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IscA Mediates Iron Delivery for Assembly of Iron-Sulfur Clusters in IscU under the Limited Accessible Free Iron Conditions*

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Increasing evidence suggests that IscS, a cysteine desulfurase, provides sulfur for assembly of transient iron-sulfur clusters in IscU. IscU appears to act as a scaffold and eventually transfers the assembled clusters to target proteins. However, the iron donor for the iron-sulfur cluster assembly largely remains elusive. Here we find that Escherichia coli IscU fails to assemble iron-sulfur clusters when the accessible “free” iron in solution is limited by an iron chelator sodium citrate. Remarkably, IscA, an iron-sulfur cluster assembly protein with an iron association constant of $3.0 \times 10^{19}$ M$^{-1}$, is able to overcome the iron limitation due to sodium citrate and deliver iron for the IscS-mediated iron-sulfur cluster assembly in IscU. Substitution of the invariant cysteine residues Cys-99 or Cys-101 in IscA with serine completely abolishes the iron binding activity of the protein. The IscA mutants that fail to bind iron are unable to mediate iron delivery for the iron-sulfur cluster assembly in IscU under the limited accessible “free” iron conditions. The results suggest that IscA is capable of recruiting intracellular iron and providing iron for the iron-sulfur cluster assembly in IscU in cells in which the accessible “free” iron content is probably restricted.

The biogenesis of iron-sulfur proteins requires a coordinated incorporation of iron and sulfur to form iron-sulfur clusters. Recently, a highly conserved gene cluster iscSUA-hscBA-fdx has been identified as critical for the iron-sulfur cluster assembly in bacteria (1–5). Among the six proteins (IscS, IscU, IscA, HscB, HscA, and ferredoxin) encoded by the gene cluster iscSUA-hscBA-fdx, IscS is a cysteine desulfurase that catalyzes the desulfurization of L-cysteine and provides sulfur for the iron-sulfur cluster assembly in IscU (6–11). IscU appears to act as a scaffold and eventually transfers the assembled iron-sulfur clusters to target proteins (6–8). IscA has also been proposed as a scaffold for the iron-sulfur cluster assembly, because purified IscA from E. coli (12) and its homologs from Azotobacter vinelandii (13), cyanobacterium Synechocystis PCC 6803 (14–16), and Schizosaccharomyces pombe (17, 18) can host iron-sulfur clusters and transfer the assembled clusters to target proteins.

Whereas both IscU and IscA can assemble transient iron-sulfur clusters when iron and sulfide are provided in solution, there is little sequence similarity between the two proteins. Recent x-ray crystal structure of the E. coli IscA (19, 20) and the NMR structure of the IscU homolog from Thermotoga maritima (21, 22) further indicate that IscU and IscA are structurally very different proteins. IscU is fluxional among widely different conformational arrangements (21, 22). IscA, on the other hand, is remarkably stable with a compact globular domain and an apparently mobile C-terminal tail (19, 20). The crystal structure also indicates that IscA probably exists as a tetramer with the three invariant cysteine residues (Cys-35, Cys-99, and Cys-101) projected to form a “cysteine pocket” within a central channel formed by the association of IscA monomers (19). The unique structure of IscA (19, 20) suggests that IscA, besides being a potential alternative scaffold, may have other functions in the biogenesis of iron-sulfur proteins.

In the previous study (23), we reported that purified E. coli IscA contains iron with an apparent iron association constant of $3.0 \times 10^{19}$ M$^{-1}$. The iron center in IscA is stable and resistant to oxygen. The electron paramagnetic resonance measurements of the iron-loaded IscA revealed an electron paramagnetic resonance signal at $g = 4–6$, which disappears when the iron-loaded IscA is reduced with dithionite (23), indicating that the iron center in IscA is in an oxidized state. The ratio of iron to IscA monomer in the iron-loaded IscA is $1:2$, suggesting a stoichiometry of one iron atom per two IscA monomers (23). Interestingly, the iron-loaded IscA can provide iron for the iron-sulfur cluster assembly in IscU in the presence of L-cysteine and cysteine desulfurase (IscS) in vitro (23). These results led us to hypothesize that IscA may be able to recruit intracellular iron and provide iron for the iron-sulfur cluster assembly in IscU. Nonetheless, kinetics characterizations indicated that the iron-loaded IscA has little advantage over “free” iron in solution as iron source for the IscS-mediated iron-sulfur cluster assembly in IscU (23), leaving the role of IscA as a physiological iron donor for the iron-sulfur cluster assembly questionable.

The concentration of the accessible “free” iron in cells is likely to be much less than what often has been used for the in vitro iron-sulfur cluster assembly, because an elevated level of the intracellular “free” iron content is highly toxic to cells due to the generation of hydroxyl free radicals via the Fenton reaction (24). Therefore, it is important to determine whether the iron-sulfur cluster assembly in IscU can occur under the limited accessible “free” iron conditions. In this study, we find that sodium citrate, a physiological iron chelator with an iron association constant of $1.0 \times 10^{17}$ M$^{-1}$ (25), can effectively block the IscS-mediated iron-sulfur cluster assembly in IscU by limiting the accessible “free” iron content in solution. Remarkably, we find that IscA is able to recruit iron from citrate and deliver iron for the IscS-mediated iron-sulfur cluster assembly in IscU. Site-directed mutagenesis studies show that, among the three invariant cysteine residues (Cys-35, Cys-99, and Cys-101) in

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† The abbreviation used is: IscS, cysteine desulfurase protein.
IscA, Cys-35 is not critical for the iron binding, although the substitution of Cys-35 with serine results in a decreased iron binding affinity of the protein. In contrast, the substitution of Cys-99 or Cys-101 with serine completely abolishes the iron binding activity of IscA. The IscA mutants (C99S and C101S) that fail to bind iron are unable to mediate iron delivery for the iron-sulfur cluster assembly in IscU under the limited accessible "free" iron condition. The potential role of IscA as an iron donor for the iron-sulfur cluster assembly in IscU will be discussed.

**Experimental Procedures**

**Site-directed Mutagenesis and Protein Purification**—The site-directed mutagenesis of *E. coli* IscA was carried out using the QuikChange™ mutagenesis kit (Stratagene) and a previously constructed plasmid, pTNI5CA (23), according to the manufacturer’s instruction. Three pairs of degenerated primers were designed to replace Cys-35, Cys-99, and Cys-101 in IscA with serine, respectively. Mutations in the iscA gene were confirmed by direct sequencing using the T7 primer (Gene Laboratory, Louisiana State University). The His-tagged IscA mutants were expressed and purified, and the His tag was subsequently removed by incubating with thrombin (0.65 unit/ml) (Fierce) overnight as described for wild type IscA (23). Recombinant E. coli IscU (23) and IscS (26) were prepared as described previously. The purity of all of the purified proteins was greater than 95% as judged by electrophoresis analysis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Blue. The concentration of apo-IscA was determined using an extinction coefficient of 2.4 mM$^{-1}$ cm$^{-1}$ at 260 nm of 2.4 mM (Pierce) overnight as described for wild type IscA (23). The same iron binding experiments were carried out for IscU and IscS were determined using extinction coefficients at 280 nm of 11.2 and 39.7 mM$^{-1}$ cm$^{-1}$, respectively. All of the protein concentrations in text are referred to the monomeric species.

Iron-Sulfur Cluster Assembly in IscU—Purified IscU (40 μM) was incubated with IscS (1 μM), dithiothreitol (2 mM), Fe(NH$_4$)$_2$(SO$_4$)$_2$ (50 μM), NaCl (200 mM), and Tris (20 mM) (pH 8.0). The reaction mixture was purged with pure argon gas and preincubated at 37°C for 5 min before l-cysteine (1 mM) was added to initiate the iron-sulfur cluster assembly reaction under anaerobic conditions. As indicated, sodium citrate and/or IscA was added prior to the 5 min preincubation. The iron-sulfur cluster assembly in IscU was monitored in a Beckman DU-640 UV-visible absorption spectrometer. The data were the averages from three independent experiments.

**RESULTS**

**Sodium Citrate Blocks the Iron-Sulfur Cluster Assembly in IscU**—When purified *E. coli* IscU (40 μM) was incubated with cysteine desulphurrase (IscS) (1 μM), ferrous iron (50 μM), dithiothreitol (2 mM), and l-cysteine (1 mM) in buffer containing NaCl (200 mM) and Tris (20 mM) (pH 8.0) anaerobically at 37°C, an absorption peak at 456 nm that has been attributed to the iron-sulfur clusters in IscU (6) gradually appeared and reached the maximum in ~30 min (Fig. 1A). As reported by others (6–11), the assembly of iron-sulfur clusters in IscU requires IscS and l-cysteine because the samples lacking any of these components failed to produce the absorption peak at 456 nm of IscU (data not shown).

Using an average extinction coefficient of 5.8 mM$^{-1}$ cm$^{-1}$ at 456 nm for the [2Fe-2S] clusters assembled in IscU (6), we estimated that up to 22% [2Fe-2S] clusters were assembled in IscU after a 30-min incubation, indicating that ~90% of ferrous iron (50 μM) in the solution was incorporated into the iron-sulfur clusters in IscU. This finding would suggest that, as the iron-sulfur cluster assembly proceeds, the concentration of iron will become very low in the incubation solution. To test this idea, we analyzed the iron-sulfur cluster assembly in IscU under the same experiment conditions with the exception that various "free" iron concentrations were used in the incubation solution. Fig. 1B shows that the yield of the IscS-mediated iron-sulfur cluster assembly in IscU had a nearly linear relation to the iron concentration in the incubation solution up to 50 μM and was apparently saturated at the concentrations necessary to reach 100% assembly.
Because an elevated level of the accessible “free” iron will promote the production of hydroxyl free radicals via the Fenton reaction (24), it is most likely that the intracellular “free” iron concentration will be much lower than what often has been used for the iron-sulfur-cluster assembly in vitro. To determine whether the iron-sulfur-cluster assembly in IscU can occur under the limited accessible “free” iron conditions, we included sodium citrate, a physiological iron chelator (25), in the incubation solution before l-cysteine was added to initiate the iron-sulfur-cluster assembly reaction. As shown in Fig. 1C, sodium citrate (5 mM) effectively blocked the IscS-mediated iron-sulfur-cluster assembly in IscU in which ferrous iron (50 μM) was used as iron source. Titration of sodium citrate revealed that the IscS-mediated iron-sulfur-cluster assembly in IscU was gradually diminished as the concentration of sodium citrate was increased (Fig. 1D).

The inhibitory effect of sodium citrate on the IscS-mediated iron-sulfur-cluster assembly in IscU was completely removed when additional ferrous iron (1 mM) was added to the incubation solution (data not shown), suggesting that sodium citrate blocks the iron-sulfur-cluster assembly in IscU by limiting the accessible “free” iron content in solution. Because the iron association constant of citrate is \(1.0 \times 10^{17} \text{ M}^{-1}\) (25), at equilibrium the accessible “free” iron concentration in the solution containing 5 mM sodium citrate and 50 μM iron will be \(1.0 \times 10^{-19} \text{ M}\). These results suggest that IscU fails to assemble iron-sulfur clusters when the accessible “free” iron concentration is decreased to \(1.0 \times 10^{-19} \text{ M}\).

IscA Restores the IscS-mediated Iron-Sulfur Cluster Assembly in IscU under the Limited Accessible “Free” Iron Conditions—We previously reported that E. coli IscA binds iron with an iron association constant of \(3.0 \times 10^{19} \text{ M}^{-1}\) (23). Because the iron association constant of citrate is \(-1.0 \times 10^{17} \text{ M}^{-1}\) (25), at equilibrium IscA should be able to compete for iron from citrate. To test this idea, apo-IscA (40 μM) or apo-IscA (40 μM) was incubated with ferrous iron (40 μM) in the presence of sodium citrate (5 mM) and dithiothreitol (2 mM) at room temperature for 15 min. The protein samples were then re-purified by passing through a HiTrap desalting column. As shown in Fig. 2A, no absorption peaks indicating iron binding in IscU were observed after IscU was incubated with ferrous iron. On the other hand, re-purified IscA showed an absorption peak at 315 nm of the iron-reconstituted IscA at 315 nm to 260 nm of the iron-reconstituted IscA was \(-1.05\), suggesting that IscA was fully loaded with iron (23). Therefore, IscA, unlike IscU, is able to recruit iron from citrate.

In the previous study, we also showed that the iron-loaded IscA can provide iron for the iron-sulfur-cluster assembly in IscU in the presence of l-cysteine and IscS in vitro (23). It is conceivable that IscA may recruit iron from citrate and deliver iron for the IscS-mediated iron-sulfur-cluster assembly in IscU under the limited accessible “free” iron conditions. To test this idea, we included IscA (40 μM) in the iron-sulfur-cluster-assembly solution containing sodium citrate (5 mM) before the reaction was initiated with l-cysteine and found that the IscS-mediated iron-sulfur-cluster assembly in IscU was largely restored by IscA (Fig. 3B). The amount of the iron-sulfur clusters assembled in IscU was further increased when more IscA (80 μM) was used (Fig. 3C). The kinetics of the IscS-mediated iron-sulfur-cluster assembly in IscU in the presence of citrate/IscA are summarized in Fig. 3D. Whereas sodium citrate effectively blocks the iron-sulfur-cluster assembly in IscU, IscA restores both the yield and rate of the IscS-mediated iron-sulfur-cluster assembly in IscU. Collectively, the results suggest that IscA is able to overcome the iron limitation due to citrate and deliver iron for the IscS-mediated iron-sulfur-cluster assembly in IscU.

To further examine whether the iron center in IscA is transferred to the iron-sulfur-clusters in IscU, we incubated the iron-loaded IscA with IscU, IscS, and dithiothreitol with or without l-cysteine anaerobically at 37°C for 30 min. After incubation, the reaction solution was loaded onto a Mono Q column to re-purify IscA and IscU as described under “Experimental Procedures.” Fig. 4A shows the elution profile of IscA and IscU from the reaction solution after incubation without l-cysteine. The SDS electrophoresis gel analysis of the eluted fractions indicated that IscA was mainly eluted in fraction 8 and IscU was eluted in fraction 10. Each eluted fraction was immediately transferred to an anaerobic cuvette for the UV-visible absorption measurements. As shown in the figure, the iron center in re-purified IscA (fraction 8) remained intact with an absorption peak at 315 nm (Fig. 4B) and IscU (fraction 10) did not have any significant absorption peaks indicating the binding of iron or iron-sulfur clusters in the protein (Fig. 4C). When l-cysteine was included in the assembly solution (Fig. 4D), the iron center in IscA (fraction 8) was largely removed (Fig. 4E) and IscU (fraction 10) had a typical absorption peak at 456 nm of the iron-sulfur clusters (Fig. 4F). From the amplitude of the absorption peak at 456 nm, we estimated that \(-23 \mu\text{M} [2\text{Fe-2S}]\) clusters were assembled in IscU in fraction 10. Although the total amount of the [2Fe-2S] clusters assembled in IscU could not be accurately obtained because IscU was also eluted in fractions 9 and 11 (Fig. 4D), the results clearly show that the iron center in IscA is transferred to the iron-sulfur clusters in IscU when...
IscA-mediated Iron-Sulfur Cluster Assembly in IscU

**DISCUSSION**

In this study, we find that *E. coli* IscU, a proposed scaffold for the iron-sulfur cluster assembly (6–8), fails to assemble iron-sulfur clusters when the accessible "free" iron in solution is restricted by sodium citrate (Fig. 1). Considering abundant iron chelators including citrate in cells, it is most likely that the assembly of iron-sulfur clusters in IscU will require the assistance of specific iron donors in vivo. Our results show that *E. coli* IscA, a key member of the iron-sulfur cluster assembly machinery (1–5), is able to overcome the iron limitation due to sodium citrate (Fig. 2) and deliver iron for the IscS-mediated iron-sulfur cluster assembly in IscU *in vitro* (Figs. 3 and 4). The results suggest that IscA may be responsible for recruiting intracellular iron and providing iron for the iron-sulfur cluster assembly in IscU *in vivo*.

IscA has previously been characterized as an alternative scaffold for the iron-sulfur cluster assembly (12–17), because purified *E. coli* IscA or its homologs can host iron-sulfur clusters when iron and sulfide are provided in solution. It is tempting to suggest that iron binding in IscA is an intermediate state during assembly of iron-sulfur clusters in IscA. However, considering that iron binding in the proposed scaffold IscU is fairly weak (6, 28) (Fig. 2), it is remarkable that IscA binds iron with an iron association constant of $3.0 \times 10^{19} \text{ M}^{-1}$ (23), which is close to that reported for human transferrin ($4.7 \times 10^{19} \text{ M}^{-1}$) (29). Such an iron association constant would allow IscA to recruit the intracellular "free" iron and make it accessible for the iron-sulfur cluster assembly in cells. Although it is possible that IscA may act as an alternative scaffold (12–17), we would like to suggest that the primary function of IscA is to recruit intracellular "free" iron and deliver iron for the iron-sulfur cluster assembly in IscU.

The mechanism by which iron is transferred from IscA to IscU may only be speculated at present. One possibility is that iron transfer from IscA to IscU may be accomplished via protein-protein interactions. An example of such has been documented for sulfur transfer from IscS to IscU in which specific cysteine residues from both proteins are involved (9–11). However, direct iron transfer from IscA to IscU may be unlikely, because the iron-loaded IscA remains intact after incubation with IscU at 37 °C for 30 min in the absence of l-cysteine (Fig. 4B). Alternatively, IscA may assemble iron-sulfur clusters and subsequently transfer the assembled clusters to IscU, since it has been reported that IscA and its homologs can transfer the assembled iron-sulfur clusters to apoferredoxin (12, 14, 18) and apoperoxidase 5'-phosphosulfate reductase (14). However, the kinetics of the iron-sulfur cluster transfer from IscA to apoperoxidoxin (13) or to apoperoxidase 5'-phosphosulfate reductase (14) was very slow (requiring 1–2 h of incubation). Under the similar experimental conditions as described previously (13), we incubated the IscA iron-sulfur clusters with apo-IscU at 37 °C for 60 min and found no iron-sulfur cluster formation in IscU, suggesting that the iron-sulfur cluster transfer from IscA to IscU may be kinetically restricted. Because the IscA-mediated iron-sulfur cluster assembly in IscU is almost complete after a 30-min incubation at 37 °C (Fig. 3D), we suppose that the iron-sulfur cluster transfer from IscA to IscU is not very analyzed for their ability to deliver iron for the IscS-mediated iron-sulfur cluster assembly in IscU. As shown in Fig. 6, mutants C99S and C101S completely failed to mediate iron delivery for the iron-sulfur cluster assembly in IscU, whereas mutant C35S showed a decreased activity. The results indicate that the IscA mutants that fail to bind iron are unable to deliver iron for the IscS-mediated iron-sulfur cluster assembly in IscU under the limited accessible "free" iron conditions.

**Fig. 3.** IscA restores the IscS-mediated iron-sulfur cluster assembly in IscU under the limited accessible "free" iron conditions. A, purified IscU (40 μM) was incubated with IscS (1 μM), NaCl (200 mM), Tris (20 mM) (pH 8.0), sodium citrate (5 mM), and Fe(NH₄)₂(SO₄)₂ (50 μM) in the presence of diithiothreitol (2 mM) anaerobically at 37 °C for 5 min before L-cysteine (1 mM) was added to initiate the iron-sulfur cluster assembly reaction. UV-visible absorption spectra were taken every 5 min after L-cysteine was added. B, same as in A with the exception that 40 μM IscA was added into the incubation solution before L-cysteine was added. C, same as in A with the exception that 80 μM IscA was added into the incubation reaction before L-cysteine was added. D, kinetics of the IscA-mediated iron-sulfur cluster assembly in IscU. The absorbance changes at 456 nm were plotted as a function of incubation time after L-cysteine was added. Closed circles, no sodium citrate; squares, with 5 mM sodium citrate; closed triangles, with 5 mM sodium citrate and 40 μM IscA; open triangles, with 5 mM sodium citrate and 80 μM IscA. The experiments were repeated at least three times, and similar results were obtained.

l-cysteine and IscS are also present in the incubation solution.

IscA Mutants That Fail to Bind Iron Are Unable to Deliver Iron for the IscS-mediated Iron-Sulfur Cluster Assembly in IscU under the Limited Accessible "Free" Iron Conditions— *E. coli* IscA contains three invariant cysteine residues (Cys-35, Cys-99, and Cys-101) that are projected to form a cysteine pocket in the IscA tetramer (23). To examine whether Cys-35, Cys-99, and Cys-101 are involved in iron binding, we carried out the site-directed mutagenesis by substituting the three cysteine residues individually with serine to generate mutants C35S, C99S, and C101S. Purified proteins then were incubated with an equivalent amount of ferrous iron in the presence of diithiothreitol at room temperature for 15 min followed by passing through a HiTrap desalting column to remove diithiothreitol and residual ferrous iron.

Fig. 5 shows that, after incubation with ferrous iron, wild type IscA had an absorption peak at 315 nm with a ratio at 315 nm to 260 nm of 1.05, indicating that IscA was fully loaded with iron (23). For mutant C35S, the absorption peak at 315 nm was also observed after incubation with ferrous iron but the ratio at 315 nm to 260 nm was only ~0.69, suggesting that mutant C35S binds iron at a reduced binding affinity. On the other hand, no absorption peak at 315 nm was observed in mutants C99S and C101S after incubation with ferrous iron. Thus, the substitution of Cys-99 or Cys-101 with serine abolishes the iron binding activity of IscA.

In the parallel experiments, the three IscA mutants were
likely under the experimental conditions. Our current working model is that L-cysteine or sulfide/sulfur released from L-cysteine by IscS may directly facilitate or promote iron transfer from IscA to IscU. This model is based on the observation that, when L-cysteine is added to the iron-sulfur cluster assembly solution, the iron in IscA is released and the iron-sulfur clusters are assembled in IscU (Fig. 4, E and F).

Evidence, additional experiments are needed to test this model. If proven to be the case, L-cysteine would have two distinct functions for the iron-sulfur cluster assembly: 1) to facilitate iron transfer from IscA to IscU and 2) to provide sulfur for the iron-sulfur clusters in IscU. It is plausible that regulation of intracellular L-cysteine content may constitute another control mechanism of the biogenesis of iron-sulfur clusters in cells.

IscA is highly conserved from bacteria to humans. All of the IscA homologs contain three invariant cysteine residues (Cys-35, Cys-99, and Cys-101, E. coli numbering), which have been shown to be important for the in vivo IscA activity in Saccharomyces cerevisiae (30, 31). An in vitro study indicated that two of the three invariant cysteine residues (equivalents of the E. coli Cys-99 and Cys-101) in the Isa1 from cyanobacterium Synechocystis PCC 6803 are required for hosting iron-sulfur clusters (14). Another in vitro study using the Isa1 from S. pombe suggested that the mutants with each cysteine replaced individually with alanine are able to host iron-sulfur clusters and transfer iron-sulfur clusters to target proteins (17, 18). The interpretation of the in vitro results could be compli-

**FIG. 4.** Iron transfer from IscA to the iron-sulfur clusters in IscU in the presence of L-cysteine and IscS. The iron-loaded IscA (50 μM) was incubated with IscU (50 μM), IscS (1 μM), dithiothreitol (2 mM), and 20 mM Tris (pH 8.0) with or without L-cysteine (1 mM) anaerobically at 37 °C for 30 min. After incubation, the reaction solution (2 ml) was loaded onto a Mono Q column pre-equilibrated with buffer containing 20 mM Tris (pH 8.0). The proteins were re-purified as described under “Experimental Procedures.” A, the elution profile of the reaction solution after incubation at 37 °C for 30 min without L-cysteine. Inset, the SDS electrophoresis gel analysis of the eluted fractions. The migration positions of purified IscA and IscU were indicated on the left side of the gel image. The absorption spectra of fractions 8 and 10 from A were shown in B and C, respectively. D, the elution profile of the reaction solution after incubation at 37 °C for 30 min with L-cysteine. Inset, the SDS electrophoresis gel analysis of the eluted fractions. The absorption spectra of fractions 8 and 10 from D were shown in E and F, respectively.

**FIG. 5.** Iron binding activity of the IscA mutants C35S, C99S, and C101S. Purified wild type IscA and mutants C35S, C99S, and C101S (40 μM each) were incubated with Fe(NH₄)₂(SO₄)₂ (40 μM) in the presence of dithiothreitol (2 mM) followed by passing through a HiTrap desalting column. The eluted protein samples were subjected to the UV-visible absorption measurements. The absorption peak at 315 nm indicates the iron binding in the protein.

**FIG. 6.** IscA mutants that fail to bind iron are unable to mediate iron delivery for the IscS-mediated iron-sulfur cluster assembly in IscU. IscA (A) or IscA mutants C35S (B) or C99S (C) or C101S (D) (40 μM each) was incubated with IscU (40 μM), IscS (1 μM), sodium citrate (5 mM), NaCl (200 mM), Tris (20 mM) (pH 8.0), Fe(NH₄)₂(SO₄)₂ (50 μM), and dithiothreitol (2 mM) anaerobically at 37 °C for 5 min before L-cysteine (1 mM) was added to initiate the assembly reaction. The absorption spectra were taken every 5 min after L-cysteine was added. The experiments were repeated at least three times, and similar results were observed.
cated by the sometimes nonspecific formation of iron-sulfur clusters by proteins containing free thiol groups (20). From the x-ray crystal structure of E. coli IscA (19, 20), the three invariants, Cys-35, Cys-99, and Cys-101, are projected to form a cysteine pocket within a central channel formed by the association of IscA monomers. The C-terminal nine residues (amino acids 99–107) including Cys-99 and Cys-101 are not visible in the reported electron density maps (19, 20), probably because the cysteine pocket is a flexible structure. Here, we find that, among the three invariant cysteine residues in E. coli IscA, Cys-35 is not critical for the iron binding because substitution of Cys-35 with serine only decreases the iron binding affinity of the protein. In contrast, Cys-99 and Cys-101 in IscA are required for the iron binding activity (Fig. 5) and for delivering iron for the IscS-mediated iron-sulfur cluster assembly in IscU under the limited accessible “free” iron conditions (Fig. 6). This result is consistent with the genetic studies of Isa1 in S. cerevisiae in which the substitution of either of the two cysteine residues (equivalents of the E. coli Cys-99 and Cys-101) with serine completely inactivates the function of Isa1 (31). It seems that the flexible C-terminal Cys-99 and Cys-101) with serine completely inactivates the assembly in IscU under the limited accessible free iron conditions of Cys-35 with serine only decreases the iron binding affinity of the protein. The iron-sulfur cluster assembly in ISU, a human homolog of the IscA homologs (Isa1 or Isa2) leads to the accumulation of iron-sulfur cluster assembly in cells. In contrast, depletion of additional proteins may be involved in providing iron for the function of Isa1 (31). It seems that the flexible C-terminal cysteine pocket is a flexible structure. Here, we find that, among the three invariant cysteine residues in E. coli IscA, Cys-35 is not critical for the iron binding because substitution of Cys-35 with serine only decreases the iron binding affinity of the protein. In contrast, Cys-99 and Cys-101 in IscA are required for the iron binding activity (Fig. 5) and for delivering iron for the IscS-mediated iron-sulfur cluster assembly in IscU.

Recently, it was reported that human frataxin, an iron-bind-

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