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Copper binding in IscA inhibits iron-sulphur cluster assembly in *Escherichia coli*

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Summary

Among the iron-sulphur cluster assembly proteins encoded by gene cluster *iscSUA-hscBA-fdx* in *Escherichia coli*, IscA has a unique and strong iron binding activity and can provide iron for iron-sulphur cluster assembly in proteins *in vitro*. Deletion of IscA and its paralogue SufA results in an *E. coli* mutant that fails to assemble [4Fe-4S] clusters in proteins under aerobic conditions, suggesting that IscA has a crucial role for iron-sulphur cluster biogenesis. Here we report that among the iron-sulphur cluster assembly proteins, IscA also has a strong and specific binding activity for Cu(I) *in vivo* and *in vitro*. The Cu(I) centre in IscA is stable and resistant to oxidation under aerobic conditions. Mutation of the conserved cysteine residues that are essential for the iron binding in IscA abolishes the copper binding activity, indicating that copper and iron may share the same binding site in the protein. Additional studies reveal that copper can compete with iron for the metal binding site in IscA and effectively inhibits the IscA-mediated [4Fe-4S] cluster assembly in *E. coli* cells. The results suggest that copper may not only attack the [4Fe-4S] clusters in dehydratases, but also block the [4Fe-4S] cluster assembly in proteins by targeting IscA in cells.

Introduction

IscA is a key member of the iron-sulphur cluster assembly machinery encoded by the housekeeping gene cluster *iscSUA-hscBA-fdx* in *Escherichia coli* (Zheng *et al.*, 1998; Roche *et al.*, 2013), and is highly conserved among aerobic organisms from bacteria to humans (Vinella *et al.*, 2009). Biochemical studies have shown that IscA may act as an alternative scaffold (Krebs *et al.*, 2001; Ollagnier-de-Choudens *et al.*, 2001) or intermediate carrier for iron-sulphur cluster biogenesis (Mapolelo *et al.*, 2012b; 2013; Vinella *et al.*, 2013). However, unlike other scaffold proteins such as IscU (Agar *et al.*, 2000; Unciuleac *et al.*, 2007; Raulfs *et al.*, 2008), *E. coli* IscA has a unique and strong iron binding activity *in vitro* (Ding and Clark, 2004; Ding *et al.*, 2005b; Yang *et al.*, 2006; Landry *et al.*, 2013) and *in vivo* (Wang *et al.*, 2010). Recent studies further showed that iron binding activity of IscA is conserved, as IscA homologues from *Azotobacter vinelandii* (Mapolelo *et al.*, 2012a), *Saccharomyces cerevisiae* (Muhlenhoff *et al.*, 2011), and humans (Lu *et al.*, 2010) also have a strong iron binding activity. Furthermore, the iron centre in IscA can be mobilized by L-cysteine (Ding *et al.*, 2005a; Landry *et al.*, 2013) and transferred for iron-sulphur cluster assembly in target proteins *in vitro* (Yang *et al.*, 2006), suggesting that IscA may act as an iron chaperone to deliver iron for iron-sulphur cluster biogenesis. In human cells, depletion of IscA1 results in deficiency of iron-sulphur cluster assembly in mitochondria and cytosol (Song *et al.*, 2009). In *A. vinelandii*, depletion of IscA homologue leads to a null growth phenotype when cells are cultured under the oxygen-elevated conditions (Johnson *et al.*, 2006). In *E. coli*, deletion of IscA and its paralogue SufA also produces a null growth phenotype in M9 minimal media under aerobic conditions (Lu *et al.*, 2008; Mettert *et al.*, 2008). Further studies revealed that deletion of IscA and SufA blocks the [4Fe-4S] cluster assembly without significant effect on the [2Fe-2S] cluster assembly in *E. coli* cells under aerobic growth conditions (Tan *et al.*, 2009), suggesting that IscA/SufA has a crucial role for the [4Fe-4S] cluster assembly in *E. coli* cells. Consistent with this idea, other research groups have also reported that IscA homologues are essential for the [4Fe-4S] cluster assembly in *S. cerevisiae* (Muhlenhoff *et al.*, 2011) and human cells (Sheftel *et al.*, 2012).

Escherichia coli IscA is a homodimer with three conserved cysteine residues (Cys-35, Cys-99 and Cys-101) from each monomer forming a 'cysteine pocket' between

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two monomers (Bilder *et al*., 2004; Cupp-Vickery *et al*., 2004). The site-directed mutagenesis studies showed that the 'cysteine pocket' is essential for the iron binding activity *in vitro* (Ding *et al*., 2004) and the physiological function of IscA in *E. coli* cells (Lu *et al*., 2008). The 'cysteine pocket' in IscA appears to be highly flexible to accommodate a mononuclear iron or an iron-sulphur cluster without significant change of the structure (Wada *et al*., 2005). The flexibility of the 'cysteine pocket' led us to postulate that IscA may also bind other transition metal ions such as copper in its metal binding site. While copper is an essential element for all living cells, excess copper is highly toxic (Rodriguez-Montelongo *et al*., 1993; Karlsson *et al*., 2008). Recent studies further suggested that excess copper may disrupt the labile [4Fe-4S] clusters in dehydratases (Macomber and Imlay, 2009) and block iron-sulphur cluster biogenesis in *E. coli* (Fung *et al*., 2013; Outten and Munson, 2013) and *Bacillus subtilis* (Chillappagari *et al*., 2010). Since iron-sulphur proteins are involved in diverse physiological processes from energy metabolism to DNA repair and replication (Johnson *et al*., 2005; White and Dillingham, 2012), the copper-mediated inhibition of iron-sulphur cluster biogenesis would have a broad impact on multiple cellular functions. Nevertheless, the molecular mechanism underlying the copper-mediated toxicity on iron-sulphur proteins has not been fully understood. Here, we report that among the iron-sulphur cluster assembly proteins encoded by the gene cluster *iscSUA-hscBA-fdx* (Zheng *et al*., 1998), IscA has a strong and specific copper binding activity in *E. coli* cells and *in vitro*. Copper and iron appear to share the same binding site in IscA, as mutation of the three conserved cysteine residues in IscA abolishes the iron and copper binding activities. Furthermore, excess copper can compete with iron for the metal binding site in IscA and effectively inhibit the IscA-mediated [4Fe-4S] cluster assembly without significant effect on the [2Fe-2S] cluster assembly in *E. coli* cells. The results suggest that copper may not only attack the labile [4Fe-4S] clusters in dehydratases as reported previously (Macomber and Imlay, 2009), but also block the [4Fe-4S] cluster assembly in *E. coli* cells by targeting the iron-sulphur cluster assembly protein IscA.

Results

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

To prevent or alleviate copper toxicity, *E. coli* has three copper homeostatic systems to maintain low intracellular copper content: CopA, a P-type ATPase that pumps copper ion out of the cytoplasm (Fan and Rosen, 2002); CueO, an oxidase that oxidizes Cu(I) to Cu(II) in the periplasm to prevent adventitious entry into the cytoplasm (Stoyanov *et al*., 2001); and a copper pump that transports copper ion from the periplasm to the extracellular environment (Munson *et al*., 2000). Deletion of CopA, CueO, and CusA, a subunit of the copper pump (Munson *et al*., 2000), results in an *E. coli* strain that is hypersensitive to copper in growth media (Grass and Rensing, 2001; Macomber and Imlay, 2009).

To explore the copper binding activity of iron-sulphur cluster assembly proteins *in vivo*, we expressed each protein encoded by the gene cluster *iscSUA-hscBA-fdx* in the constructed *E. coli copA/cueO/cusA* mutant cells grown in LB media under aerobic conditions. $CuSO_4$ (200 μM) was added to the cell cultures 10 min before the expression of recombinant protein was induced. $CuSO_4$ at 200 μM was chosen as it reduced cell growth of the *E. coli copA/cueO/cusA* mutant in LB by about 20% and did not significantly affect protein synthesis in the cells. Each of the iron-sulphur cluster assembly proteins was produced in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with or without 200 μM CuSO4. Purified proteins were then subjected to the UV-visible absorption measurements and metal content analyses. As shown in Fig. 1A, addition of $CuSO_4$ (200 μM) to LB media had little or no effect on the UV-visible absorption spectrum of IscS, a cysteine desulphurase that catalyses desulphurization of L-cysteine and provides sulphide for iron-sulphur cluster assembly in proteins (Smith *et al*., 2001; Marinoni *et al*., 2012). Similarly, addition of $CuSO_4$ (200 μM) to LB media did not significantly change the UV-visible absorption spectra of the iron-sulphur cluster assembly scaffold protein IscU (Agar *et al*., 2000; Unciuleac *et al*., 2007; Raulfs *et al*., 2008) (Fig. 1B), and heat shock cognate proteins HscB (Fig. 1D) and HscA (Fig. 1E) (Kim *et al*., 2012). Ferredoxin (Fdx) [2Fe-2S] cluster (Ta and Vickery, 1992; Chandramouli *et al*., 2007; Kim *et al*., 2013; Yan *et al*., 2013) was also not affected by addition of $CuSO_4$ (200 μM) in LB media (Fig. 1F). Only IscA showed a new absorption peak at 258 nm (Fig. 1C) when $CuSO_4$ (200 μM) was added to LB media. As the absorption peak at 258 nm has been attributed to the Cu(I) binding with two cysteine residues as ligands in the Parkinsonism-associated protein DJ-1 (Puno *et al*., 2013), we propose that IscA may bind Cu(I) via cysteine residues.

The copper content analyses using neocuproine or ICP-ES (the Inductively Coupled Plasma-Emission Spectrometry) showed that IscA purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $CuSO_4$ (200 μM) contained 0.60 ± 0.09 copper atoms per IscA dimer, while IscS, HscA and HscB had very little or no detectable amounts of copper (Fig. 2A). IscU and ferredoxin retained a small but reproducible amount of copper (~ 0.1 copper atoms per dimer). However, unlike IscA, the copper content in IscU and ferredoxin was further decreased after additional purification steps, indicating

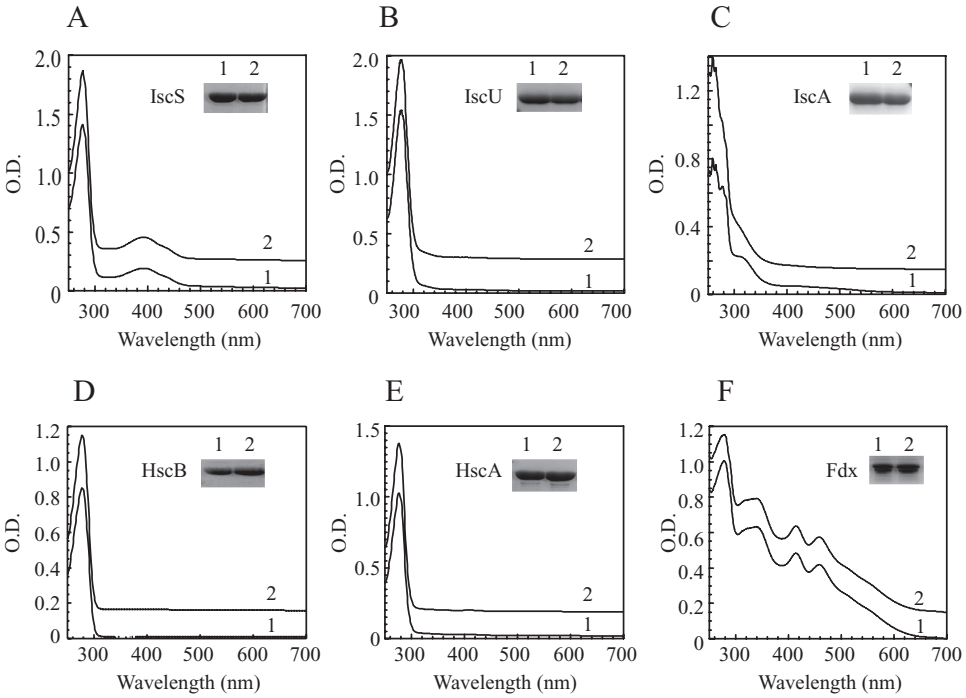

Fig. 1. IscA has a unique copper binding activity among the iron-sulphur cluster assembly proteins. Each protein encoded by the gene cluster *iscSUA-hscBA-fdx* was expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with or without 200 μM $CuSO_4$. Proteins were purified from the cells and subjected to UV-visible absorption measurements. (A) IscS; (B) IscU; (C) IscA; (D) HscB; (E) HscA; (F) ferredoxin. In each panel: spectrum 1, without $CuSO_4$ in LB media; spectrum 2, with $CuSO_4$ in LB media. Insert in each panel is a photograph of SDS-PAGE gel of purified proteins. The results are representatives of three independent protein preparations.

that copper binding in IscU and ferredoxin was not stable. Thus, among the iron-sulphur cluster assembly proteins encoded by *iscSUA-hscBA-fdx*, IscA has a unique and strong copper binding activity.

As copper-binding proteins often have electron paramagnetic resonance (EPR) signals (Ve *et al.*, 2012), purified IscA was subjected to EPR measurements. As shown in Fig. 2B, IscA purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $CuSO_4$ (200 μM) had no EPR signal. However, when purified IscA was treated with 2.5% (v/v) nitric acid to oxidize Cu(I) in the protein as described in Ve *et al.* (2012), an EPR signal representing a typical Cu(II) centre (Smith *et al.*, 2008; Ve *et al.*, 2012) appeared (Fig. 2B), demonstrating that purified IscA indeed binds Cu(I) that can be oxidized by the strong oxidant nitric acid. Quantification of the Cu(II) EPR signal showed that purified IscA contained 0.55 ± 0.12 copper atoms per IscA dimer, which is close to that determined using neocuproine (0.60 ± 0.09 copper atoms per IscA dimer).

To further determine the copper binding activity of IscA *in vivo*, recombinant IscA was expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with increased concentrations of $CuSO_4$. Figure 2C shows that as the concentration of $CuSO_4$ in LB media was gradually increased from 0 to 1.0 mM, the copper binding of IscA was progressively increased from 0 to about 1.4 copper atoms per IscA dimer. On the other hand, the cell growth of the *E. coli copA/cueO/cusA* mutant was gradually decreased to about 30% when the concentration of $CuSO_4$ in LB media was increased from 0 to 1.0 mM (Fig. 2C). Because the cell growth of the *E. coli copA/cueO/cusA* mutant in LB media was severely inhibited by 1.0 mM $CuSO_4$ (Fig. 2C), we were unable to obtain the maximum copper binding in IscA expressed in the *E. coli copA/cueO/cusA* mutant cells. Nevertheless, the results suggest that the copper binding in IscA inversely correlates with the cell growth when the concentration of $CuSO_4$ in LB media is increased from 0 to 1.0 mM.

The in vitro *copper binding activity of IscA*

To determine the *in vitro* copper binding activity of IscA, we prepared apo-IscA as described previously (Landry *et al.*, 2013) and incubated apo-IscA (50 μM dimer) with increasing concentrations of $CuSO_4$ (0 to 200 μM) in the presence of dithiothreitol. Dithiothreitol was used to reduce Cu(II) to Cu(I) (Banci *et al.*, 2003) to emulate the intracellular redox state of copper ion (Macomber and Imlay, 2009) and to reduce thiol groups in IscA for copper binding. After incubation at room temperature for 15 min, IscA was re-purified by passing through a HiTrap Desalting

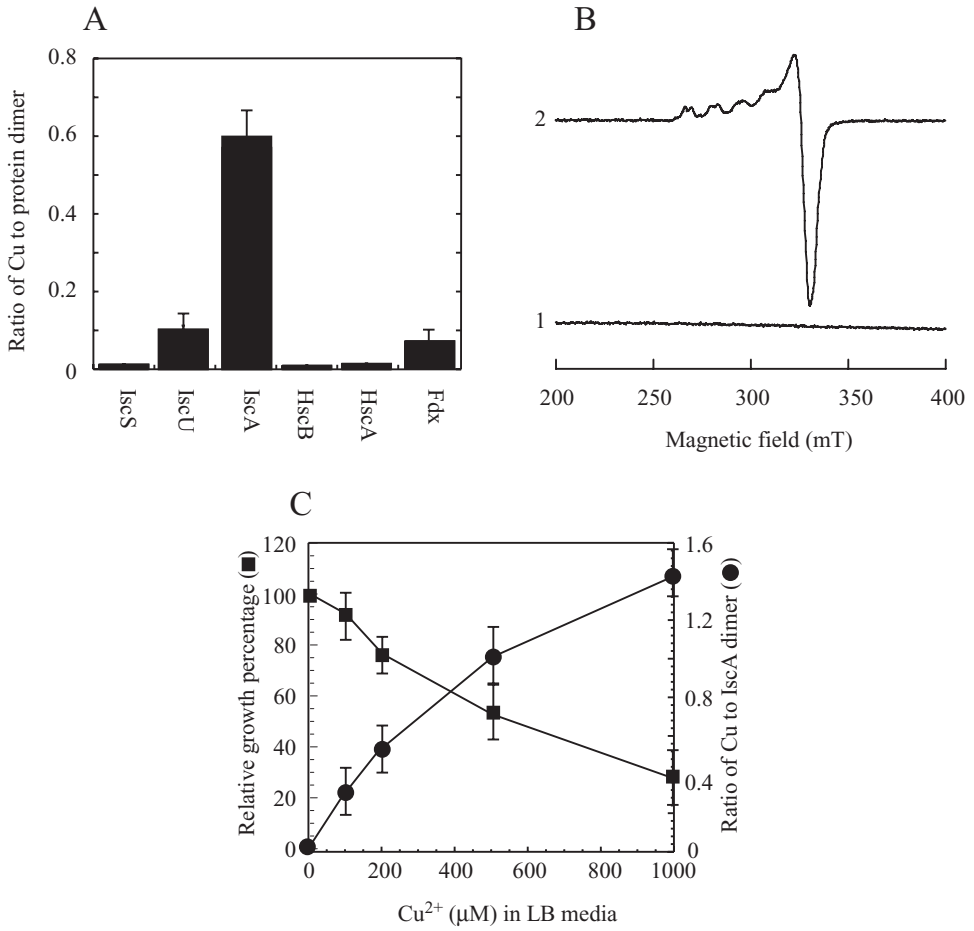

Fig. 2. Relative copper binding activity of IscA in the *E. coli copA/cueO/cusA* mutant cells.
A. The copper content of the iron-sulphur cluster assembly proteins encoded by the gene cluster *iscSUA-hscBA-fdx* purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with 200 μM $CuSO_4$.
B. The EPR spectra of IscA purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with 200 μM $CuSO_4$. Purified IscA (50 μM) (spectrum 1) was treated with 2.5% nitric acid (spectrum 2).
C. Correlation of the copper binding in IscA and the relative cell growth in LB media supplemented with 0, 100, 200, 500, and 1000 μM $CuSO_4$. The copper content in purified IscA was plotted as a function of the $CuSO_4$ concentration in LB media (closed circles). The relative cell growth was defined as the percentage of the cell growth in LB media with $CuSO_4$ over that without $CuSO_4$, and was plotted as a function of the $CuSO_4$ concentration in LB media (closed squares). The 100% cell growth represented the cell density of O.D. at 600 nm of ~ 1.0 after 3 h at 37°C in LB media with aeration. The results are the mean ± standard deviations from three independent experiments.

column. As the $CuSO_4$ concentration in the incubation solution was increased, the amplitude of the absorption peak at 258 nm (Fig. 3A) and the copper content (Fig. 3B) of re-purified IscA were gradually increased and saturated at about twofold excess of $CuSO_4$ in the incubation solution. It was reported that dithiothreitol and copper may generate hydroxyl free radicals in solution (Kachur *et al.*, 1997). However, production of hydroxyl free radicals in solution had a long delay (up to 5 h) after dithiothreitol was mixed with copper under aerobic conditions (Kachur *et al.*, 1997). Furthermore, similar results were obtained when apo-IscA was incubated with $CuSO_4$ (0 to 200 μM) and dithiothreitol (2 mM) under anaerobic conditions (data not shown). Thus, it is unlikely that oxygen or hydroxyl free radicals are involved in the copper binding in IscA. Because the copper binding in IscA was almost linear at low concentrations of $CuSO_4$ (Fig. 3B), we were unable to estimate the copper binding constant of IscA. Nevertheless, the result clearly showed that the maximum copper binding in IscA is 2.2 ± 0.4 copper atoms per IscA dimer (Fig. 3B).

IscA proteins re-purified after incubation with $CuSO_4$ and dithiothreitol were also subjected to EPR measurements. Without any treatments, re-purified IscA proteins were EPR silent, similar to that purified from *E. coli* cells (Fig. 2B). However, when re-purified IscA proteins were treated with 2.5% (v/v) nitric acid to oxidize Cu(I) (Ve *et al.*, 2012), a typical Cu(II) EPR signal appeared,

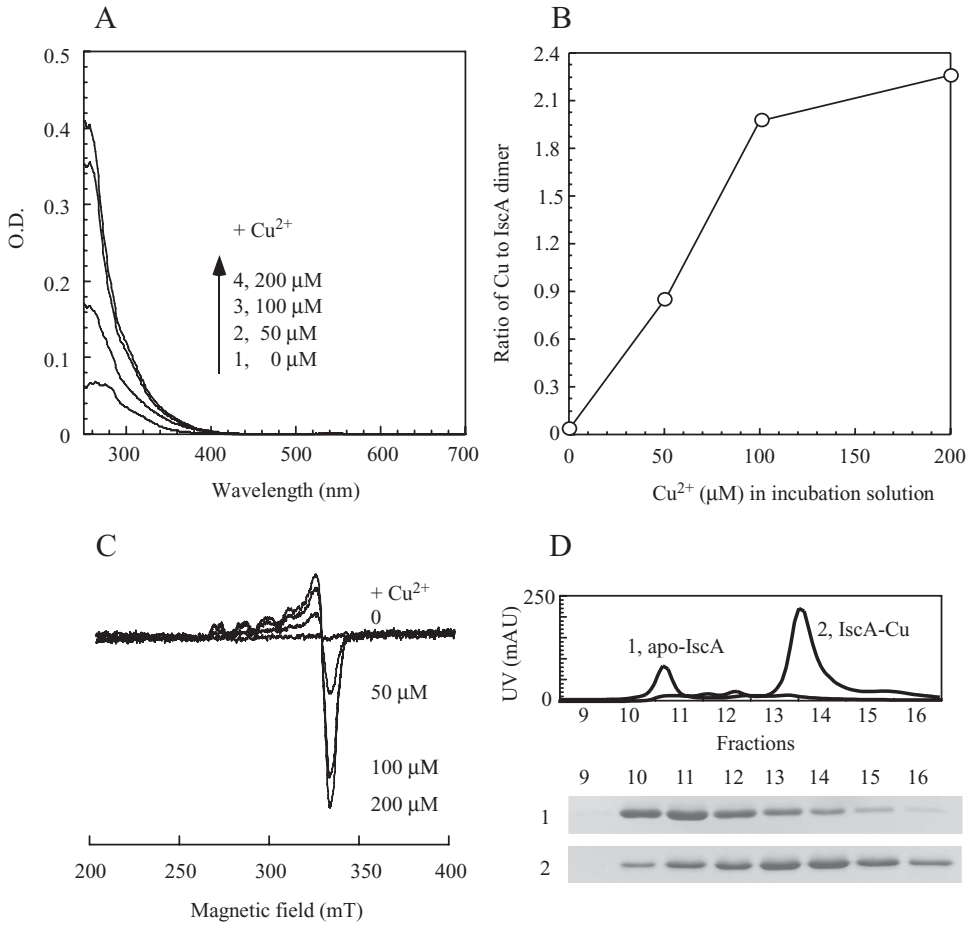

Fig. 3. *In vitro* copper binding activity of IscA. Apo-IscA (50 µM dimer) was incubated with 0, 50, 100, and 200 µM $CuSO_4$ in the presence of dithiothreitol (2 mM). Protein was re-purified from incubation solutions by passing through a Hi-trap Desalt column.
A. UV-visible spectra of re-purified IscA after reconstitution with the indicated concentration of $CuSO_4$.
B. Relative copper binding activity of IscA. The copper content of re-purified IscA was analysed and plotted as a function of the $CuSO_4$ concentration in the incubation solution.
C. The EPR spectra of the copper-bound IscA. Re-purified IscA proteins were treated with 2.5% (v/v) nitric acid and subjected to the EPR measurements.
D. Elution profiles of apo-IscA and the copper-bound IscA from a Mono-Q column. The copper-bound IscA was prepared after apo-IscA (50 µM dimer) was incubated with 200 µM $CuSO_4$ in the presence of dithiothreitol (2 mM). Top, elution profiles of apo-IscA (trace 1) or the copper-bound IscA (trace 2) using a linear gradient of NaCl (0 to 0.5 M). Bottom, photographs of the SDS-PAGE gel of the eluted fractions of apo-IscA (sample 1) and the copper-bound IscA (sample 2).

indicating that IscA also binds Cu(I) *in vitro*. The amplitude of the Cu(II) EPR signal of IscA was proportionally increased as the concentration of $CuSO_4$ was increased in the incubation solution (Fig. 3C), and was closely correlated with the copper content in IscA (Fig. 3B).

To further explore the property of the copper-bound IscA, re-purified IscA proteins were analysed using an anion exchange Mono-Q column which separates proteins based on their net charge and protein conformation. Figure 3D shows that copper binding in IscA shifted the protein elution profile from fractions 10 and 11 (apo-IscA) to fractions 13 and 14 (the copper-bound IscA), indicating that copper binding may have significantly changed the conformation of IscA dimer.

The conserved cysteine residues in IscA are required for the copper binding activity

The three conserved cysteine residues (Cys-35, Cys-99 and Cys-101) are essential for the iron binding activity of IscA *in vitro* (Ding *et al.*, 2004) and for its physiological function in *E. coli* (Lu *et al.*, 2008). If copper and iron share the same binding site in IscA, mutation of the conserved cysteine residues would disrupt both the iron and copper binding in the protein. To test this idea, we constructed an IscA mutant in which all three cysteine residues (Cys-35, Cys-99 and Cys-101) were replaced with serine. Prepared apo-IscA and IscA mutant were then incubated with twofold excess of $Fe(NH_4)_2(SO_4)_2$ or

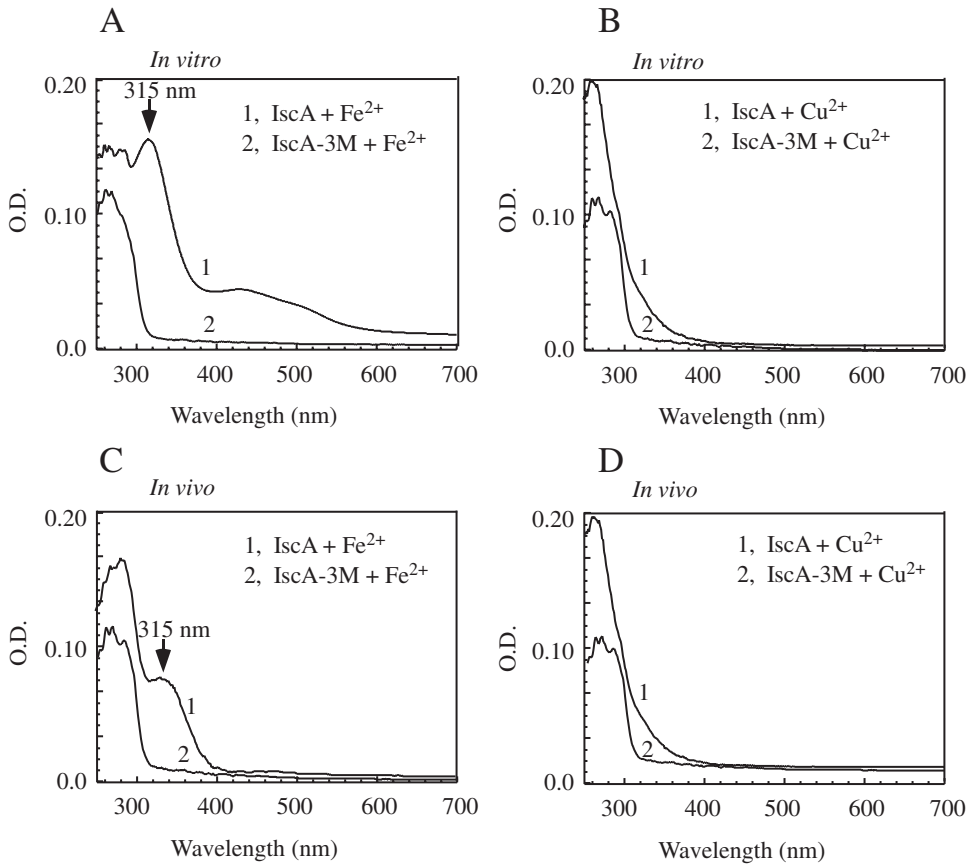

Fig. 4. The conserved cysteine residues are essential for the copper binding in IscA.
A. UV-visible spectra of IscA and the IscA mutant (IscA-3 M) after incubation with iron *in vitro*. Apo-IscA and the IscA triple mutant (50 μM dimer) were incubated with $Fe(NH_4)_2(SO_4)_2$ (100 μM) in the presence of dithiothreitol (2 mM) at room temperature for 15 min, followed by protein re-purification.
B. UV-visible spectra of IscA and the IscA mutant (IscA-3 M) after incubation with copper *in vitro*. Apo-IscA and the IscA mutant (50 μM dimer) were incubated with $CuSO_4$ (100 μM) in the presence of dithiothreitol (2 mM) at room temperature for 15 min, followed by protein re-purification.
C. UV-visible spectra of IscA and the IscA mutant (IscA-3 M) purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $Fe(NH_4)_2(SO_4)_2$ (200 μM).
D. UV-visible spectra of IscA and the IscA mutant (IscA-3 M) purified from the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $CuSO_4$ (200 μM).

$CuSO_4$ in the presence of dithiothreitol, followed by protein re-purification to remove residual metal ions and dithiothreitol. As reported previously, incubation of the wild-type apo-IscA with twofold excess of iron or copper produced the iron-bound IscA (with an absorption peak at 315 nm) (Fig. 4A) (Ding and Clark, 2004) or the copper-bound IscA (with an absorption peak at 258 nm) (Fig. 4B) respectively. In contrast, incubation of the IscA mutant with iron or copper did not produce any absorption peaks of the iron or copper binding in the protein. The iron and copper content analyses confirmed that unlike the wild-type IscA, the IscA mutant did not bind any detectable amounts of iron or copper under the same experimental conditions (data not shown).

The wild-type IscA and IscA mutant proteins were also expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $Fe(NH_4)_2(SO_4)_2$ (200 μM) or $CuSO_4$ (200 μM). Again, unlike the wild-type IscA, the IscA mutant failed to bind any iron (Fig. 4C) or copper (Fig. 4D) in the *E. coli* cells. Taken together, the results suggest that the three conserved cysteine residues in IscA are essential for its iron and copper binding activity *in vitro* and *in vivo*.

Copper competes with iron for the metal binding site in IscA

If iron and copper share the metal binding site, excess copper may compete with iron for the same binding site in IscA. Indeed, addition of $CuSO_4$ (200 μM) to LB media largely eliminated the iron binding peak at 315 nm of IscA expressed in the *E. coli copA/cueO/cusA* mutant cells

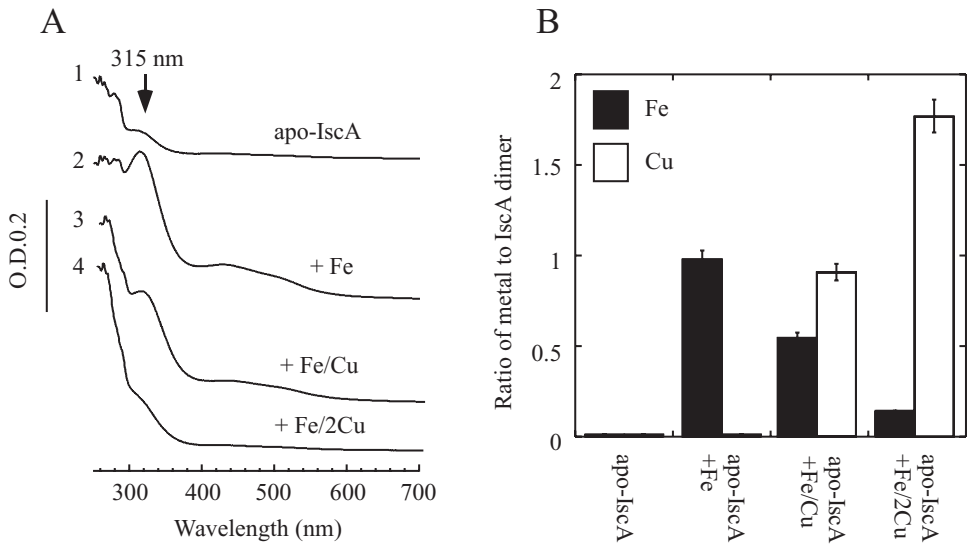

Fig. 5. Excess copper competes with iron for the metal binding sites in IscA.
A. Copper competes for the iron binding site in IscA. Apo-IscA (50 μM dimer) (spectrum 1) was incubated with $Fe(NH_4)_2(SO_4)_2$ (100 μM) (spectrum 2), $Fe(NH_4)_2(SO_4)_2$ (100 μM) and $CuSO_4$ (100 μM) (spectrum 3), or $Fe(NH_4)_2(SO_4)_2$ (100 μM) and $CuSO_4$ (200 μM) (spectrum 4) in the presence of dithiothreitol (2 mM) at room temperature for 15 min. IscA was re-purified from the incubation solutions and subjected to UV-visible absorption measurements.
B. Iron and copper content analyses of re-purified IscA. The iron and copper content of re-purified IscA proteins from panel (A) were analysed and plotted as the ratio of metal to IscA dimer. The protein concentration was determined from the samples after incubation without metal ions. The results are presented as the mean ± standard deviations.

(Fig. 1C). The metal content analyses further showed that the iron content of IscA was decreased from 0.21 ± 0.04 to less than 0.04 iron atoms with concomitant increase of the copper content from 0 to 0.60 ± 0.09 copper atoms per IscA dimer when LB media were supplemented with 200 μM $CuSO_4$.

To further explore the copper and iron binding competition in IscA, purified apo-IscA was incubated with a fixed amount of iron and increasing amounts of copper in the presence of dithiothreitol, followed by re-purification of IscA. As shown in Fig. 5A, incubation of apo-IscA (50 μM dimer) with 100 μM $Fe(NH_4)_2(SO_4)_2$ produced an iron-bound IscA with an absorption peak at 315 nm as reported above. However, when apo-IscA (50 μM dimer) was incubated with 100 μM $Fe(NH_4)_2(SO_4)_2$ and 100 μM $CuSO_4$, the absorption peak at 315 nm of IscA was significantly diminished (Fig. 5A), and the iron content of IscA was decreased with concomitant increase of the copper content in the protein (Fig. 5B). When apo-IscA (50 μM dimer) was incubated with 100 μM $Fe(NH_4)_2(SO_4)_2$ and 200 μM $CuSO_4$, the absorption peak at 315 nm of re-purified IscA was largely eliminated (Fig. 5A), and the iron content of IscA was further decreased with the copper content increased to 1.81 ± 0.15 copper atoms per IscA dimer (Fig. 5B). Thus, excess copper can effectively compete with iron for the metal binding site in IscA.

Copper inhibits the [4Fe-4S] cluster assembly in the E. coli *cells*

Previous studies revealed that IscA and its paralogue SufA are essential for the [4Fe-4S] cluster assembly in *E. coli* cells under aerobic conditions (Tan *et al*., 2009). IscA and SufA share the similar crystal structure (Bilder *et al*., 2004; Cupp-Vickery *et al*., 2004; Wada *et al*., 2005) and iron binding activity (Lu *et al*., 2008). Parallel experiments showed that *E. coli* SufA also has a similar copper binding activity as IscA in the *E. coli copA/cueO/cusA* mutant cells grown in LB media (Supplementary Fig. S1). Thus, if copper competes with iron for the metal binding site in IscA and SufA, copper may inhibit the IscA/SufA-mediated [4Fe-4S] cluster assembly in *E. coli* cells. To test this idea, we decided to analyse the [4Fe-4S] cluster assembly in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with or without $CuSO_4$ (200 μM).

Escherichia coli dihydroxyacid dehydratase (IlvD) requires an intact [4Fe-4S] cluster for its catalytic activity (Flint *et al*., 1993). Relative activity of IlvD has previously been used to assess the [4Fe-4S] cluster assembly in the protein in *E. coli* cells (Ren *et al*., 2008). In this set of experiments, recombinant IlvD was expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with or without $CuSO_4$ (200 μM). The cell extracts were prepared after the cells were passed through French

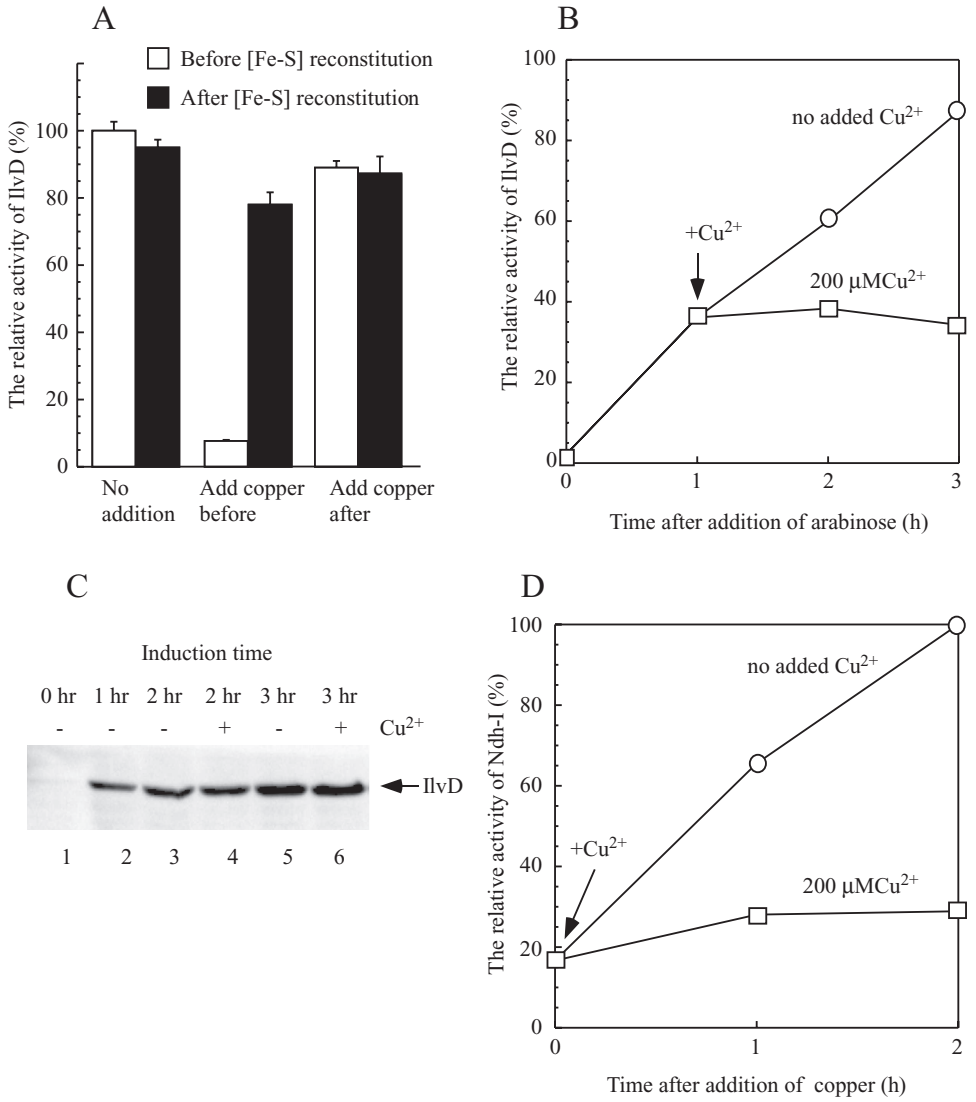

Fig. 6. Copper largely blocks the [4Fe-4S] cluster assembly in the *E. coli* cells.
A. Inhibition of the [4Fe-4S] cluster assembly in recombinant dihydroxyacid dehydratase (IlvD) in the *E. coli* cells by copper. Recombinant IlvD was expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media. $CuSO_4$ (200 μM) was added to the cell culture before or after recombinant IlvD was produced in the cells. Cell extracts were prepared, and the enzyme activity of IlvD in the cell extracts was measured before (open bars) and after (closed bars) the cell extracts were incubated with L-cysteine (1 mM), cysteine desulphurase IscS (1 μM), $Fe(NH_4)_2(SO_4)_2$ (100 μM) and dithiothreitol (2 mM) under anaerobic conditions.
B. Effect of copper on the IlvD [4Fe-4S] cluster assembly in the *E. coli copA/cueO/cusA* mutant cells. $CuSO_4$ (200 μM) was added to the cell culture after recombinant IlvD was expressed for 1 h. Cell extracts were prepared at indicated time points, and the enzyme activity of IlvD in the cell extracts was measured and plotted as a function of cell growth time.
C. Effect of copper on the recombinant IlvD expression in the *E. coli copA/cueO/cusA* mutant cells. The cell extracts were prepared and analysed by Western blotting using the antibody against His-tag. Lane 1, at time 0; lane 2, after 1 h induction; lanes 3 and 4, after 2 h induction; lanes 5 and 6, after 3 h induction. Lanes 3 and 5, with no copper addition; lanes 4 and 6, with $CuSO_4$ (200 μM).
D. Effect of copper on NADH dehydrogenase I in the *E. coli* cells. Overnight cells of the *E. coli copA/cueO/cusA* mutant were grown for 3 h, and treated with or without $CuSO_4$ (200 μM). The enzyme activity of NADH dehydrogenase I in the *E. coli* cells was measured at 0, 1 and 2 h after addition of $CuSO_4$. The relative enzyme activity of NADH dehydrogenase I was plotted as a function of cell growth time. The results are the representatives from three independent experiments.

press once, and immediately used for the IlvD activity assay. Figure 6A shows that addition of $CuSO_4$ (200 μM) to LB media before recombinant IlvD was expressed in the *E. coli* cells decreased the IlvD enzyme activity to about 10%. However, the enzyme activity of IlvD was largely recovered when the cell extracts were incubated with L-cysteine, cysteine desulphurase IscS, $Fe(NH_4)_2(SO_4)_2$, and dithiothreitol to reconstitute the [4Fe-4S] clusters in IlvD under anaerobic conditions (Fig. 6A), suggesting that $CuSO_4$ (200 μM) in LB media blocks the [4Fe-4S] cluster

assembly in IlvD without inhibiting protein synthesis in the *E. coli* cells. Interestingly, when $CuSO_4$ (200 μM) was added to LB media after recombinant IlvD was expressed in the *E. coli* cells, over 85% of the enzyme activity of IlvD remained, indicating that copper at 200 μM in LB media does not significantly affect the pre-assembled [4Fe-4S] clusters in IlvD in the *E. coli* cells.

To further explore the copper-mediated inhibition of the [4Fe-4S] cluster assembly in IlvD in the *E. coli copA/cueO/cusA* mutant cells, $CuSO_4$ (200 μM) was added to LB media when recombinant IlvD was expressed for 1 h. As shown in Fig. 6B, without any addition of $CuSO_4$, the active IlvD (IlvD with an intact [4Fe-4S] cluster) linearly accumulated in the cells. However, addition of $CuSO_4$ (200 μM) to LB media effectively blocked the new [4Fe-4S] cluster assembly in IlvD in the *E. coli* cells. Pre-assembled [4Fe-4S] clusters in IlvD decreased only slightly in the *E. coli* cells during incubation with LB media supplemented with $CuSO_4$ (200 μM). The Western blotting analyses of the cell extracts further confirmed that $CuSO_4$ (200 μM) did not significantly affect the protein expression of IlvD in the *E. coli* cells (Fig. 6C). These results further suggest that $CuSO_4$ (200 μM) can effectively inhibit the [4Fe-4S] cluster assembly in IlvD without significant effect on the pre-assembled [4Fe-4S] clusters in the protein in the *E. coli copA/cueO/cusA* mutant cells grown in LB media.

If copper blocks the [4Fe-4S] cluster assembly in the *E. coli* cells, it should also inhibit the activities of other proteins that contain [4Fe-4S] clusters. Indeed, similar results were obtained when recombinant aconitase B [4Fe-4S] cluster (Varghese *et al*., 2003) or phosphogluconate dehydratase [4Fe-4S] cluster (Jang and Imlay, 2007) was expressed in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $CuSO_4$ (200 μM) (data not shown). We also examined the effect of $CuSO_4$ (200 μM) on native NADH dehydrogenase I which requires multiple iron-sulphur clusters for its catalytic activity (Nakamaru-Ogiso *et al*., 2008). Using deamino-NADH as specific substrate for NADH dehydrogenase I (Matsushita *et al*., 1987), we found that addition of $CuSO_4$ (200 μM) dramatically inhibited the enzyme activity of NADH dehydrogenase I but without significantly affecting the activity of pre-existed NADH dehydrogenase I in the *E. coli copA/cueO/cusA* mutant cells (Fig. 6D). Thus, copper can effectively inhibit the [4Fe-4S] cluster assembly in *E. coli* cells.

Excess copper emulates the phenotype of an E. coli *mutant with deletion of IscA/SufA*

Deletion of IscA and its paralogue SufA results in an *E. coli* mutant that fails to assemble [4Fe-4S] clusters (Fig. 7A) but not [2Fe-2S] clusters in proteins (Fig. 7B) under aerobic growth conditions (Tan *et al*., 2009). If the copper binding in IscA/SufA blocks iron-sulphur cluster biogenesis in *E. coli* cells, excess copper may emulate a phenotype of the *E. coli* mutant with deletion of IscA/SufA. To test this idea, we expressed the *E. coli* endonuclease III in the *E. coli copA/cueO/cusA* mutant cells grown in LB media supplemented with $CuSO_4$ (200 μM). Endonuclease III hosts a stable [4Fe-4S] cluster (Thayer *et al*., 1995) which should be resistant to disruption by copper (Macomber and Imlay, 2009). As shown in Fig. 7C, addition of $CuSO_4$ (200 μM) to LB media largely blocked the [4Fe-4S] cluster assembly in recombinant endonuclease III in the *E. coli* cells. In contrast, addition of $CuSO_4$ (200 μM) to LB media had very little or no effect on the [2Fe-2S] cluster assembly in ferric iron reductase FhuF (Muller *et al*., 1998) in the *E. coli copA/cueO/cusA* mutant cells (Fig. 7D). Similarly, addition of $CuSO_4$ (200 μM) to LB media did not affect the [2Fe-2S] cluster assembly in ferredoxin expressed in the *E. coli copA/cueO/cusA* mutant cells (Fig. 1F). The selective inhibition of the [4Fe-4S] cluster assembly by $CuSO_4$ (200 μM) in the *E. coli* cells strongly suggests that copper in LB media may emulate the phenotype of the *E. coli* cells with deletion of IscA/SufA.

IscA acts as a primary target of copper toxicity

The proteins encoded by the gene cluster *iscSUA-hscBA-fdx* represent the house-keeping machinery for iron-sulphur cluster assembly under normal physiological conditions (Zheng *et al*., 1998). SufA and other proteins encoded by the alternative iron-sulphur cluster assembly gene cluster *sufABCDSE* (Takahashi and Tokumoto, 2002) are induced only under iron starvation (Outten *et al*., 2004) or oxidative stress (Lee *et al*., 2004). Thus, we postulate that IscA could be the primary target of copper toxicity in *E. coli* cells under normal growth conditions. To test this idea, we deleted the gene encoding IscA in the wild-type *E. coli* cells and explored the copper toxicity on iron-sulphur cluster assembly in these mutant cells. Recombinant endonuclease III was expressed in the wild-type and the IscA-deleted mutant cells grown in LB media supplemented with or without $CuSO_4$ (2 mM). Protein was purified from the cells and subjected to UV-Visible absorption measurements. As shown in Fig. 8A, deletion of IscA severely blocked the [4Fe-4S] cluster assembly in endonuclease III expressed in *E. coli* cells. In contrast, deletion of SufA had no noticeable effect on the [4Fe-4S] cluster assembly in endonuclease III expressed in *E. coli* cells (data not shown). While $CuSO_4$ (2 mM) in LB media largely inhibited the [4Fe-4S] cluster assembly in endonuclease III in the wild-type *E. coli* cells, $CuSO_4$ (2 mM) had very little or no additional inhibitory effect on the [4Fe-4S] cluster assembly in endonuclease III in the IscA-deleted cells. We also performed the same experiments in the *E. coli copA/cueO/cusA* mutant cells, and found that deletion of IscA also significantly inhibited the [4Fe-4S] cluster assembly in

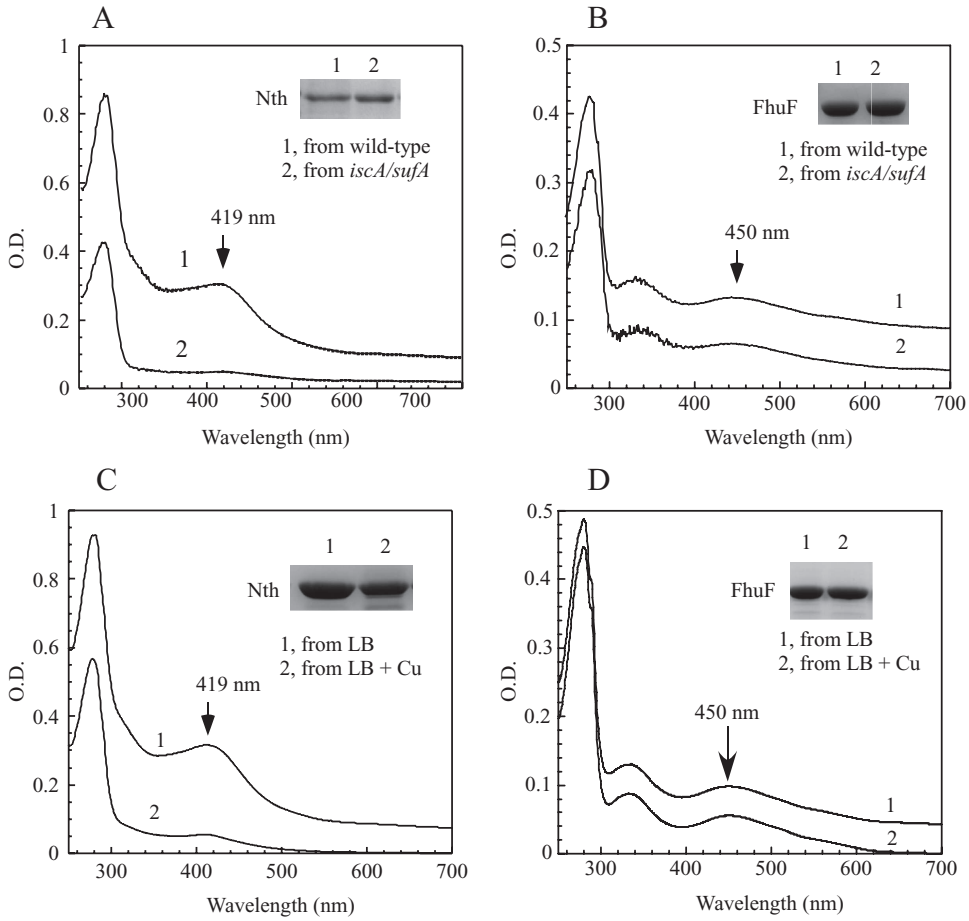

Fig. 7. Copper selectively inhibits the [4Fe-4S] cluster assembly in the *E. coli* cells.
A. UV-visible absorption spectra of recombinant endonuclease III (Nth) (~ 30 μM) purified from the *E. coli* wild-type cells (spectrum 1) and the mutant cells with deletion of IscA/SufA (spectrum 2) grown in LB media under aerobic growth conditions. The absorption peak at 419 nm indicates the [4Fe-4S] cluster of endonuclease III.
B. UV-visible absorption spectra of recombinant FhuF (~ 8 μM) purified from the *E. coli* wild-type cells (spectrum 1) and the mutant cells with deletion of IscA/SufA (spectrum 2) grown in LB media under aerobic growth conditions. The absorption peak at 450 nm indicates the [2Fe-2S] cluster of FhuF.
C. UV-visible absorption spectra of recombinant endonuclease III (Nth) (~ 22 μM) purified from the *E. coli copA/cueO/cusA* mutant cells supplemented with 0 (spectrum 1) or 200 μM (spectrum 2) $CuSO_4$ in LB media under aerobic growth conditions.
D. UV-visible absorption spectra of recombinant ferric iron reductase FhuF (~ 5 μM) purified from the *E. coli copA/cueO/cusA* mutant cells supplemented with 0 (spectrum 1) or 200 μM (spectrum 2) $CuSO_4$ in LB media under aerobic growth conditions. Insert in each panel is a photograph of SDS-PAGE gel of purified proteins.

endonuclease III in the cells. Again, while $CuSO_4$ (200 μM) largely inhibited the [4Fe-4S] cluster assembly in endonuclease III in the *E. coli copA/cueO/cusA* mutant cells, $CuSO_4$ (200 μM) had very little or no additional inhibitory effect on the [4Fe-4S] cluster assembly in endonuclease III in the IscA-deleted *copA/cueO/cusA* cells (Fig. 8B).

Discussion

It was previously postulated that copper may mediate production of reactive oxygen species via Fenton reaction and contribute to copper toxicity in cells (Rodriguez-Montelongo *et al.*, 1993; Karlsson *et al.*, 2008). However, recent studies argued that copper does not promote oxidative damage in *E. coli* cells even under aerobic conditions (Macomber *et al.*, 2007). Instead, copper may directly disrupt the labile [4Fe-4S] clusters of dehydratases, inactivate these enzymes, and contribute to copper toxicity in cells (Macomber and Imlay, 2009). In this study, we expand this view by showing that copper can effectively block the [4Fe-4S] cluster assembly in *E. coli* cells by targeting the iron-sulphur cluster assembly protein IscA. Among the iron-sulphur cluster assembly proteins encoded by the gene cluster *iscSUA-hscBA-fdx* (Zheng *et al.*, 1998), IscA has a strong and specific copper binding activity *in vivo* (Figs 1 and 2) and *in vitro* (Fig. 3). Substitution of the three

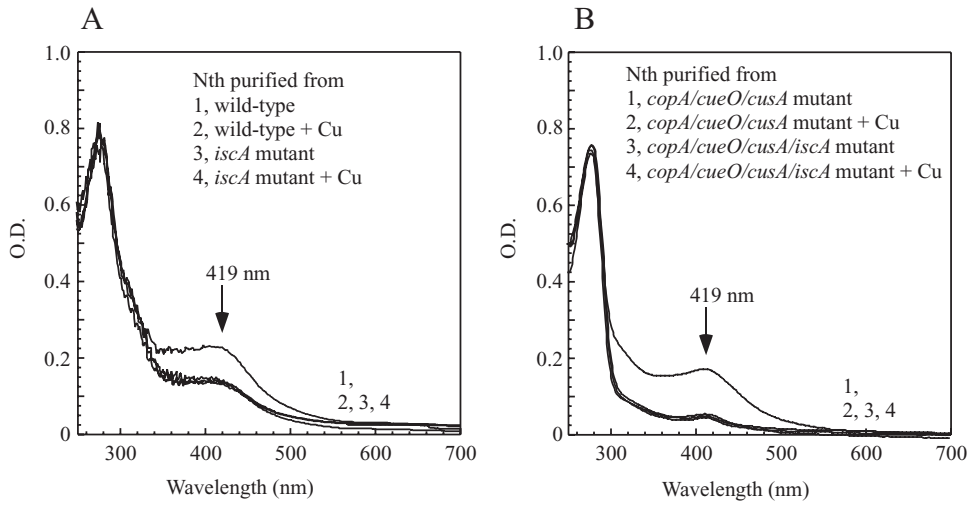

Fig. 8. IscA is a primary target of copper toxicity. Recombinant endonuclease III was expressed in the *E. coli* cells grown in LB media supplemented with or without $CuSO_4$. Protein was purified from the *E. coli* cells and subjected to UV-visible absorption measurements.
A. Effect of copper on the [4Fe-4S] cluster assembly in endonuclease III in the wild-type and the *iscA* mutant cells. The *E. coli* cells were grown at 37°C in LB media with aeration for 3 h. As indicated, $CuSO_4$ (2 mM) was added 10 min before expression of recombinant endonuclease III was induced.
B. Effect of copper on the [4Fe-4S] cluster assembly in endonuclease III in the *E. coli copA/cueO/cusA* mutant and the *copA/cueO/cusA/iscA* mutant cells. The *E. coli* cells were grown at 37°C in LB media with aeration for 3 h. As indicated, $CuSO_4$ (200 μM) was added 10 min before expression of recombinant endonuclease III was induced. The protein concentration was calibrated to ~ 40 μM for the UV-visible absorption measurements.

conserved cysteine residues with serine in IscA abolishes the iron and copper binding activities (Fig. 4), indicating that copper and iron may share the same binding sites in the protein. In addition, excess copper can compete with iron for the metal binding site in IscA (Fig. 5) and inhibit the IscA-mediated [4Fe-4S] cluster assembly in *E. coli* cells grown in LB media (Figs 6 and 7). Furthermore, deletion of IscA severely inhibits the [4Fe-4S] cluster assembly in *E. coli* cells under normal growth conditions and addition of copper to the IscA-deleted mutant cells has very little or no additional inhibitory effect on the [4Fe-4S] cluster assembly in the *E. coli* cells (Fig. 8). These results led us to propose that copper can effectively block the [4Fe-4S] cluster assembly in proteins and that IscA is a primary target of copper toxicity in *E. coli* cells.

The crystallographic studies revealed that IscA has a 'cysteine pocket' formed by the three conserved cysteine residues which may accommodate a mononuclear iron or iron-sulphur cluster (Bilder *et al.*, 2004; Cupp-Vickery *et al.*, 2004; Wada *et al.*, 2005). The results from this study suggest that the 'cysteine pocket' in IscA can also facilitate the copper binding, as mutation of the cysteine residues to serine abolishes both the copper and iron binding activities of IscA. While each IscA dimer can bind one ferric iron atom (Ding and Clark, 2004; Mapolelo *et al.*, 2012a), IscA appears to bind two Cu(I) atoms per IscA dimer (Fig. 3), suggesting that copper and iron may have different ligand binding co-ordination in IscA. Perhaps, unlike the iron binding in IscA which likely has five co-ordinates with two or three cysteinate ligands (Ding and Clark, 2004; Mapolelo *et al.*, 2012a), the copper binding may only need two cysteine residues to form linear biscysteinate co-ordination geometry as seen in the Parkinsonism-associated protein DJ-1 protein (Puno *et al.*, 2013). Although we are unable to estimate the copper binding affinity of IscA from the copper titration experiments (Fig. 3B), the results from the competition of iron and copper binding in IscA (Fig. 5) suggests that IscA has similar binding affinities for iron and copper. Evidently, elucidation of the copper binding ligands in IscA requires additional experimentations. Nevertheless, the observed competition of iron and copper binding for the metal binding sites in IscA represents a dynamic interplay between intracellular copper content and iron-sulphur cluster biogenesis in cells.

The salient finding of this study is that copper can effectively inhibit the [4Fe-4S] cluster assembly (Figs 5 and 6) without significant effect on the pre-assembled clusters in proteins in *E. coli* cells grown in LB media. In *E. coli*, iron-sulphur clusters are assembled by two major iron-sulphur cluster assembly machineries, the housekeeping *iscSUA-hscBA-fdx* (Zheng *et al.*, 1998; Roche *et al.*, 2013) and the redundant *sufABCDSE* (Takahashi and Tokumoto, 2002) which is induced under iron starvation (Outten *et al.*, 2004) or oxidative stress (Lee *et al.*, 2004). Since increased expression of the second iron-

sulphur cluster assembly gene cluster *sufABCDSE* is an indication of the iron-sulphur cluster assembly deficiency in *E. coli* cells (Jang and Imlay, 2010), we analysed the expression of the *sufA::lacZ* operon in the wild-type *E. coli* cells (Jang and Imlay, 2010) in response to $CuSO_4$ in LB media, and found that expression of the *sufA::lacZ* operon was indeed induced by $CuSO_4$ (Supplementary Fig. S2), suggesting that copper has a general inhibitory effect on iron-sulphur cluster biogenesis. This is in agreement with the recent observation that inactivation of the copper efflux pump CusCFBA stimulates expression of the *sufA::lacZ* operon in *E. coli* cells (Fung *et al*., 2013). The apparent contradiction with the previous report that copper disrupts the labile [4Fe-4S] clusters in dehydratases (Macomber and Imlay, 2009) is likely due to the different growth media used in the experiments. While the wild-type *E. coli* cells are highly sensitive to copper in M9 minimal media (32 μM $CuSO_4$ completely inhibits the cell growth) (Macomber and Imlay, 2009), the wild-type *E. coli* cells are highly resistant to copper in LB media [cell growth is decreased by only about 20% when LB media are supplemented with 2 mM $CuSO_4$ (Supplementary Fig. S2)]. One possible consideration is that copper in M9 minimal media and LB media may interfere the iron transportation in *E. coli* cells differently. It is also possible that iron deficiency in M9 minimal media (Wang *et al*., 2010) may have already limited iron-sulphur cluster biogenesis in *E. coli* cells in such that the effect of copper on iron-sulphur cluster biogenesis could not be observed.

Among the iron-sulphur cluster assembly proteins encoded by *iscSUA-hscBA-fdx* (Zheng *et al*., 1998), only IscA has a specific and strong copper binding activity (Fig. 1). The inverse correlation between the copper binding in IscA and the cell growth of the *E. coli copA/cueO/cusA* mutant cells in LB media supplemented with increased concentration of $CuSO_4$ (Fig. 2C) indicates that the copper binding in IscA may directly contribute to the cell growth inhibition. More importantly, the observation that excess copper in LB media selectively inhibits the [4Fe-4S] cluster assembly without affecting the [2Fe-2S] cluster assembly in *E. coli* cells (Fig. 7C and D) suggests that copper in LB media may emulate the phenotype of an *E. coli* mutant with deletion of IscA/SufA (Tan *et al*., 2009). Although SufA can also bind copper (Supplementary Fig. S1), the gene encoding SufA is induced only under iron starvation (Outten *et al*., 2004) or oxidative stress (Lee *et al*., 2004). Thus, IscA could be the major target of copper-toxicity in *E. coli* cells under normal growth conditions. Indeed, we find that deletion of IscA largely inhibits the [4Fe-4S] cluster assembly in endonuclease III expressed in *E. coli* cells. Furthermore, while copper in LB media effectively inhibits the [4Fe-4S] cluster assembly in endonuclease III in the *E. coli* cells, copper has very little or no additional inhibitory effect on the [4Fe-4S] cluster assembly in the IscA-deleted cells (Fig. 8), supporting the notion that IscA is the primary target of copper toxicity. Interestingly, copper has also been shown to inhibit iron-sulphur cluster assembly in *B. subtilis* by binding to a *Bacillus*-specific iron-sulphur cluster assembly protein SufU (Chillappagari *et al*., 2010). We noticed the small amount of copper binding in IscU (Fig. 2A) which could also contribute to the copper-mediated inhibition of iron-sulphur cluster assembly in *E. coli* cells. However, considering the relatively weak binding of copper in IscU, it is plausible that the copper-bound IscA may accidently transfer the copper to IscU in cells. The question on how the copper binding in IscA inhibits iron-sulphur cluster biogenesis can only be speculated. One explanation is that excess copper may compete with iron for the metal binding site in IscA, thus preventing the turnover of IscA for iron-sulphur cluster assembly. Alternatively, the copper-bound IscA may poison the iron-sulphur cluster assembly machinery and inhibit iron-sulphur cluster biogenesis in cells. Additional experiments are required to illustrate the mechanism underlying copper-mediated inhibition of iron-sulphur cluster assembly in cells.

Experimental procedures

Gene knockout in E. coli *cells*

Genes encoding three major proteins regulating intracellular copper homeostasis: CopA, CueO and CusA, were deleted following the procedures described in Datsenko and Wanner (2000). Gene encoding IscA was also deleted from the wild-type and the *copA/cueO/cusA* mutant cells. The gene deletion was confirmed by PCR (Vazyme Biotech). All primers for the gene deletion and confirmation were synthesized by Takara co. (Dalian, China). The constructed *E. coli copA/cueO/cusA* mutant cells grow normally in LB media and are hypersensitive to copper in the media, as reported previously (Grass and Rensing, 2001; Macomber and Imlay, 2009).

Enzyme activity assay for dihydroxyacid dehydratase (IlvD)

The cell extracts were prepared by passing the cells containing recombinant IlvD through French press once, followed by centrifugation at 34 000 *g* for 45 min. The specific enzyme activity was measured using substrate DL-2,3-dihydroxy-isovalerate which was synthesized according to the method of Cioffi *et al*. (1980). In the enzyme assay, 10 μl of the cell extracts (~ 5.0 mg total protein ml^{-1}) prepared from the *E. coli* cells containing recombinant IlvD were added to 390 μl pre-incubated solutions containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 10 mM D,L-2,3-dihydroxy-isovalerate (Flint *et al*., 1993). The reaction product (keto acids) was monitored at 240 nm using an extinction coefficient of 0.19 cm^{-1} mM^{-1} (Flint *et al*., 1993). The amount of recombinant IlvD in cell extracts was quantified by Western blotting using the antibody against His-tag (abcam co.) For the reconstitution of iron-sulphur

clusters in recombinant IlvD, cell extracts were incubated with cysteine desulphurase (IscS) (1 μM), $Fe(NH_4)_2(SO_4)_2$ (100 μM), L-cysteine (1 mM), and dithiothreitol (2 mM) at 37°C under anaerobic conditions for 30 min.

Enzyme activity assay for the NADH dehydrogenase I

NADH dehydrogenase I activity of *E. coli* cells was measured following the procedures described in Xu and Imlay (2012). Briefly, the *E. coli copA/cueO/cusA* mutant cells were grown in LB media at 37°C with aeration for 3 h before being treated with or without $CuSO_4$ (200 μM). Aliquots of the cells were taken at 0, 1, and 2 h after addition of $CuSO_4$, washed once with buffer containing Tris (20 mM, pH 8.0) and NaCl (200 mM), and re-suspended to OD of 2.5 at 600 nm. Inverted membrane vesicles were prepared by passing the cells through French press once. Inverted membrane vesicles (10 μl) was added to the reaction solution (290 μl) containing Tris (20 mM, pH 8.0), NaCl (200 mM) and deamino-NADH (50 μM). Damino-NADH was chosen as NADH dehydrogenase II does not act on this NADH analogue (Matsushita *et al.*, 1987). The NADH dehydrogenase I activity was determined by measuring oxidation of deamino-NADH at 340 nm at room temperature.

Protein purification

Genes encoding the housekeeping iron-sulphur cluster assembly proteins IscS, IscU, IscA, HscB, HscA, and ferredoxin from *E. coli* were amplified using PCR, and cloned to an expression plasmid pBAD as described previously (Yang *et al.*, 2006). The IscA mutant was constructed by the site-directed mutagenesis QuikChange kit (Agilent co.). The cloned genes and mutants were confirmed by direct sequencing. The plasmids expressing *E. coli* dihydroxyacid dehydratase (IlvD), endonuclease III, and FhuF were previously prepared (Tan *et al.*, 2009). Each plasmid was introduced into the *E. coli copA/cueO/cusA* mutant cells. Cells containing the protein expression plasmid were grown to OD of 0.6 at 600 nm. $CuSO_4$ was then added to cell cultures 10 min before recombinant protein was induced with 0.02% arabinose. After 3 h of induction at 37°C with aeration, cells were harvested and washed twice with protein purification buffer [NaCl (500 mM), Tris (20 mM, pH 8.0)]. Proteins were purified to a single band on the SDS-PAGE gel as described in Yang *et al.* (2006). The concentration of purified protein was determined from the absorption peak at 280 nm using the published extinction coefficients.

Copper and iron content analyses

The iron content of protein samples was determined using ferroZine (Cowart *et al.*, 1993) as described previously (Ding *et al.*, 2005b). The copper content of protein samples was determined following the procedure described in Tutem *et al.* (1991) with slight modification. Briefly, protein samples (160 μl) were incubated with 10 μl neocuproine [5 mM in H_2O:ethanol (1:1)], 20 μl SDS (20%) and 10 μl sodium ascorbate (5 mM). Mixtures were incubated at room temperature for 20 min, followed by centrifugation. The amplitude of the absorption peak at 450 nm of supernatants was used for quantification of copper content in each protein sample. Freshly prepared $CuSO_4$ (10 μM) was used as a standard. The metal contents in proteins were also analysed by the Inductively Coupled Plasma-Emission Spectrometry (ICP-ES). Both metal content analyses produced similar results.

EPR measurements

The EPR spectra were recorded at X-band on a Bruker ESP-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. The routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 30 K; receive gain, 1.0×10^5. A solution containing Cu(II)-EDTA (10 μM) was used as a standard for quantification of Cu(II) in the protein.

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