheux B, Trouillas P, Authier FJ, Durr A, Mandel JL, Vescovi A, Pandolfo M, Koenig M. Frataxin is reduced in Friedreich ataxia patients and is associated with mitochondrial membranes. Hum Mol Genet 1997;6:1771–1780. [PubMed: 9302253]
59. Angelini S, Gerez C, Ollagnier-de Choudens S, Sanakis Y, Fontecave M, Barras F, Py B. NfuA, a new factor required for maturing Fe/S proteins in Escherichia coli under oxidative stress and iron starvation conditions. J Biol Chem 2008;283:14084–14091. [PubMed: 18339628]
60. Bandyopadhyay S, Naik SG, O'Carroll IP, Huynh BH, Dean DR, Johnson MK, Dos Santos PC. A proposed role for the Azotobacter vinelandii NfuA protein as an intermediate iron-sulfur cluster carrier. J Biol Chem 2008;283:14092–14099. [PubMed: 18339629]
61. Jin Z, Heinnickel M, Krebs C, Shen G, Golbeck JH, Bryant DA. Biogenesis of iron-sulfur clusters in photosystem I: holo-NfuA from the cyanobacterium Synechococcus sp. PCC 7002 rapidly and efficiently transfers [4Fe-4S] clusters to apo-PsaC in vitro. J Biol Chem 2008;283:28426–28435. [PubMed: 18694929]

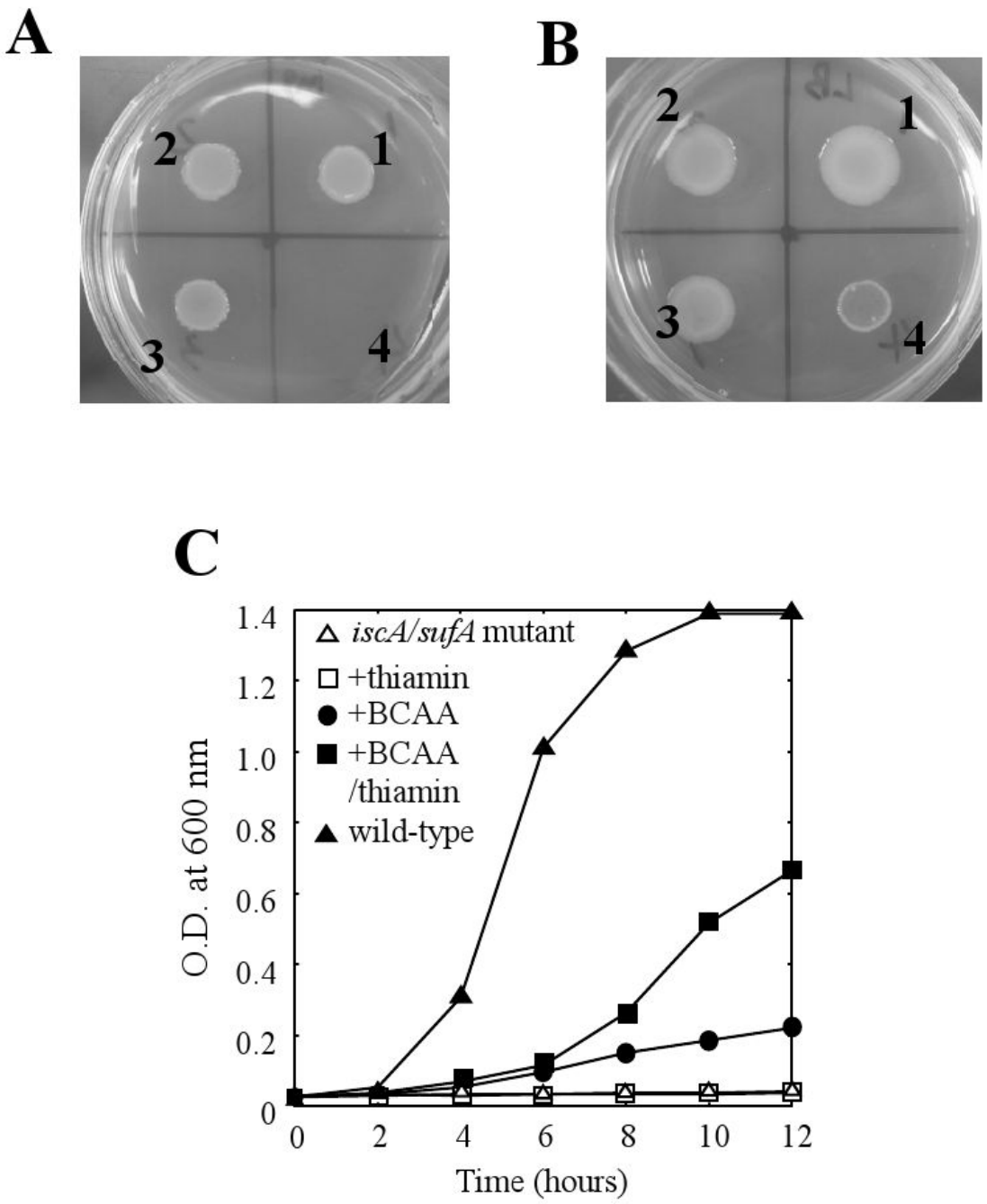

Figure 1. Deletion of IscA/SufA paralogs in *E. coli* results in deficiency of the branched-chain amino acids (BCAA) and thiamin under aerobic growth conditions

E. coli cells (~10^7) of the wild-type (1), the *sufA* mutant (2), the *iscA* mutant (3), and the *iscA/sufA* double mutant (4) were inoculated either on a minimal medium plate containing 0.2% glucose **(A)** or on a LB plate **(B)**. The pictures were taken after incubation at 37°C overnight under aerobic growth conditions. **C)**, growth curves of the wild-type (closed triangles) and the *iscA/sufA* mutant (open triangles) in minimal medium containing 0.2% glucose under aerobic growth conditions. Open squares: the *iscA/sufA* double mutant in minimal medium supplemented with thiamin (1.0 μg/mL). Closed circles: the *iscA/sufA* double mutant in minimal medium supplemented with three branched-chain amino acids (90 μg/mL). Closed

squares: cell growth of the *iscA/sufA* double mutant in minimal medium supplemented with both BCAA (90 μg/mL) and thiamin (1.0 μg/mL). The results are representatives of three independent experiments.

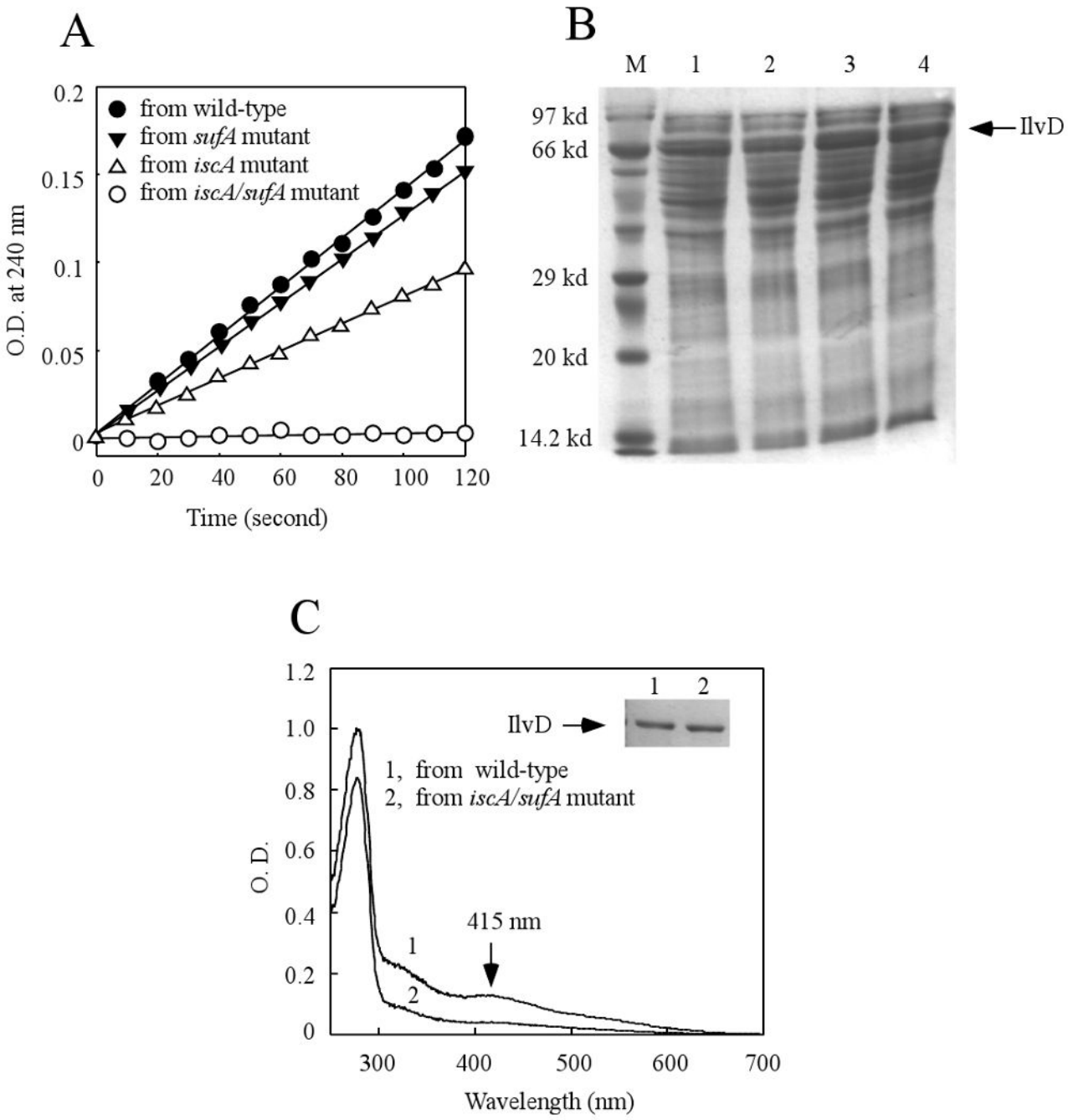

Figure 2. IscA/SufA paralogs are required for the [4Fe-4S] cluster assembly in IlvD in *E. coli* under aerobic growth conditions

A), specific activity of IlvD in the cell extracts prepared from the wild-type (closed circles), the *sufA* mutant (closed triangles), the *iscA* mutant (open triangles), and the *iscA/sufA* double mutant (open circles) grown in LB medium under aerobic growth conditions. The enzyme activity of IlvD was monitored at 240 nm as described in the Experimental. **B)**, SDS polyacrylamide gel electrophoresis analysis of the cell extracts prepared from the wild-type (1), the *sufA* mutant (2), the *iscA* mutant (3), and the *iscA/sufA* double mutant (4) cells containing recombinant IlvD. The molecular weight markers are indicted on left. **C)**, UV-visible absorption spectra of recombinant IlvD purified from the wild-type (spectrum 1) and

the *iscA/sufA* double mutant (spectrum 2). The concentrations of IlvD were ~ 20 μM. The insert shows a photograph of the SDS/PAGE gel of IlvD purified from the wild-type (lane 1) and the *iscA/sufA* double mutant (lane 2). Abbreviation: O.D. absorbance (A).

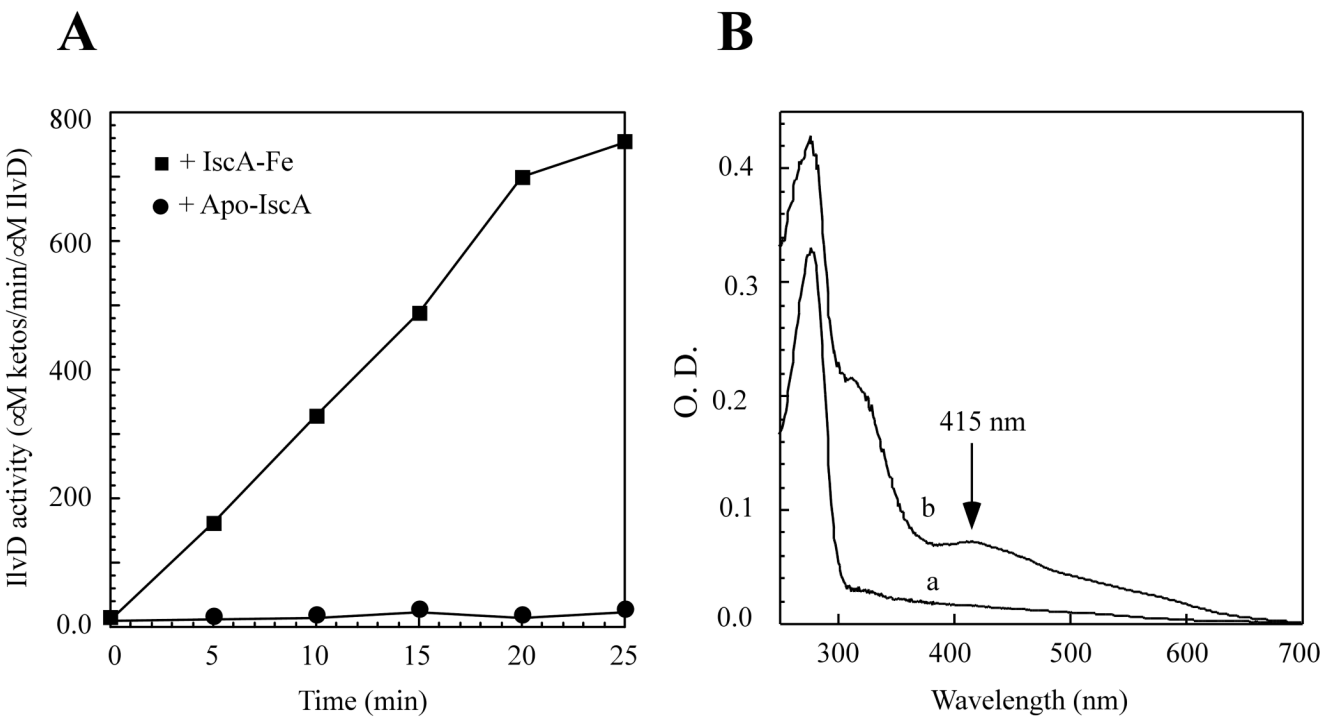

Figure 3. The iron-bound IscA can efficiently provide iron for the [4Fe-4S] cluster assembly in apo-IlvD under aerobic conditions

A), re-activation of apo-IlvD by the iron-bound IscA. Apo-IlvD (10 μM) prepared from the *iscA/sufA* double mutant was incubated with the iron-bound IscA (containing 75 μM iron), L-cysteine (0.5 mM), cysteine desulfurase IscS (0.5 μM), and dithiothreitol (2 mM) (closed squares) at 37°C aerobically for indicated time. Apo-IlvD (10 μM) incubated with apo-IscA, L-cysteine (1 mM), IscS (0.5 μM) and dithiothreitol (2 mM) at 37°C was used as a control (closed circles). The enzyme activity of IlvD was measured as described in the Experimental. **B)**, UV-visible absorption spectra of IlvD before (a) and after (b) incubation with the iron-bound IscA, L-cysteine, IscS and dithiothreitol at 37°C for 30 min under aerobic conditions. IlvD was re-purified from the incubation solutions using a Mono-Q column. The concentrations of IlvD were ~10 μM.

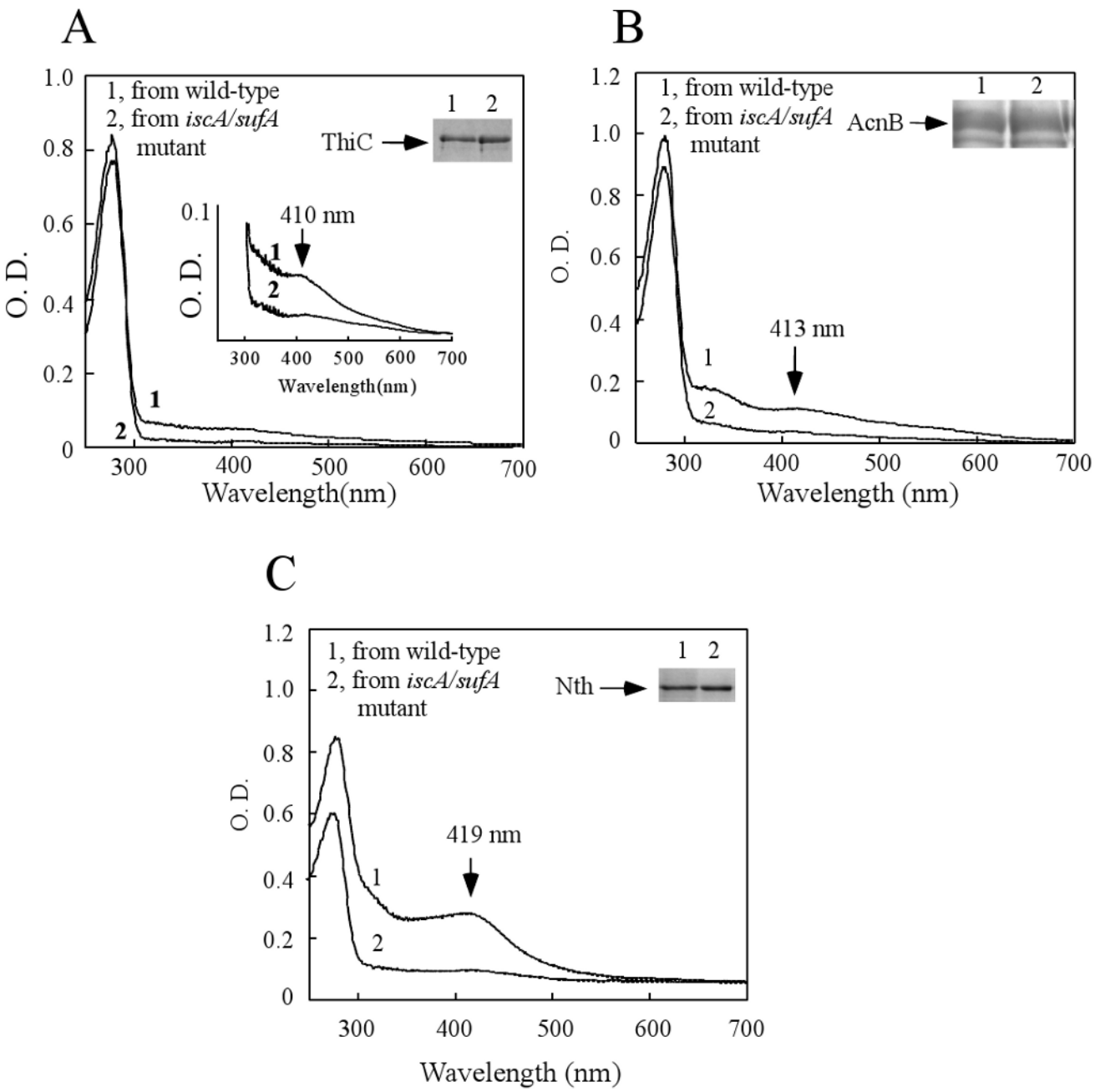

Figure 4. IscA/SufA paralogs are required for the [4Fe-4S] cluster assembly in ThiC, aconitase B, and endonuclease III in *E. coli* under aerobic growth conditions

A), UV-visible absorption spectra of ThiC purified from the wild-type (1) and the *iscA/sufA* double mutant (2) grown in LB medium under aerobic growth conditions. The concentrations of purified ThiC were ~11 μM. **B)**, UV-visible absorption spectra of aconitase B purified from the wild-type (1) and the *iscA/sufA* double mutant (2) grown in LB medium under aerobic growth conditions. The concentrations of purified aconitase B were ~12 μM. **C)**, UV-visible absorption spectra of endonuclease III (Nth) purified from the wild-type (1) and the *iscA/sufA* double mutant (2) grown in LB medium under aerobic growth conditions. The concentrations of purified endonuclease III were ~15 μM. Inserts in each panel are photographs

of the SDS/PAGE gel of purified protein from the wild-type (1) and the *iscA/sufA* double mutant (2), respectively.

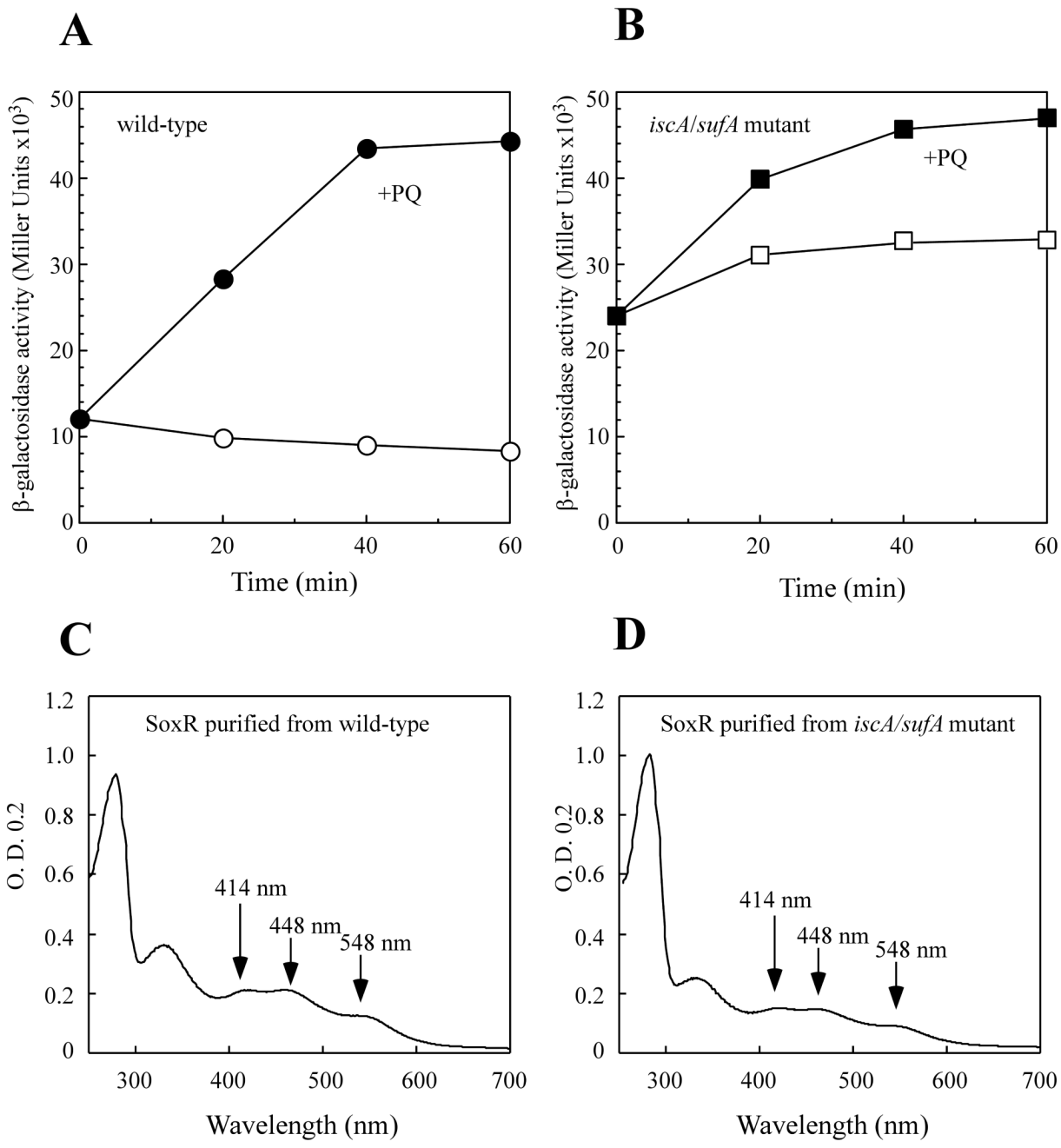

Figure 5. Redox activation of the transcription factor SoxR by paraquat in the wild-type and the *iscA/sufA* double mutant *E. coli* cells

A), activation of SoxR by paraquat in the wild-type *E. coli* cells. Cells hosting a plasmid pTN1530 (containing a reporter gene *soxS::lacZ*) were incubated in the absence (open circles) or presence (closed circles) of paraquat (100 μM) with vigorous aeration (250 rpm) in LB medium. Aliquots (20 μL) were taken from cell cultures at 20-min intervals for the β-galactosidase measurements as described in the Experimental. **B)**, activation of SoxR by paraquat in the *iscA/sufA* double mutant under aerobic growth conditions. The *iscA/sufA* double mutant cells hosting a plasmid pTN1530 (containing a reporter gene *soxS::lacZ*) were incubated in the absence (open squares) or presence (closed squares) of paraquat (100 μM)

with vigorous aeration (250 rpm) in LB medium. Aliquots (20 μL) were taken from cell cultures at 20-min intervals for the β-galactosidase measurements as described in the Experimental. **C)**, UV-visible absorption spectrum of recombinant SoxR purified from the wild-type *E. coli* cells grown in LB medium under aerobic conditions. **D)**, UV-visible absorption spectrum of recombinant SoxR purified from the *iscA/sufA* double mutant *E. coli* cells grown in LB medium under aerobic conditions. The protein concentrations of SoxR in panel C and D were ~ 60 μM.

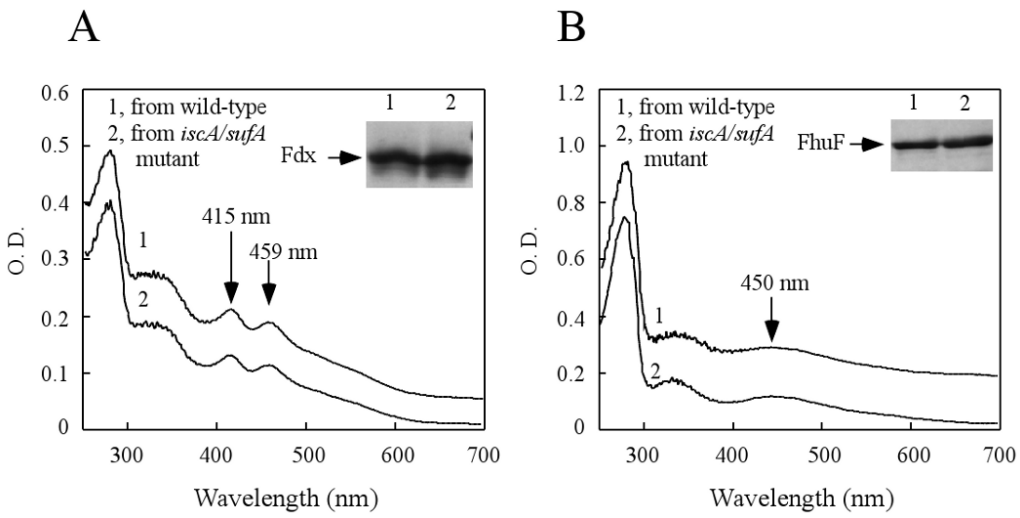

Figure 6. IscA/SufA paralogs are dispensable for the [2Fe-2S] cluster assembly in ferredoxin and FhuF in *E. coli* under aerobic growth conditions

Ferredoxin or FhuF was expressed in the wild-type and the *iscA/sufA* double mutant cells grown in LB medium under aerobic growth conditions. Proteins were purified as described in the Experimental. **A)**, UV-visible absorption spectra of ferredoxin purified from the wild-type (spectrum 1) and the *iscA/sufA* double mutant (spectrum 2). The concentrations of purified ferredoxin were ~50 μM. Spectrum 1 was up-shifted by 0.05 O.D. Inserts are photographs of the SDS/PAGE gel of purified ferredoxin from the wild-type (1) and the *iscA/sufA* double mutant (2). **B)**, UV-visible spectra of FhuF purified from the wild-type (spectrum 1) and the *iscA/sufA* double mutant (spectrum 2). The concentrations of purified FhuF were ~13 μM. Spectrum 1 was up-shifted by 0.2 O.D. Inserts are photographs of the SDS/PAGE gel of purified FhuF from the wild-type (1) and the *iscA/sufA* double mutant (2).

Table 1

PCR primers for the gene cloning

gene	Primer-1	Primer-2
ilvD	5′-atagagctcatgcctaagtaccgttccgccacc-3′	5′-cgcgaattcttaacccccccagtttcgatttatc-3′
thiC	5′-ataccatggctgcaacaaaactgacccg-3′	5′-tctaagcttcgcttcctccttacgcaggtag-3′
acnB	5′-gaaccgccatggtagaagaatacc-3′	5′-tgacttttttaaagcttagtctgga-3′
nth	5′-aatgtcccatggataaagcaaaac-3′	5′-ttcttcaaagcttaactttctctt-3′
fdx	5′-aggtttaccatggcaaagattgtt-3′	5′-cctctgttaaagcttacgcgcatg-3′
soxR	5′-atagagctcatggaaaagaaattacccc-3′	5′-cgcgaattcttagttttgttcatcttcc-3′
fhuF	5′-tatccatggcctatcgttccgcaccgctctatg-3′	5′-gcgaagctttttcagcgtacaatcgccacattg-3′

ilvD, dihydroxyacid dehydratase; *thiC*, thiamin synthase C; *acnB*, aconitase B; *nth*, endonuclease III; *fdx*, ferredoxin; *soxR*, redox transcription factor SoxR; *fhuF*, siderophore-iron reductase.