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Iron binding activity is essential for the function of IscA in iron-sulphur cluster biogenesis

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Abstract

Iron-sulphur cluster biogenesis requires coordinated delivery of iron and sulphur to scaffold proteins, followed by transfer of the assembled clusters from scaffold proteins to target proteins. This complex process is accomplished by a group of dedicated iron-sulphur cluster assembly proteins that are conserved from bacteria to humans. While sulphur in iron-sulphur clusters is provided by L-cysteine via cysteine desulfurase, the iron donor(s) for iron-sulphur cluster assembly remains largely elusive. Here we report that among the primary iron-sulphur cluster assembly proteins, IscA has a unique and strong binding activity for mononuclear iron *in vitro* and *in vivo*. Furthermore, the ferric iron centre tightly bound in IscA can be readily extruded by L-cysteine, followed by reduction to ferrous iron for iron-sulphur cluster biogenesis. Substitution of the highly conserved residue tyrosine 40 with phenylalanine (Y40F) in IscA results in a mutant protein that has a diminished iron binding affinity but retains the iron-sulphur cluster binding activity. Genetic complementation studies show that the IscA Y40F mutant is inactive *in vivo*, suggesting that the iron binding activity is essential for the function of IscA in iron-sulphur cluster biogenesis.

Introduction

Throughout evolution, iron-sulphur clusters have become integral parts of diverse physiological processes including photosynthesis, nitrogen fixation, sugar metabolism, co-factor biogenesis, RNA modification and translation, DNA replication and repair, and gene expression regulation.[1-4] While iron-sulphur clusters can be assembled in proteins *in vitro* with ferrous iron and sulphide, iron-sulphur cluster assembly *in vivo* requires a group of dedicated proteins that are conserved from bacteria to humans.[5] In *Escherichia coli*, the proteins encoded by a gene cluster *iscSUA-hscBA-fdx* are primarily responsible for iron-sulphur cluster assembly under normal growth conditions.[5] The homologues of the six proteins encoded by *iscSUA-hscBA-fdx* have been identified in eukaryotic organisms.[2] Among them, IscS is a cysteine desulfurase that catalyzes desulfurization of L-cysteine[6-8] and provides sulphur for iron-sulphur cluster assembly in a scaffold protein IscU.[9-11] IscU in turn transfers the assembled clusters to target proteins.[12, 13] IscA has been characterized as an alternative scaffold or intermediate carrier for iron-sulphur cluster assembly.[14, 15] A comprehensive characterization of iron-sulphur cluster binding in an IscA homolog NifIscA from *Azotobacter vinelandii* has recently been reported.[16] Two heat shock cognate proteins, HscB and HscA, have specific interactions with IscU[17] and promote the transfer of assembled clusters from IscU to target proteins in an ATP-dependent reaction.[18, 19] Ferredoxin, which contains a stable [2Fe-2S] cluster,[20] is likely involved in the reductive formation of a [4Fe-4S] cluster in scaffold protein IscU [21, 22].

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While the sulphur donor for iron-sulphur cluster assembly has been well established,[6-8] the iron donor(s) remains largely elusive. Frataxin, a mitochondrial protein that is linked to neurodegenerative disease Friedreich's ataxia,[23] has previously been characterized as a likely iron donor for iron-sulphur cluster assembly.[24, 25] Deficiency of frataxin results in diminished activity of iron-sulphur enzymes in yeast cells,[26] and frataxin has physical interactions with iron-sulphur cluster assembly protein IscU,[27-29] the IscU/IscS complex,[30, 31] and with the iron-sulphur enzyme aconitase in mitochondria.[32] However, frataxin has a weak iron binding activity with an iron binding constant of 4 to 55 μM.[24, 33, 34] Such a low iron binding affinity may preclude frataxin from binding any significant amounts of iron in mitochondria under normal physiological conditions. Furthermore, deletion of frataxin homologue CyaY in *E. coli* does not affect iron-sulphur cluster biogenesis,[35] and frataxin-deficient yeast cells can be rescued by either expressing ferritin[36] or vacuolar iron transporter CCC1.[37] In fact, scavenging H_2O_2 can effectively restore iron-sulphur enzyme activities in a *Drosophila* model of Friedreich's ataxia.[38] Thus, the primary function of frataxin could be to maintain iron homeostasis under oxidative stress[39, 40] or to modulate overall iron-sulphur cluster assembly in cells.[30, 31]

In previous studies, we reported that unlike other iron-sulfur cluster assembly scaffold proteins such as IscU, IscA has a strong iron binding activity with an apparent iron association constant of 1.0×10^{19} M^{-1}.[41-45] A similar strong iron binding activity has been observed for IscA homologues from humans,[46] yeast cells,[47] and *A. vinelandii* ^{Nif}IscA.[48] In this study, we present new evidence showing that among the primary iron-sulphur cluster assembly proteins, IscA is unique in binding iron, and that the tightly bound ferric iron in IscA can be readily extruded by L-cysteine, followed by reduction to ferrous iron for iron-sulphur cluster assembly. Site-directed mutagenesis studies show that the iron-binding activity is crucial for the physiological function of IscA in iron-sulphur cluster biogenesis.

Experimental

Protein preparation

The iron-sulphur cluster assembly proteins IscS, IscU, IscA, HscB, and HscA from *E. coli* were prepared as described previously.[43] The IscA mutant in which tyrosine 40 was substituted with phenylalanine (Y40F) was constructed using the site-directed mutagenesis kit (Stratagene co). The mutation of gene *iscA* in the cloned plasmid was confirmed by direct sequencing. Human frataxin was subcloned from plasmid pETHF2[49] to expression plasmid pET28b$^+$ and prepared as described in.[46] The *E. coli* frataxin homologue CyaY was prepared as described in.[44] The protein concentration was determined from the absorption peak at 260 nm or 280 nm using the previously published extinction coefficient for each protein.

In vitro iron binding and iron-sulphur cluster assembly in IscA

For the iron binding experiments, each of purified iron-sulphur cluster assembly proteins (50 μM in monomer) was incubated with $Fe(NH_4)_2(SO_4)_2$ (50 μM) and sodium citrate (5 mM) in buffer containing NaCl (200 mM), Tris (20 mM, pH 8.0) in the presence of dithiothreitol (2 mM) at room temperature for 20 min, followed by passing through a High-trap Desalting column. Total iron contents in re-purified protein samples were determined using an iron indicator FerroZine as described in.[50] For iron-sulphur cluster assembly, purified IscA was incubated with IscS (0.5 μM), Tris (20 mM, pH 8.0), NaCl (200 mM), $Fe(NH_4)_2(SO_4)_2$ (50 μM), and dithiothreitol (2 mM) at 37°C for 5 min under anaerobic conditions. L-cysteine (1 mM) was then added to initiate the iron-sulphur cluster assembly reaction. The amount of iron-sulphur clusters assembled in protein was monitored in a Beckman DU-640 UV-Visible spectrophotometer.

EPR measurements

EPR (electron paramagnetic resonance) spectra were recorded at X-band on a Bruker ESP-300 EPR spectrometer using an Oxford Instruments ESR-9 flow cryostat. The routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; temperature, 10 K; receive gain, 1.0×10^5.

In vivo activity assay of the IscA Y40F mutant

For the *in vivo* activity of the IscA Y40F mutant, gene encoding Y40F was cloned into a plasmid pBAD (Invitrogen co.). The constructed plasmid pBAD/*Y40F* was introduced into an *E. coli* mutant in which gene *iscA* and its paralogue *sufA* were in-frame deleted.[51] The *E. coli iscA/sufA* double mutant was viable in rich LB medium, but had a null-growth phenotype in M9 minimal medium under aerobic conditions.[51] The *E. coli iscA/sufA* double mutant cells containing pBAD/*iscA* and pBAD served as positive and negative control, respectively.

Results and discussion

IscA has a unique iron binding activity among the iron-sulphur cluster assembly proteins

To explore the iron binding activity of the primary iron-sulphur cluster assembly proteins encoded by the gene cluster *iscSUA-hscBA-fdx* from *E. coli*,[5] we expressed each protein in wild-type *E. coli* cells grown in LB media. Each protein was purified to a single band on the SDS-PAGE gel.

Because ferredoxin contained a stable [2Fe-2S] cluster,[20] ferredoxin was not included for the iron binding analysis. Among the other iron-sulphur cluster assembly proteins encoded by *iscSUA-hscBA-fdx*, only purified IscA contains 0.09±0.02 iron atoms per IscA dimer (Fig. 1a). No acid-labile sulphur is detectable in purified IscA, indicating that purified IscA does not contain any significant amounts of iron-sulphur clusters.

The iron binding activity of purified iron-sulphur cluster assembly proteins is also analyzed *in vitro*. Each purified protein is incubated with freshly prepared ferrous iron in the presence of dithiothreitol under aerobic conditions, followed by re-purification of protein Fig. 1b) shows that while the iron content in IscA is increased to 1.05±0.10 iron atoms per IscA dimer after incubation with an equivalent amount of ferrous iron (relative to protein monomer), the iron content in other proteins is less than 0.1 iron atoms per protein dimer, demonstrating that IscA has a unique iron binding activity.

The only other candidate proposed for the iron donor to iron-sulphur cluster assembly in scaffold protein IscU is frataxin and its bacterial homologue CyaY.[24, 25] To explore the iron binding activity of frataxin/CyaY *in vitro* under the same experimental conditions, purified *E. coli* CyaY and human frataxin are incubated with an equivalent amount of ferrous iron in the presence of dithiothreitol. Very little or no iron binding is found in re-purified CyaY and human frataxin (data not shown), suggesting that unlike IscA from *E. coli* [41] and humans,[46] CyaY/frataxin has a very weak iron binding activity in the presence of dithiothreitol.

The ferric iron centre in IscA can be extruded b y L-cysteine, followed by reduction to ferrous iron

To access the iron in IscA for iron-sulphur cluster biogenesis, the iron stored in the protein must be quickly released when there is such a demand. Previously, we reported that L-cysteine, but not other related biological thiols including *N*-acetyl-L-cysteine or reduced

glutathione, can efficiently release iron from the iron-bound IscA.[52] However, the mechanism underlying the L-cysteine-mediated iron release from IscA was not clear.

The iron-bound IscA has an unusual EPR signal at g = 4.3 and 6.0.[41] The signal is completely eliminated upon removal of iron from the protein, and fully restored after reconstitution with iron, indicating that the EPR signal represents the iron binding in IscA.[41] Interestingly, similar EPR signal was previously reported for the S = 3/2, reduced [4Fe-4S] cluster in proteins such as nitrogenase[53] and 2-hydroxyglutaryl-CoA dehydratase.[54] While further biophysical studies will be important to determine the spin state of the iron center in *E. coli* IscA, recent optical and magnetic spectroscopic characterization of ^{Nif}IscA, an IscA homologue from *A. vinelandii*, provided comprehensive and convincing evidence for the assignment of the iron site in ^{Nif}IscA to a ferric iron in an unusual rhombic spin S = 3/2 state.[48] The EPR spectra of the iron-bound ^{Nif}IscA from *A. vinelandii*[48] and the iron-bound IscA from *E. coli*[41] are essentially the same. Furthermore, the iron center in both *E. coli* IscA and ^{Nif}IscA is redox active: the EPR signal at g = 4.3 and 6.0 disappears when the iron centre in IscA is reduced by sodium dithionite.[41, 48]

To follow the process of the L-cysteine-mediated iron release from IscA, we used EPR to monitor the redox state of the iron centre in IscA when the iron-bound IscA was incubated with L-cysteine under aerobic conditions. At different time points, aliquots were taken from the incubation solution after addition of L-cysteine for EPR measurements.

Fig. 2 shows that upon addition of L-cysteine, the EPR signal at g = 4.3 and 6.0 of the iron-bound IscA is quickly replaced with a new EPR signal at g = 4.3, representing “free” ferric iron in solution.[55] After further incubation (over 20 min), the newly formed EPR signal at g = 4.3 is gradually decreased, indicating that the “free” ferric iron is reduced to the EPR-silent ferrous iron. As a control, incubation of the iron-bound IscA with *N*-acetyl-L-cysteine or reduced glutathione does not affect the EPR signal at g = 4.3 and 6.0 of the iron-bound IscA (data not shown). On the other hand, D-cysteine has the same activity as L-cysteine in releasing iron from IscA,[52] suggesting that the redox property, but not stereochemistry, of L-cysteine is responsible for the iron release from IscA.

The transition of the EPR signal of the iron-bound IscA from g = 4.3 and 6.0 to g = 4.3 upon addition of L-cysteine indicates that the L-cysteine-mediated iron release from IscA may have two distinctive steps. In the first step, L-cysteine extrudes ferric iron from IscA via ligand exchange to form the L-Cys-Fe^{3+}-IscA and/or the L-cys-Fe^{3+} complex which attributes to the observed EPR signal at g = 4.3 (Fig. 2). In the second step, the L-Cys-Fe^{3+}-IscA and/or L-cys-Fe^{3+} complex is reduced by L-cysteine to the EPR-silent ferrous iron which will be accessible for iron-sulphur cluster biogenesis.[43]

Mutation at residue tyrosine 40 to phenylalanine diminishes the iron binding activity of IscA

In crystal structure, *E. coli* IscA is a homodimer with the three conserved cysteine residues (Cys-35, Cys-99 and Cys-101) projected to form a “cysteine pocket” between two monomers.[56, 57] Mutation of any of the three conserved cysteine residues to serine produced IscA mutant proteins that have a diminished iron binding activity [58] and are inactive in *E. coli* cells.[51] However, because the cysteine residues may accommodate either a mononuclear iron or iron-sulphur cluster in IscA, mutation of cysteine residues would have affected both the iron and iron-sulphur cluster binding in the protein.

Among the amino acid residues that are in the vicinity of the putative iron binding site in IscA dimer, tyrosine 40 is highly conserved and located in the interface between two IscA monomers (Fig. 3). Unlike other nearby conserved residues that form a hydrophobic packing

between IscA monomers,[56] the hydroxyl group of tyrosine 40 may act as a possible oxygenic ligand for iron binding in IscA dimer.[48]

To explore the role of tyrosine 40 in the iron binding and iron-sulphur cluster binding of IscA, we constructed an IscA mutant in which tyrosine 40 was substituted with phenylalanine (Y40F). The IscA mutant Y40F protein was purified (Supplementary Figure A), and incubated with increasing concentrations of ferrous iron, followed by re-purification of the protein. Fig. 4 shows that unlike wild-type IscA, the absorption peak at 315 nm of the iron binding in Y40F is only slightly increased after incubation with ferrous iron in the presence of dithiothreitol. The iron content analyses revealed that after incubation with two-fold excess of iron per protein dimer, the ratio of iron to the wild-type IscA dimer is 1.05±0.10, while the ratio of iron to the IscA mutant Y40F dimer is less than 0.15 (Supplementary Figure B). The wild-type IscA and Y40F mutant proteins after reconstitution with two fold excess of iron were re-purified concentrated, and subjected to the EPR measurements. While wild-type IscA has a typical EPR spectrum of the iron-bound protein with g = 4.3 and 6.0, Y40F only has a small EPR signal at g = 4.3 region (data not shown). In the light of recent publication on the iron binding property of ^{Nif}IscA,[48] it would be interesting to determine whether tyrosine 40 actually provides the oxygenic ligand for the iron binding in IscA. Regardless, the results shown in Figure 4 and Supplementary Figure clearly demonstrate that mutation at Y40F severely diminishes the iron binding activity of *E. coli* IscA *in vitro*.

Next, we explored the iron-sulphur cluster binding activity of the wild-type IscA and IscA mutant Y40F. Purified IscA and the IscA mutant Y40F proteins were incubated with ferrous iron, L-cysteine, and a catalytic amount of IscS in the presence of dithiothreitol under anaerobic conditions. After 20 min incubation, the iron-sulphur cluster assembly in IscA was analyzed. Fig. 5 shows that like wild-type IscA, the IscA mutant Y40F retains the iron-sulphur cluster binding activity, suggesting that mutation of Y40F does not significantly affect the iron-sulphur cluster assembly in IscA *in vitro*.

IscA mutant Y40F is inactive in *E. coli* cells

E. coli has at least two IscA paralogs: ErpA and SufA. ErpA, which maps at a distance from any iron-sulphur cluster assembly-related genes, has been characterized as a dedicated scaffold for maturation of the key iron-sulphur enzymes in the isoprenoids biosynthesis pathway.[59] SufA, on the other hand, is a member of second iron-sulphur cluster assembly gene cluster *sufABCDSE* in *E. coli*.[60] Purified SufA, like IscA, has a strong iron binding activity and provides iron for iron-sulphur cluster assembly in IscU *in vitro*.[51] While deletion of IscA or SufA in *E. coli* cells only has a mild effect on cell growth, deletion of both IscA and SufA results in a null-growth phenotype in M9 minimal media under aerobic conditions.[51, 61] Re-introducing IscA or SufA restores the cell growth of the *E. coli iscA/sufA* double mutant,[51, 62] suggesting that IscA and SufA are complementary to each other.

To determine the *in vivo* activity of the IscA mutant Y40F, a plasmid expressing Y40F was introduced into the *E. coli iscA/sufA* double mutant cells. The cells were inoculated in M9 minimal media and grown under aerobic conditions. Fig. 6 shows that unlike wild-type IscA, Y40F failed to restore the cell growth of the *E. coli iscA/sufA* double mutant in M9 minimal media under aerobic conditions. Since the IscA mutant Y40F has a diminished iron binding activity but retains the iron-sulphur cluster binding activity, we propose that the iron binding activity is essential for the physiological function of IscA in iron-sulphur cluster biogenesis.

Phylogenomic analyses revealed that IscA is highly conserved from prokaryotic to eukaryotic organisms.[63] In *A. vinelandii*, depletion of IscA produces a null-growth

phenotype in modified Burks minimal medium under elevated oxygen conditions.[64] In *E. coli*, deletion of IscA and its paralog SufA results in a mutant that fails to grow in M9 minimal media under aerobic conditions.[51, 61] In *S. cerevisiae*, depletion of IscA homologues leads to iron accumulation in mitochondria and dependency on lysine and glutamate in media.[65] In human Hela cells, RNAi knockdown of IscA homologue results in decreased activities of iron-sulphur enzymes in both mitochondria and cytosol.[66] Evidently, IscA has a crucial role for iron-sulphur cluster biogenesis, especially under aerobic conditions. However, the physiological function of IscA remains controversial. One hypothesis stated that IscA acts as an alternative scaffold protein or intermediate carrier for iron-sulphur cluster biogenesis, as IscA is able to bind an iron-sulphur cluster and transfer the cluster to target proteins.[14-16] However, unlike other scaffold proteins such as IscU,[9] IscA has a strong iron binding activity with a maximum binding of one iron per IscA dimer.[41-45] Recent spectroscopic characterization of ^{Nif}IscA from *A. vinelandii* confirms that ^{Nif}IscA is able to bind one iron per dimer, and that the the iron center in IscA is in an unusual intermediate $S = 3/2$ spin state.[48] The results presented in this study provide new evidence for the hypothesis that IscA is a bona fide iron binding protein. Among the primary iron-sulphur cluster assembly proteins encoded by the gene cluster *iscSUA-hscBA-fdx*[5] and the putative iron donor frataxin/CyaY,[24, 25] only IscA has a strong binding activity for mononuclear iron in the presence of dithiothreitol. Furthermore, the ferric iron centre tightly bound in IscA can be readily extruded by L-cysteine, followed by reduction to ferrous iron. Additional studies further reveal that mutation at tyrosine 40, a possible oxygenic ligand for the iron binding in IscA,[48] diminishes iron binding activity but retains iron-sulphur cluster binding activity of IscA. Genetic complementation studies show that Y40F is inactive *in vivo*, suggesting that the iron binding activity is essential for the physiological function of IscA. Collectively, the results support the hypothesis that IscA may act as an iron donor for iron-sulphur cluster biogenesis. Nevertheless, since IscA can also bind iron-sulphur clusters [14, 15] and transfer the assembled clusters to target proteins,[16] the role of IscA as an alternative scaffold protein cannot be excluded. Perhaps, the iron binding in IscA could be the initial step for iron-sulphur cluster assembly either in IscA or in other scaffold proteins such as IscU.[43] Evidently, additional experiments are required to illustrate the role of IscA in iron-sulphur cluster biogenesis.

It is worth pointing out that IscA/SufA is dispensable for cell growth of *E. coli* in M9 minimal media under anaerobic conditions.[45] Interestingly, most anaerobic organisms contain no IscA homologues and almost all aerobic organisms contain at least one copy of IscA in their genomes.[63] It may be envisioned that under anaerobic conditions intracellular “free” iron is more freely available for iron-sulphur cluster biogenesis, and IscA is unnecessary. In aerobic organisms, a functional IscA will be required to recruit intracellular iron and deliver iron or iron-sulphur clusters for iron-sulphur cluster biogenesis under aerobic conditions.

Conclusions

Among the iron-sulphur cluster assembly proteins, IscA is unique in binding mononuclear iron. The tightly-bound iron centre in IscA can be readily extruded by L-cysteine, followed by reduction to ferrous iron. An IscA mutant that has a diminished iron binding activity but retains iron-sulphur cluster binding *in vitro* is inactive *in vivo*. The results suggest that IscA is a bona fide iron binding protein and that the iron binding activity is essential for physiological function of IscA in iron-sulphur cluster biogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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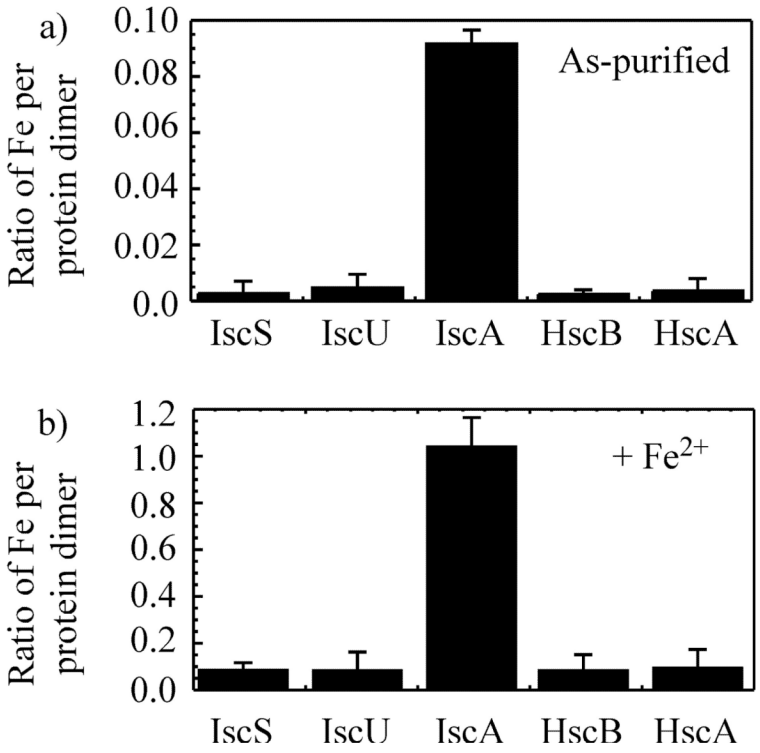

Fig. 1.

Iron binding of primary iron-sulphur cluster assembly proteins. Iron-sulphur cluster assembly proteins IscS, IscU, IscA, HscB and HscA were purified from *E. coli* cells grown in LB media. **a)**, iron content of purified iron-sulphur cluster assembly proteins. Iron content was presented as the ratio of iron atoms per protein dimer. **b)**, iron content of iron-sulphur cluster assembly proteins (50 μM in monomer) after incubation with $Fe(NH_4)_2(SO_4)_2$ (50 μM), sodium citrate (5 mM) and dithiothreitol (2 mM). Proteins were re-purified from incubation solutions. Iron content was presented as the ratio of iron atoms per protein dimer. The data are averages plus standard deviations from three independent experiments.

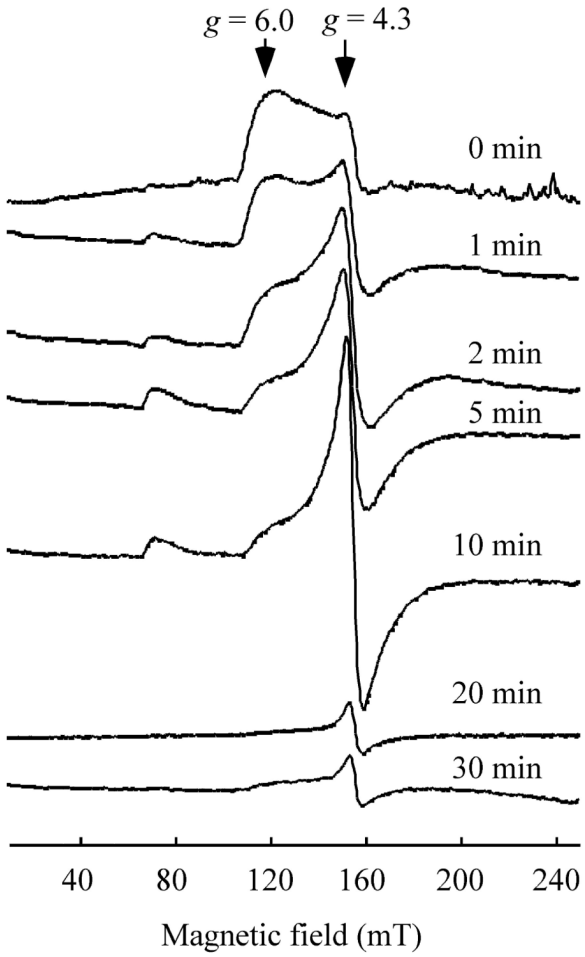

Fig. 2.

L-cysteine-mediated iron release from IscA. Iron-bound IscA dimer (300 μM) was incubated with 2 mM L-cysteine under aerobic conditions. Aliquots were taken at indicated time and frozen immediately for the liquid helium-temperature EPR measurements.

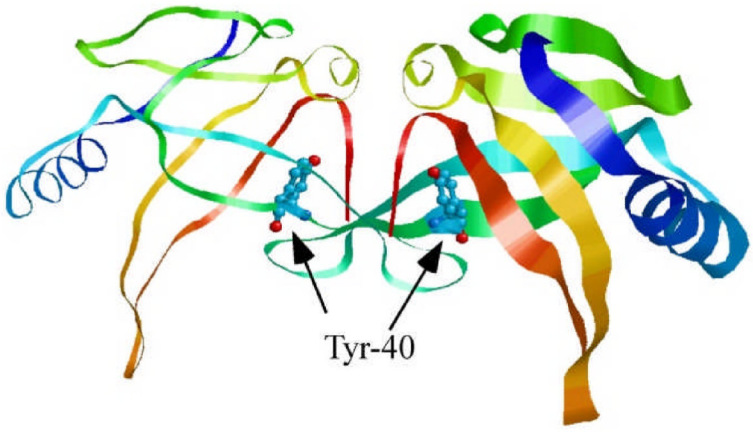

Fig. 3.

Location of Tyr-40 in *E. coli* IscA dimer. Tyr-40 is located in the interface between two IscA monomers, and is in the vicinity of the putative iron binding site (adapted from [56]). The conserved iron binding residues (Cys-99 and Cys-101) of IscA are not visible in the electron density map, likely because of the high flexibility.

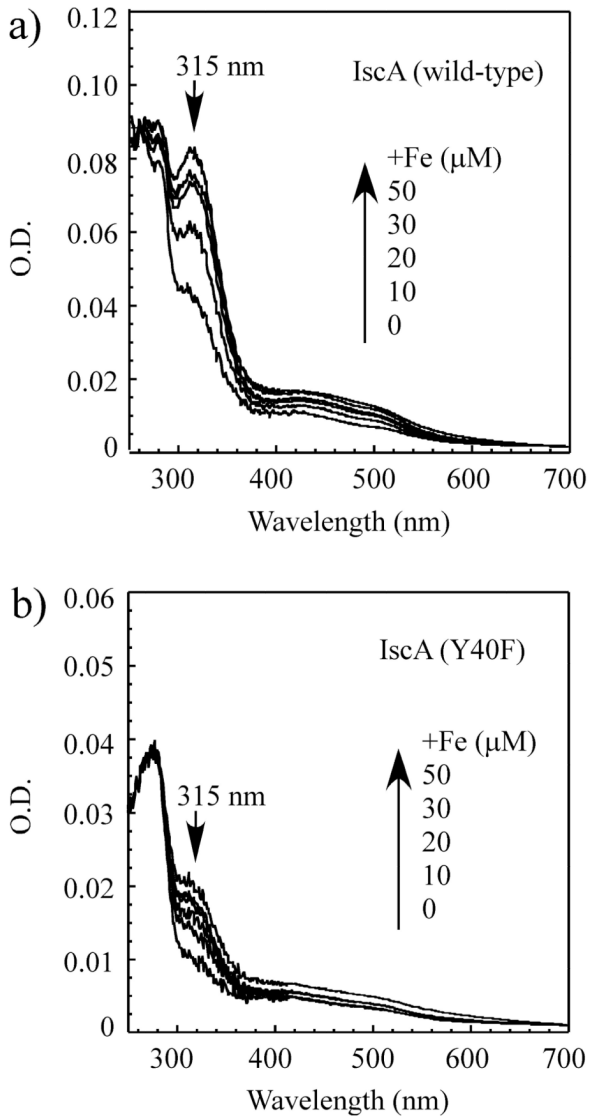

Fig. 4.

IscA mutant Y40F has a diminished iron binding activity. a), purified wild-type IscA dimer (25 μM) was incubated with indicated concentrations of $Fe(NH_4)_2(SO_4)_2$ in the presence of dithiothreitol (2 mM) at room temperature for 20 min, followed by re-purification of protein. Spectra were calibrated to the same amplitude of the absorption peak at 260 nm of the IscA sample after reconstitution with two-fold excess of iron. b), same as in a), except IscA mutant Y40F dimer (25 μM) was used. Spectra were calibrated to the same amplitude of the absorption peak at 260 nm of the Y40F sample after reconstitution with two-fold excess of iron.

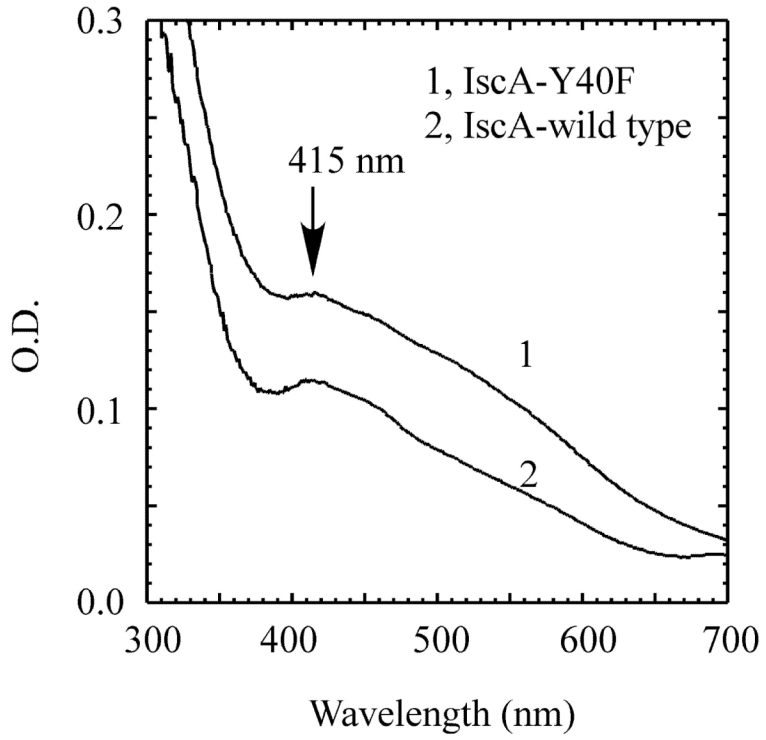

Fig. 5.

In vitro iron-sulphur cluster assembly in IscA and IscA mutant Y40F. Purified IscA mutant Y40F (spectrum 1) or wild-type IscA dimer (spectrum 2) (25 μM) was incubated with IscS (0.5 μM), $Fe(NH_4)_2(SO_4)_2$ (100 μM), dithiothreitol (2 mM) in buffer containing Tris (20 mM, pH 8.0) and NaCl (200 mM) at 37°C under anaerobic conditions. L-cysteine (1 mM) was then added to the incubation solutions to initiate the iron-sulphur cluster assembly reaction. Spectra were taken 20 min after addition of L-cysteine. The absorption peak at 415 nm reflects the iron-sulphur cluster formation in IscA.

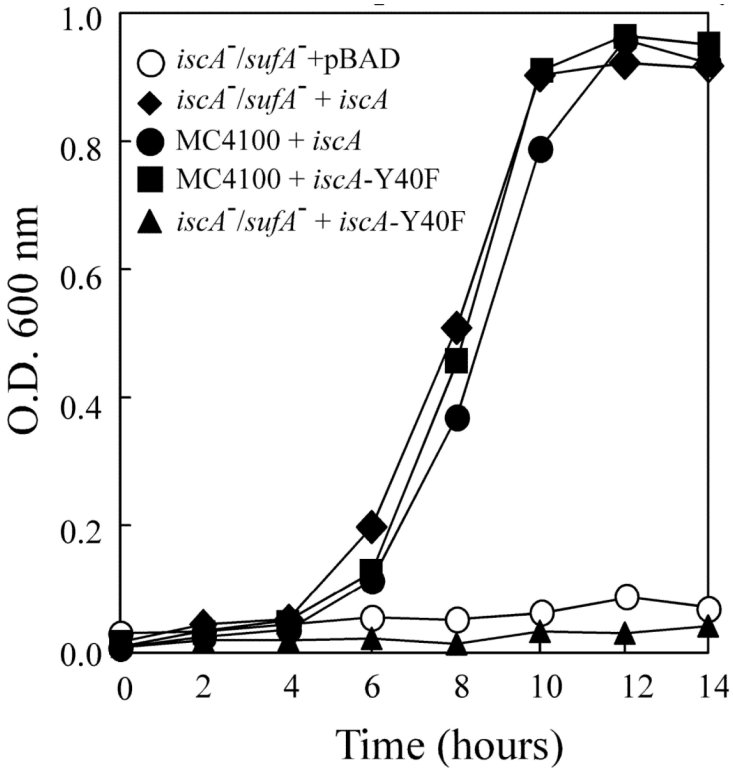

Fig. 6.

Complementary activity of the IscA mutant Y40F in *E. coli* mutant with deletion of IscA and its paralog SufA. The *E. coli* wild-type (MC4100) and the mutant cells with deletion of IscA/SufA (*iscA*⁻/*sufA*⁻) containing expression plasmids were grown in M9 minimal media at 37°C under aerobic conditions. Cell growth was monitored at O.D. at 600 nm for 14 hours after inoculation of 1:100 dilutions.