Complementary roles of SufA and IscA in the biogenesis of iron–sulfur clusters in Escherichia coli

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INTRODUCTION

Iron–sulfur proteins represent one of the major iron-containing proteins in cells. Over 200 unique iron–sulfur proteins have been characterized so far in diverse physiological processes [1–3]. Despite the ubiquitous presence of iron–sulfur proteins, the mechanism underlying iron–sulfur cluster assembly is still not fully understood [1–3]. Genetic studies have revealed a gene cluster iscSUA-HscBA-fdx that is responsible for general biogenesis of iron–sulfur clusters in Escherichia coli [4,5]. The six proteins encoded by the gene cluster are highly conserved from bacteria to humans [3–7]. IscS is a cysteine desulfurase that catalyses desulfurization of L-cysteine [8,9] and transfers sulfane sulfur to a scaffold protein IscU [10,11]. IscU assembles transient iron–sulfur clusters and delivers the clusters to target proteins [12,13]. Two heat-shock cognate proteins HscB and HscA have specific protein–protein interactions with IscU [14–16] and stimulate the transfer of the assembled clusters from IscU to apo-fdx (ferredoxin) in an ATP-dependent reaction [17]. The function of fdx is still not clear, although recent studies indicated that fdx could be a physiological reductant for the formation of [4Fe–4S] clusters in IscU [18]. IscA has been characterized as an alternative scaffold [19–25] and as a regulatory protein [26]. However, unlike IscU [12], IscA has a strong iron-binding affinity with an iron-association constant of 2.0 × 10^19 M\(^{-1}\) [27,28], and the iron-bound IscA can provide iron for the iron–sulfur cluster assembly in IscU in the presence of a thioredoxin reductase system [28–32]. These results suggest that the primary function of IscA may be to recruit intracellular iron and deliver iron for the iron–sulfur cluster assembly in IscU [28].

IscA or SufA is re-introduced into the iscA−/sufA− double mutant, demonstrating further that either IscA or SufA is sufficient for their functions in vivo. Purified SufA, like IscA, is an iron-binding protein that can provide iron for the iron–sulfur cluster assembly in IscU in the presence of a thioredoxin reductase system which emulates the intracellular redox potential. Site-directed mutagenesis studies show that the SufA/IscA variants that lose the specific iron-binding activity fail to restore the cell growth of the iscA−/sufA− double mutant. The results suggest that SufA and IscA may constitute the redundant cellular activities to recruit intracellular iron and deliver iron for the iron–sulfur cluster assembly in E. coli.

Key words: cysteine desulfurase IscS, iron donor, iron–sulfur cluster biogenesis, IscA, IscU, SufA.

Biogenesis of iron–sulfur clusters requires a concerted delivery of iron and sulfur to target proteins. It is now clear that sulfur in iron–sulfur clusters is derived from L-cysteine via cysteine desulfurases. However, the specific iron donor for the iron–sulfur cluster assembly still remains elusive. Previous studies showed that IscA, a member of the iron–sulfur cluster assembly gene cluster in Escherichia coli, is a novel iron-binding protein, and that the iron-bound IscA can provide iron for the iron–sulfur cluster assembly in a proposed scaffold IscU in vitro. However, genetic studies have indicated that IscA is not essential for the cell growth of E. coli. In the present paper, we report that SufA, an IscA paralogue in E. coli, may represent the redundant activity of IscA. Although deletion of IscA or SufA has only a mild effect on cell growth, deletion of both IscA and SufA in E. coli results in a severe growth phenotype in minimal medium under aerobic growth conditions. Cell growth is restored when either

Abbreviations used: fdx, ferredoxin; LB, Luria–Bertani.

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phenotype in minimal medium under aerobic growth conditions. The cell growth is restored when IscA or SufA is re-introduced into the iscA−/sufA− double mutant, demonstrating further that either IscA or SufA is sufficient for the functions in vivo. Like IscA, purified SufA has a strong iron-binding activity, and the iron-bound SufA can efficiently provide iron for the iron–sulfur cluster assembly in IscU in the presence of a thioredoxin reductase system. Finally, the SufA/IscA variants that lose the specific iron-binding activity in vitro fail to restore the cell growth of the iscA−/sufA− double mutant. The results led us to propose that SufA and IscA may represent the redundant cellular activities to recruit intracellular iron and deliver iron for iron–sulfur cluster assembly in E. coli.

EXPERIMENTAL

Gene knockout

The in-frame gene knockout was achieved following the procedure developed by Church’s group [48]. Briefly, the in-frame deleted DNA fragments were obtained by using crossover PCR using two pairs of primers obtained from the website http://arep.med.harvard.edu/labc/adnan/projects/EcoliKOprimers/EcoliKOprimers.html: IscA-Ni, 5′-caagcaatcaacttcacctcactaatttataaccatcgatttacctcacttcatcgctt-3′; IscA-No, 5′-aaggaaagccgcccttcctcctcggtgatgtggaatgtc-3′; IscA-Ci, 5′-cttttatagtagtggctttagtggtgtatcgcaatcgataaccecc-3′; IscA-Co, 5′-cgcaacgcgtcgcaccatctagctgagctggggtat-3′; SufA-Ni, 5′-caagcaaatcacttcacctaatttataaccatcgatttacctcacttcatcgctt-3′; SufA-No, 5′-aaggaaagccgcccttcctcctcggtgatgtggaatgtc-3′; SufA-Ci, 5′-cttttatagtagtggctttagtggtgtatcgcaatcgataaccecc-3′; SufA-Co, 5′-cgcaacgcgtcgcaccatctagctgagctggggtat-3′; and SufA-Co, 5′-cgcaacgcgtcgcaccatctagctgagctggggtat-3′.

The PCR products were cloned into a low-copy number temperature-sensitive vector pKOV that has chloramphenicol-resistant and sucrose-sensitive markers [48]. The cloned plasmid was transformed into a recombination-proficient E. coli strain (MC4100). The transformants were selected on the LB (Luria–Bertani) plates containing 20 µg/ml chloramphenicol at 42°C. The obtained colonies were immediately plated on the LB plates containing 5% (w/v) sucrose at 30°C to select the colonies that underwent the second recombination event. The colonies that were sucrose-resistant and chloramphenicol-sensitive were selected for further analysis. The positive colonies were confirmed with PCR using the primers flanking the target genes. For the iscA−/sufA− double mutant, the clones were selected on the LB plates containing 5% (w/v) sucrose and 50 µM Fe(NH4)2(SO4)2 under anaerobic conditions. For the controlled expression of SufA, IscA was incubated with freshly prepared 0–100 µM Fe(NH4)2(SO4)2 in the presence of a thioredoxin reductase system (5 µM thioredoxin–1, 0.5 µM thioredoxin reductase and 500 µM NADPH) at 37°C for 30 min, followed by re-purification of SufA using a Mono-Q column. The amplitudes of the absorption peak at 315 nm were used to estimate the iron binding in SufA as described in [27]. The total amounts of iron and sulfide in the protein samples were analysed using the methods of Fischer [51] and Siegel [52] respectively.

Iron-binding assay

The iron-depleted SufA (apo-SufA) was prepared by incubation with 10 mM EDTA and 2 mM dithiothreitol at 37°C for 30 min, followed by re-purification of the protein using a Mono-Q column. For the iron-binding experiments, 40 µM apo-SufA was incubated with freshly prepared 0–100 µM Fe(NH4)2(SO4)2 in the presence of a thioredoxin reductase system (5 µM thioredoxin–1, 0.5 µM thioredoxin reductase and 500 µM NADPH) at 37°C for 30 min, followed by re-purification of SufA using a Mono-Q column. The purity of proteins was greater than 95% as judged by electrophoretic analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The protein concentration of apo-SufA was determined using a molar absorption coefficient at 280 nm of 7.3 mM−1·cm−1. All protein concentrations in the text refer to the monomeric species.

Site-directed mutagenesis

The site-directed mutagenesis was carried out using the QuikChange™ site-directed mutagenesis kit (Stratagene) [29]. Three pairs of degenerated primers were designed to replace Cys-50, Cys-114 and Cys-116 each with serine in SufA. The mutations in the sufA gene were confirmed by direct sequencing. The SufA variants in which the cysteine residue was replaced with serine were overproduced and purified as for wild-type SufA. The IscA variants in which the conserved cysteine residues were replaced with serine were prepared as described previously [31].

Iron–sulfur cluster assembly in IscU

In typical iron–sulfur cluster assembly reactions, 50 µM E. coli IscU was incubated with 1 µM cysteine desulfurase IscS, 200 mM NaCl and 20 mM Tris/HCl (pH 8.0) in the presence of dithiothreitol or a thioredoxin reductase system as described in [29]. Either the iron-bound SufA or IscA was included as the iron donor in the pre-incubation solution. The reaction mixture was purged with pure argon gas and pre-incubated at 37°C for 5 min. The iron–sulfur cluster assembly in IscU was initiated by adding L-cysteine (1 mM) and was monitored at 456 nm [12] in a Beckman DU-640 UV–visible absorption spectrometer equipped with a temperature controller.

EPR measurements

The EPR spectra were recorded at X-band on a Bruker ESP-300 spectrometer using an Oxford Instruments ESR-900 flow cryostat (Chemistry Department/LSU). The EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 4.5 K; receive gain, 1.0 × 105.

RESULTS

Complementary roles of IscA and SufA in E. coli

Previous biochemical studies have shown that E. coli IscA is an iron-binding protein that can deliver iron for the iron–sulfur
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Three in-frame deletion mutants, iscA<sup>−</sup>, sufA<sup>−</sup> and iscA<sup>−</sup>/sufA<sup>−</sup>, were prepared from wild-type E. coli strain MC4100 as described in the Experimental section. (A) Effect of the IscA and SufA deletion on the cell growth in liquid LB medium. E. coli cells were inoculated in LB medium overnight, diluted in freshly prepared LB medium, and grown at 37°C with aeration. Cell growth was determined by measuring the attenuation at 600 nm (OD at 600 nm). (B) Effect of the IscA and SufA deletion on the cell growth in liquid M9 minimal medium. E. coli cells were inoculated in LB medium overnight, washed twice with M9 minimal medium containing 0.2% glucose, and grown at 37°C with aeration. Cell growth was determined by measuring the attenuation at 600 nm (OD at 600 nm). (C) Cell growth under aerobic and anaerobic conditions. Overnight cultures were washed twice with M9 minimal medium. A 2 µl aliquot of cell culture (~10<sup>6</sup> cells) was inoculated on the LB plates (a and b) or on the M9 minimal medium plates (c and d). Plates a and c were incubated at 37°C under aerobic growth conditions. Plates b and d were incubated at 37°C inside a GasPak anaerobic jar. The picture was taken after overnight incubation. Key: 1, wild-type (WT); 2, iscA<sup>−</sup> mutant; 3, sufA<sup>−</sup> mutant; 4, iscA<sup>−</sup>/sufA<sup>−</sup> mutant. (D) PCR verification of the deletion mutants. Two pairs of primers (IscA-No/IscA-Co, SufA-No/SufA-Co) were used for PCR verification of the iscA and sufA gene deletion respectively. The PCR products were run on a 1% agarose gel. Lane M, a 2 kb DNA ladder; lanes 1–4, wild-type, iscA<sup>−</sup> mutant, sufA<sup>−</sup> mutant and iscA<sup>−</sup>/sufA<sup>−</sup> mutant PCR-amplified using the IscA primers respectively. The PCR product of the iscA deletion (MT) is 1062 bp and that of wild-type (WT) is 1347 bp. Lanes 5–8, wild-type, iscA<sup>−</sup> mutant, sufA<sup>−</sup> mutant and iscA<sup>−</sup>/sufA<sup>−</sup> mutant PCR-amplified using the SufA primers respectively. The PCR product of the sufA deletion (MT) is 1063 bp and that of wild-type (WT) is 1335 bp. Results are representative of at least three experiments.

However, genetic studies have indicated that IscA is not essential for the cell growth of E. coli [39,40]. Using the in-frame gene-deletion approach [48], we replicated the observation that deletion of the iscA gene in E. coli has only a mild effect on the cell growth in LB medium (Figure 1A) and in minimal medium (containing 0.2% glucose) (Figure 1B) under aerobic growth conditions.

The genome-wide search in E. coli revealed two IscA paralogues: ErpA [41] and SufA [42]. Because ErpA has been identified as a dedicated iron–sulfur cluster assembly scaffold required for maturation of the key enzymes in the isoprenoid biosynthesis pathway [41] and SufA is a member of the second iron–sulfur cluster assembly gene cluster sufABCDSE [44–46], we chose to pursue the function of SufA further by deleting the sufA gene. As shown in Figures 1(A) and 1(B), deletion of SufA did not significantly affect the cell growth of E. coli either, indicating that SufA, like IscA, is not essential for the cell growth of E. coli. However, the iscA<sup>−</sup>/sufA<sup>−</sup> double mutant grew slowly in LB medium (Figure 1A) and showed a severe growth phenotype in minimal medium (Figure 1B) under aerobic growth conditions.

We then compared the cell growth of the E. coli mutants under anaerobic and aerobic conditions. Cells were inoculated on LB plates or minimal medium plates, and incubated under aerobic and anaerobic growth conditions overnight. Figure 1(C) shows that the single-deletion mutants (iscA<sup>−</sup> or sufA<sup>−</sup>), like the wild-type, grew on both the LB and minimal medium plates under aerobic and anaerobic growth conditions. On the other hand, the iscA<sup>−</sup>/sufA<sup>−</sup> double mutant grew slowly on the LB plate and did not grow...
on the minimal medium plate under aerobic growth conditions, consistent with the growth curves observed in the liquid medium (Figures 1A and 1B). Interestingly, under anaerobic conditions, the iscA−/sufA− double mutant grew on the minimal medium plate (Figure 1C), suggesting that IscA/SufA is required for the cell growth only under aerobic conditions. The potential mechanisms underlying the necessity of IscA/SufA for the aerobic growth of E. coli in minimal medium are discussed below. Nevertheless, these results clearly show that deletion of both IscA and SufA in E. coli resulted in a severe growth phenotype in minimal medium under aerobic growth conditions.

To explore further the complementary roles of IscA and SufA in E. coli cells, we re-introduced SufA or IscA into the iscA−/sufA− double mutant using the arabinose-controlled expression plasmid pBAD as described in the Experimental section. Figure 2 shows that IscA or SufA was able to restore the cell growth of the iscA−/sufA− double mutant in minimal medium under aerobic growth conditions, demonstrating further that either IscA or SufA is sufficient for their functions in E. coli cells.

Purified SufA is an iron-binding protein

The complementary activities of IscA and SufA to restore the cell growth of the iscA−/sufA− double mutant (Figure 2) suggest that SufA may have a similar iron-binding activity to that of IscA. To test this idea, we purified recombinant E. coli SufA as described in the Experimental section. Figure 3(A) shows that the as-purified SufA has a clear absorption peak at 315 nm, indicative of iron binding in the protein [27,29]. The total iron and sulfide content analysis showed that as-purified SufA contains approx. 0.12 ± 0.03 iron and 0.02 ± 0.02 sulfide per SufA monomer (n = 3). After incubation with the iron chelator EDTA (10 mM) and dithiothreitol (2 mM) at 37 °C for 30 min, the absorption peak at 315 nm of SufA was mostly eliminated, and the iron and sulfide contents in the protein were not detectable. The iron-depleted SufA (apo-SufA) (40 µM) was then incubated with ferrous iron (0–100 µM) in the presence of a thioredoxin reductase system which emulates the intracellular redox potential [53]. As shown in Figures 3(B) and 3(C), the absorption peak at 315 nm of SufA was almost linearly increased and saturated at approx. 20 µM iron. The absorption amplitude at 315 nm closely correlates with the total iron content in SufA. Approx. 0.56 ± 0.16 iron per SufA monomer (n = 3) was found in the iron-saturated SufA samples, which is similar to that found for E. coli IscA [27].

Previous studies also showed that the iron-bound IscA has a unique EPR signal at g = 4–6 reflecting a high-spin S = 3/2 mononuclear iron centre [27]. In this experiment, we compared the EPR spectra of IscA and SufA at liquid helium temperature. Figure 4(A) shows that as-purified SufA and IscA have an almost identical EPR signal at g = 4–6. The EPR signal is completely eliminated after incubation with EDTA (10 mM) and dithiothreitol (2 mM) to remove the iron from the proteins. When apo-SufA or apo-IscA was re-incubated with an equivalent amount of ferrous iron in the presence of a thioredoxin reductase system, the EPR signal at g = 4–6 was fully recovered with a much higher amplitude (Figure 4B). In contrast, no EPR signal at g = 4–6 was observed for the scaffold protein IscU before and after reconstitution with ferrous iron (Figure 4). Thus SufA, like IscA, has a specific iron-binding activity.

The iron-bound SufA provides iron for the iron–sulfur cluster assembly in IscU

Because the iron-bound IscA can efficiently provide iron for the iron–sulfur cluster assembly in IscU in vitro [27–32], we speculated that the iron-bound SufA may also act as an iron donor for the iron–sulfur cluster assembly. In these experiments, apo-IscU was pre-incubated with a catalytic amount of cysteine desulfurase IscS in the presence of a thioredoxin reductase system. The iron–sulfur cluster assembly reaction was initiated by adding L-cysteine. Figure 5(A) shows that no iron–sulfur clusters were assembled without iron donors in the incubation solution. When the iron-bound IscA (Figure 5B) or the iron-bound SufA (Figure 5C) was included in the pre-incubation solution, the absorption peak at 456 nm, reflecting the formation of the IscU [2Fe–2S] clusters [12], quickly appeared. The kinetics of the iron–sulfur cluster assembly in IscU are almost indistinguishable when the iron-bound IscA or the iron-bound SufA was used as the iron donor (Figure 5D). Re-purification of IscU from the incubation solutions demonstrated further that the iron centre in SufA and IscA is transferred for the iron–sulfur cluster assembly in IscU (results not shown). Thus both SufA and IscA are able to provide iron for the iron–sulfur cluster assembly in IscU in the presence of a thioredoxin reductase system.

The conserved cysteine residues in SufA/IscA are required for the iron binding in vitro and for their functions in vivo

The site-directed mutagenesis studies revealed that the three conserved cysteine residues (Cys-35, Cys-99 and Cys-101) in IscA are essential for its specific iron-binding activity [31]. It would be pertinent to examine whether the conserved cysteine residues in SufA are also required for its specific iron-binding activity. Three SufA variants in which each cysteine residue (Cys-50, Cys-114 and Cys-116) was replaced with serine were constructed. Purification of the SufA variants produced similar
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Figure 3  Purified SufA is an iron-binding protein

E. coli SufA was purified as described in the Experimental section. (A) UV–visible absorption spectra of SufA before (a) and after (b) iron was depleted. The protein concentration in each sample was 50 µM. OD 0.2 = 0.2 absorbance unit. (B) UV–visible absorption spectra of apo-SufA after incubation with different concentrations of iron. Apo-SufA (40 µM) was incubated with Fe(NH₄)₂(SO₄)₂ (0–100 µM) in the presence of a thioredoxin reductase system at 37°C for 30 min, followed by re-purification of SufA. OD 0.1 = 0.1 absorbance unit. (C) Iron-binding curve of SufA. The amplitudes at 315 nm (B) were plotted as a function of the iron concentration in the incubation solutions, determined as the absorbance at 315 nm (OD at 315 nm).

protein yields as wild-type SufA, indicating that the SufA variants, like the IscA variants [29], are stable in E. coli cells.

Figure 6 shows the UV–visible absorption spectra of the SufA variants (C50S, C114S and C116S) before and after incubation with an equivalent amount of ferrous iron in the presence of a thioredoxin reductase system. Whereas replacing Cys-50 with serine significantly decreased the iron-binding activity of SufA, replacing Cys-114 or Cys-116 with serine completely abolished iron binding in the protein. The iron binding (or lack of it) in the SufA variants was determined further by the total iron content analysis. Approx. 65% of the iron binding (relative to wild-type SufA) was found for the SufA-C50S variant, and little or no iron binding was found for the SufA-C114S and SufA-C116S variants.

The severe growth phenotype of the E. coli iscA/sufA double mutant in minimal medium under aerobic growth conditions (Figure 1) provides an opportunity to evaluate the function of the SufA/IscA variants in vivo. The arabinose-controlled expression plasmids containing the SufA/IscA variants were introduced into the iscA/sufA double mutant. Because the iscA/sufA double mutant containing the IscA/SufA variants did not grow in minimal medium under aerobic growth conditions, we were unable to verify the protein expression of the IscA/SufA variants in the cells. Nevertheless, we found that the IscA/SufA variants were expressed in the iscA/sufA double mutant grown in LB medium under aerobic growth conditions (results not shown). It is reasonable to assume that the protein expression of the IscA/SufA variants in the iscA/sufA double mutant is not affected by the substitution of serine for the cysteine residues. Taken together, the results suggest that the conserved cysteine residues in IscA/SufA are essential for their specific iron-binding activity in vitro and for their functions in vivo.

DISCUSSION

Among the six proteins encoded by the iron–sulfur cluster assembly gene cluster iscSUA-hscBA-fdx in E. coli [4,5], only IscA is found to be a novel iron-binding protein with an iron-association constant of 2.0 × 10¹⁹ M⁻¹ [27]. Whereas the iron-bound
Figure 4  EPR spectra of E. coli SufA and IscA

(A) EPR spectra of as-purified SufA (a), IscA (b) and IscU (c). Each protein was purified as described in the Experimental section. The protein concentration in each sample was 400 µM. (B) EPR spectra of SufA (a), IscA (b) and IscU (c) after incubation with ferrous iron. Apo-proteins were incubated with an equivalent amount of Fe(NH₄)₂(SO₄)₂ in the presence of a thioredoxin reductase system at 37°C for 30 min, followed by re-purification of the protein. The protein concentration was ∼600 µM.

Figure 5  Iron-bound SufA can provide iron for iron–sulfur cluster assembly in IscU

IscU (50 µM) was incubated with IscS (1 µM), NaCl (200 mM) and Tris/HCl (20 mM) (pH 8.0) in the presence of dithiothreitol (2 mM) at 37 °C for 5 min. l-Cysteine (1 mM) was added to initiate the iron–sulfur cluster assembly reaction. The absorption spectra were taken every 2 min for 20 min after the addition of l-cysteine. O.D., absorbance units. (A) No iron donor was included. (B) Iron-bound IscA (200 µM) was included in the pre-incubation solution. (C) Iron-bound SufA (200 µM) was included in the pre-incubation solution. (D) Kinetics of the iron–sulfur cluster assembly in IscU when IscA (●) or SufA (▲) was used as the iron donor. The iron–sulfur cluster assembly in IscU was monitored at 456 nm (OD at 456 nm) [12].

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Figure 6 The conserved cysteine residues in SufA are essential for its specific iron-binding activity

The conserved cysteine residues in SufA are essential for its specific iron-binding activity. The UV-visible absorption spectra of SufA and the SufA variants (C50S, C114S and C116S) before (A) and after (B) incubation with an equivalent amount of Fe(NH$_4$)$_2$(SO$_4$)$_2$ in the presence of a thioredoxin reductase system. The protein concentration in each sample was 50 µM. The absorption peak at 315 nm indicates the iron binding in the protein. OD 0.2 = 0.2 absorbance unit.

IscA is stable under both aerobic and anaerobic conditions, the iron centre in IscA can be readily mobilized by L-cysteine [30] and transferred for the iron–sulfur cluster assembly in IscU in the presence of a thioredoxin reductase system [27–32]. These results led us to propose that IscA may act as an iron donor for the iron–sulfur cluster assembly [28]. However, the physiological significance of IscA for the biogenesis of iron–sulfur clusters has been challenged by the observation that deletion of IscA in *E. coli* [39,40] has only a mild effect on the cell growth [41]. In the present paper, we report that SufA, a member of the second iron–sulfur cluster assembly gene cluster *sufABCDSE* found in *E. coli* [44–46], may represent the redundant activity of IscA. While the in-frame deletion of IscA or SufA did not significantly affect the cell growth of *E. coli*, deletion of both IscA and SufA resulted in a severe growth phenotype in minimal medium under aerobic growth conditions (Figure 1). Re-introduction of either IscA or SufA into the *iscA*/*sufA* double mutant restored cell growth (Figure 2), demonstrating further that either IscA or SufA is sufficient for their functions in vivo. Like IscA, purified SufA has a specific iron-binding activity (Figures 3 and 4) and the iron-bound SufA can efficiently provide iron for the iron–sulfur cluster assembly in IscU (Figure 5). Finally, the SufA/IscA variants that lose the specific iron-binding activity (Figure 6) fail to restore the cell growth of the *iscA*/*sufA* double mutant (Figure 7). These results suggest that SufA/IscA may represent the redundant iron donors for the biogenesis of iron–sulfur clusters.

A number of studies also indicate that IscA/SufA may act as an alternative scaffold for the iron–sulfur cluster assembly [19–25,42,43]. This notion is supported by recent studies of an *E. coli* IscA parologue ErpA which binds an iron–sulfur cluster [41]. In addition, the IscA homologues in *Thermosynechococcus elongatus* [25] and *Acidithiobacillus ferrooxidans* [54] expressed in *E. coli* are found containing an iron–sulfur cluster. However, since the conserved cysteine residues in the IscA/SufA crystal structure models are positioned close enough to potentially accommodate either a mononuclear iron or an iron–sulfur cluster [37,38,47], it should not be surprising that some IscA homologues can bind an iron–sulfur cluster. A subtle structural change in IscA homologues may switch between a mononuclear iron-binding site and an iron–sulfur cluster-binding site. Indeed, in the cyanobacterium *Synechocystis* PCC 6803, one IscA homologue SLR1417 prefers to bind a mononuclear iron and the other IscA homologue SLR1565 to bind a [2Fe–2S] cluster [55]. In a parallel experiment, we have also found that, whereas purified *E. coli* IscA and SufA contain a mononuclear iron (Figure 4), purified *E. coli* ErpA [41] binds an iron–sulfur cluster (results not shown). Therefore, even with the same conserved cysteine residues, proteins may prefer to bind a mononuclear iron or an iron–sulfur cluster, depending on other structural factors. The high iron-binding affinity of IscA/SufA and ease of mobilizing the iron bound in IscA/SufA for the biogenesis of iron–sulfur clusters strongly suggest that the primary function of IscA/SufA is to provide iron for the biogenesis of iron–sulfur clusters.

Figure 7 The conserved cysteine residues in SufA/IscA are required for their functions in vivo

The SufA/IscA variants in which the conserved cysteine residues were replaced with serine were introduced into the *iscA*/*sufA* double mutant. The cells were inoculated in LB medium overnight, washed twice with M9 minimal medium, and diluted in freshly prepared M9 minimal medium containing 0.2 % glucose and 0.002 % arabinose. The cells were grown at 37°C with aeration. The cell growth was determined by measuring the attenuation at 600 nm (OD at 600 nm). (A) Growth curves of the *iscA*/*sufA* double mutant containing different IscA variants. (B) Growth curves of the *iscA*/*sufA* double mutant containing different SufA variants.
recruit intracellular iron and deliver iron for the iron–sulfur cluster assembly in a scaffold protein IscU [28].

It is intriguing that IscA/SufA are not required for the cell growth of *E. coli* in LB medium under both aerobic and anaerobic growth conditions (Figure 1). Perhaps LB medium can provide accessible iron or other essential metabolites to support the cell growth of the *iscA* */sufA* double mutant. Specific factors in LB medium that contribute to the cell growth of the *E. coli iscA* */sufA* double mutant remain to be identified. In minimal medium, the *iscA* */sufA* double mutant did not grow under aerobic growth conditions (Figure 1). Nevertheless, under anaerobic conditions, the cell growth of the *iscA* */sufA* double mutant in minimal medium was significantly restored, suggesting that IscA/SufA is essential for cell growth only under aerobic growth conditions. This is consistent with a previous observation that depletion of IscA in *Azotobacter vinelandii* results in a null-growth phenotype only when cells are cultured in minimal medium under conditions of elevated oxygen [36]. It is worth pointing out that, under aerobic conditions, ‘free’ ferrous iron could not support the iron–sulfur cluster assembly in IscU because of rapid oxidation of ferrous iron by oxygen. However, addition of apo-IscA effectively prevents the formation of the biologically inaccessible ferric hydroxide and facilitates iron–sulfur cluster assembly in IscU under aerobic conditions [28]. Thus IscA/SufA may have a more prominent role for the biogenesis of iron–sulfur–clusters in the presence of oxygen. In this context, we propose that under aerobic growth conditions, the intracellular ‘free’ iron content accessible for the iron–sulfur cluster assembly is exceedingly low, as ferrous iron can react with oxygen to promote the production of hydroxyl free radicals [56]. Under such conditions, IscA/SufA is essential to recruit intracellular iron, maintain the soluble form of iron, and deliver iron for the biogenesis of iron–sulfur–clusters. Under anaerobic growth conditions, however, the intracellular ‘free’ iron content which will not generate any hydroxyl free radicals could be sufficient for iron–sulfur–cluster assembly. Under such conditions, IscA/SufA is largely dispensable for the biogenesis of iron–sulfur–clusters in cells. Although we are aware that other models can also explain the conditional requirement of IscA/SufA for the cell growth of *E. coli* in minimal medium under aerobic conditions, we believe that this is one of the simplest models that can be tested. The constructed *E. coli* mutants will allow us and other colleagues to delineate further the physiological roles of IscA/SufA in the biogenesis of iron–sulfur–clusters under different growth conditions.

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