Characterization of iron binding in IscA, an ancient iron–sulphur cluster assembly protein

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Iron–sulphur clusters are one of the most common types of redox centre in biology. At least six proteins (IscS, IscU, IscA, HscB, HscA and ferredoxin) have been identified as being essential for the biogenesis of iron–sulphur proteins in bacteria. It has been shown that IscS is a cysteine desulphurase that provides sulphur for iron–sulphur clusters, and that IscU is a scaffold for the IscS-mediated assembly of iron–sulphur clusters. The iron donor for iron–sulphur clusters, however, remains elusive. Here we show that IscA is an iron binding protein with an apparent

iron association constant of $3.0 \times 10^{19} \text{ M}^{-1}$, and that iron-loaded IscA can provide iron for the assembly of transient iron–sulphur clusters in IscU in the presence of IscS and L-cysteine *in vitro*. The results suggest that IscA is capable of recruiting intracellular iron and delivering iron for iron–sulphur clusters in proteins.

Key words: cysteine desulphurase, iron donor, IscA, IscU, iron–sulphur protein, SufA.

INTRODUCTION

Iron–sulphur clusters are considered to be one of the cofactors that arose first in evolution [1,2]. They exist in the form of rhombic [2Fe–2S] clusters, cuboidal [3Fe–4S] clusters, cubane-type [4Fe–4S] clusters and other more complicated clusters. Throughout evolution, iron–sulphur clusters have become integral parts of diverse biological processes, including energy conversion, nitrogen fixation, haem and biotin biosynthesis, iron homeostasis, DNA synthesis and repair, and regulation of gene expression [3–5]. Although iron–sulphur clusters can be assembled in proteins *in vitro* with ferrous iron and sulphide, it is now clear that the formation of iron–sulphur clusters is not spontaneous *in vivo*. The pioneering work by Dean and colleagues revealed that at least two proteins, NifS and NifU, are important for iron–sulphur cluster assembly in nitrogenase in *Azotobacter vinelandii* [6–8]. Additional studies led to the discovery of a highly conserved gene cluster, *iscSUA-hscBA-fdx*, that is essential for the general biogenesis of iron–sulphur proteins in bacteria [9–12]. Homologues of the proteins encoded by the gene cluster *iscSUA-hscBA-fdx* have also been identified in eukaryotic organisms [13–16], suggesting that the mechanism for the biogenesis of iron–sulphur proteins is highly conserved.

The bacterial gene cluster *iscSUA-hscBA-fdx* encodes six proteins: IscS, IscU, IscA, HscB, HscA and ferredoxin. IscS, a homologue of NifS [7], is a homodimer, with each monomer containing a pyridoxal 5-phosphate [17–19]. The enzyme catalyses the removal of sulphur from L-cysteine and generates sulphane sulphur via formation of a protein-bound cysteine persulphide intermediate on a conserved cysteine residue (Cys-328) [20,21]. Deletion of IscS in *Escherichia coli* dramatically decreases the specific activities of proteins containing iron–sulphur clusters [22]. IscU is a truncated version of NifU, containing the N-terminal domain of NifU [8]. Both IscU and NifU are capable of hosting transient iron–sulphur clusters, and are likely to provide a scaffold for IscS-mediated iron–sulphur cluster assembly [23–27]. The transient iron–sulphur clusters in IscU are subsequently transferred to target proteins [26,27].

The function of IscA in the biogenesis of iron–sulphur proteins remains controversial. Genetic studies in *Saccharomyces cerevisiae* suggested that IscA could be the iron donor for iron–sulphur clusters, as deletion of IscA homologues resulted in the accumulation of iron in mitochondria and a deficiency of iron–sulphur proteins [28–30]. On the other hand, biochemical studies indicated that IscA might function as an alternative scaffold for iron–sulphur cluster assembly, as IscA, like IscU, can host a transient [2Fe–2S] cluster [31–34]. While IscA and IscU both contain three invariant cysteine residues that are believed to be important for their function, the arrangements of these cysteines in the proteins are very different. In IscA, the three cysteine residues are positioned as X₃₄CX₆₃CGCX₆, while in IscU they are distributed almost evenly throughout the polypeptide sequence (X₃₆CX₂₅CX₄₂CX₂₂). The recent X-ray crystal structure of *E. coli* IscA [35] and the NMR structure of the IscU homologue from *Thermotoga maritima* [36] further revealed that IscA and IscU are structurally very different proteins. NMR data for the IscU homologue [36] suggest that IscU is fluxional among widely different conformational arrangements, with two sub-structures: a three-stranded anti-parallel β -sheet and a small α -helical domain. Neither of these structural motifs appears to correspond directly to structural elements of the IscA protein [35]. Instead, IscA protein assembly in the crystal structure is remarkably stable, with a compact globular domain and an apparently mobile C-terminal tail which includes two conserved cysteine residues (Cys-99 and Cys-101). The crystal structure also suggests that IscA may exist as a tetramer, and that the IscA monomers are arranged as a dimer of dimers about a central channel, with the conserved Cys-35 located in this channel [35]. Nevertheless, the current crystal structure of IscA appears to be the apo-form, which does not provide information on whether IscA binds iron or transient [2Fe–2S] clusters [35].

In the present study, we show that IscA is an iron binding protein with an apparent iron association constant of $3.0 \times 10^{19} \text{ M}^{-1}$, and that iron-loaded IscA can provide iron for the assembly of transient iron–sulphur clusters in IscU in the presence of IscS and

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L-cysteine. These results suggest that IscA may act as an iron donor for iron–sulphur clusters in *E. coli*.

EXPERIMENTAL

Gene cloning and protein preparation

The coding regions of *E. coli* IscA and IscU were amplified from wild-type *E. coli* genomic DNA by PCR using PCR Ready-to-go beads (Amersham Biosciences). Two pairs of primers were designed to contain an *Nco*I restriction site in one primer and a *Hind*III site in the other. The sequences of the primers are as follows: IscA-1, 5'-AGGTTTGGCCATGGCGATTACACT-3'; IscA-2, 5'-TGCGCATCAAAGCTTGAAGCTTTC-3'; IscU-1, 5'-GAATTTACCATGGCTTACAGCGAA-3'; IscU-2, 5'-TTTGCTTCAAGCTTGCTTTTATAG-3'. The *Nco*I/*Hind*III-digested PCR products were ligated to an expression vector pET28b+ to yield pTISCA (for IscA) and pTISCU (for IscU). The plasmids were introduced into *E. coli* strain BL21(DE3) (Novagene), and the C-terminally His-tagged IscA and IscU were overproduced in the *E. coli* cells and purified using a nickel–agarose column (Qiagen) followed by a HiTrap desalting column (Amersham Biosciences), as described previously for IscS [37]. The purity of the proteins was > 95 %, as judged by electrophoresis analysis on a 15 % (w/v) polyacrylamide gel containing SDS followed by staining with Coomassie Blue.

Purified C-terminally His-tagged IscA was used previously for the X-ray crystal structure determination [35]. N-terminally His-tagged IscA was also constructed by using two additional primers designed to contain an *Nde*I restriction site in one primer and a *Bsp*I restriction site in the other. Their sequences are: IscA-N1, 5'-TGAGGTTTGCATATGTCGATTACA-3'; IscA-N2, 5'-AACCCACGCTCAGCCGACCACGG-3'. The N-terminally His-tagged IscA was expressed and purified as described for the C-terminally His-tagged IscA. The N-terminal His tag was subsequently removed by incubating purified IscA with 0.65 unit/ml thrombin overnight, followed by removal of thrombin according to the manufacturer's instructions (Pierce). Removal of the His tag from IscA was verified by the failure of the protein to bind Ni–agarose and by SDS/PAGE analysis.

Determination of protein concentration

The deduced *E. coli* IscU sequence has one tryptophan, four tyrosine and two phenylalanine residues. The concentration of IscU was calculated based on a molar absorption coefficient at 280 nm of $11.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, using average absorption values for tryptophan and tyrosine of 5.6 and $1.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ respectively [38]. *E. coli* IscA has no tryptophan, one tyrosine and nine phenylalanine residues. As expected, iron-depleted IscA (apo-IscA) has a major absorption peak around 260 nm. The concentration of apo-IscA was calculated based on a molar absorption coefficient at 260 nm of $2.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, using average absorption values for tyrosine and phenylalanine at 260 nm of 0.40 and $0.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ respectively [38].

Iron binding analysis in IscA

Iron-depleted IscA (apo-IscA) was prepared by incubating IscA (200 μM) with 10 mM EDTA and 2 mM dithiothreitol at 37 °C for 60 min, followed by passing the samples through a HiTrap desalting column (5 ml; Amersham Biosciences) to remove EDTA and the released iron. The HiTrap desalting column was attached to an FPLC system controlled by UNICORN software (Amersham Biosciences) that allows reproducible elution profiles. For the

iron binding experiments, apo-IscA (50 μM) was incubated with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0–100 μM) in the presence of 2 mM dithiothreitol in open-to-air microtubes at room temperature for 5 min. IscA samples were then repurified by passing them through a HiTrap desalting column. The apparent iron association constant of IscA was determined using sodium citrate as a competing iron chelator [39]. Iron-loaded IscA (100 μM) was incubated with sodium citrate (0–200 mM) in the presence of 2 mM dithiothreitol in open-to-air microtubes at room temperature for 30 min before IscA was repurified using a HiTrap desalting column. The amplitudes of the absorption peaks at 315 nm of the protein samples were used for determining relative iron binding in IscA. The amounts of iron and sulphide in protein samples were analysed according to Fischer's method [40] and Siegel's method [41] respectively.

EPR measurements

Iron-loaded IscA was prepared by mixing purified IscA with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in a 1:2 molar ratio in the presence of dithiothreitol, followed by passing the sample through a HiTrap desalting column twice. For the reduced samples, freshly prepared sodium dithionite was added (at a final concentration of 4 mM) before the samples were transferred to EPR tubes and frozen immediately in liquid nitrogen. EPR spectra were recorded at X-band on a Bruker ESP-300 spectrometer using an Oxford Instruments ESR-9 flow cryostat (Chemistry Department, Louisiana State University). The EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 4.5 K; receive gain, 1.0×10^5 .

Iron–sulphur cluster assembly in IscU

Purified IscU (200 μM) was mixed with 1 μM IscS in buffer containing 2 mM dithiothreitol, 500 mM NaCl and 20 mM Tris (pH 8.0). Either $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ or iron-loaded IscA was added as iron source for the assembly of transient iron–sulphur clusters in IscU. The reaction mixture was purged with pure argon three times, and then incubated under a pure argon atmosphere at 37 °C for an additional 5 min before L-cysteine (at a final concentration of 1 mM) was added to initiate the iron–sulphur cluster assembly reaction. The amount of transient iron–sulphur clusters assembled in IscU was estimated from the amplitude change of the absorption peak at 456 nm using a molar absorption coefficient of $10.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [23].

RESULTS

Purified *E. coli* IscA contains iron

Although both IscA and IscU have been considered to be scaffolds for the iron–sulphur cluster assembly [31–34], recent studies indicate that IscA [35] and IscU [36] are structurally different proteins. To explore the function of IscA in the biogenesis of iron–sulphur proteins, we purified recombinant IscA and IscU from *E. coli* using the same protein expression system and purification protocol, as described in the Experimental section. Figure 1(A) shows that purified IscU does not have any absorption features that would indicate the binding of iron or iron–sulphur clusters in the protein, consistent with the previous reports [23–27]. Unexpectedly, purified IscA has a reddish colour, with a clear absorption peak at 315 nm and a broad absorption peak around 480 nm, indicative of iron binding in the protein [42]. Analysis of total iron and sulphide contents in purified IscA samples showed

Figure 1 UV-visible absorption spectra of purified *E. coli* IscA and IscU

(A) UV-visible absorption spectra of recombinant IscA and IscU. Purified IscA (200 μ M) or IscU (50 μ M) was dissolved in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl. (B) Effects of iron content in the *E. coli* culture on the UV-visible absorption spectrum of IscA. IscA was purified from *E. coli* cultures treated with 200 μ M $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (trace a), no addition (trace b) or 2 mM α, α' -dipyridyl (trace c) before protein expression was induced. OD, absorbance.

Figure 2 Iron binding in apo-IscA

(A) UV-visible absorption spectra of apo-IscA after reconstitution with iron. Apo-IscA (50 μ M) was incubated with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (0–100 μ M) in the presence of dithiothreitol (2 mM) in open-to-air microtubes at room temperature for 5 min. Protein was repurified by passing through a HiTrap desalting column. Spectra were calibrated to an absorbance (OD) at 260 nm of 1.0. (B) Iron binding curve of IscA. The amplitudes of the absorption peak at 315 nm of apo-IscA were obtained from the spectra in (A), and plotted as a function of the ferrous iron concentration in the incubation solution. Similar results were obtained from three independent experiments.

that there was 20.5 ± 2.1 μ M iron and 1.0 ± 0.5 μ M sulphide in 200 μ M IscA, further suggesting that purified IscA binds iron, not iron–sulphur clusters.

The iron centre in IscA is remarkably stable and resistant to oxygen, as incubation of purified IscA at 37 °C for 30 min aerobically had little effect on the absorption peak at 315 nm (results not shown). Nevertheless, the amplitude of the absorption peak at 315 nm of IscA could be modulated by adding either iron or the membrane-permeable iron chelator α, α' -dipyridyl to the *E. coli* cultures before IscA expression was induced. Figure 1(B) shows that addition of exogenous iron to the *E. coli* culture significantly increased the amplitude of the absorption peak at 315 nm of purified IscA (trace a), while addition of the iron chelator α, α' -dipyridyl completely removed the absorption peak at 315 nm (trace c). These results suggest that the iron centre in IscA is in equilibrium with the intracellular iron content.

IscA used in the above experiments contained a C-terminal His tag. It is possible that the His tag may contribute to the iron binding of IscA. Consequently, we constructed an expression system that exploits a removable N-terminal His tag. The N-terminally His-tagged IscA was purified, and the His tag was subsequently removed with thrombin as described in the Experimental section.

UV-visible absorption measurements showed that the peak at 315 nm of IscA was unchanged after the His tag was removed from the protein (results not shown), indicating that the His tag has no effect on iron binding in IscA. To avoid any potential contribution from the His tag, IscA lacking a tag was utilized for the subsequent experiments.

IscA is an iron binding protein

To determine if IscA is an iron binding protein, we prepared apo-IscA as described in the Experimental section. Analysis of the prepared apo-IscA showed that the protein had little absorption at 315 nm and contained an undetectable amount of iron. Apo-IscA was then incubated with increasing concentrations of ferrous iron in the presence of dithiothreitol in open-to-air microtubes at room temperature for 5 min, followed by passing the samples through a desalting column. Figure 2(A) shows that incubation of apo-IscA with ferrous iron restored the absorption peak at 315 nm of the protein. As the iron concentration was increased, the amplitude of the absorption peak at 315 nm of IscA increased almost linearly until the molar ratio of iron to IscA was approx. 0.4 (Figure 2B). Analysis of the iron content in the IscA samples showed that

Figure 3 Determination of the apparent iron association constant of IscA

(A) Effect of sodium citrate on iron binding in IscA. Iron-loaded IscA (100 μ M) was incubated with increasing concentrations of sodium citrate (0–200 mM) in buffer containing 500 mM NaCl and 20 mM Tris (pH 8.0) in open-to-air microtubes at room temperature for 30 min. Spectra were calibrated to an absorbance (OD) at 260 nm of 1.0. (B) Competition for IscA-bound iron by sodium citrate. The amplitudes of the absorption peak at 315 nm of IscA were obtained from the spectra in (A), and plotted as a function of the citrate concentration in the incubation solution. Similar results were obtained from at least three independent experiments.

the total amount of iron in IscA was closely proportional to the amplitude of the absorption peak at 315 nm observed with the samples. The maximum iron content in iron-loaded IscA samples was approx. 0.45 ± 0.10 mol of iron per mol of IscA, indicative of a stoichiometry of one iron per two IscA. In the same iron-loaded IscA sample, less than 0.01 mol of labile sulphide per mol of IscA was detected, showing that iron–sulphur clusters were not assembled in IscA after incubation with ferrous iron and dithiothreitol.

The iron content (Fe/IscA $\sim 0.45:1.0$) and the relative amplitude of the absorption peak at 315 nm of iron-loaded IscA (Figure 2A) were much greater than those observed with purified IscA (Fe/IscA $\sim 0.1:1.0$) (Figure 1A), suggesting that purified IscA is not fully saturated with iron. It is possible that the iron centre in IscA may be lost during purification processes. Alternatively, since the intracellular concentration of ‘loosely’ bound iron in *E. coli* cells is approx. 10 μ M [43] and recombinant IscA is artificially overproduced, the intracellular iron content may not be sufficient for all recombinant IscA proteins to bind iron inside cells.

Determination of the apparent iron association constant of IscA

To examine further iron binding by IscA, we determined the iron association constant of IscA by using sodium citrate as a competing iron chelator [39]. We chose sodium citrate because its iron association constant is close to those of other iron binding proteins [39]. Figure 3(A) shows that the absorption peak at 315 nm of the iron-loaded IscA decreased progressively as the concentration of sodium citrate in the incubation solution was increased. The concentration of sodium citrate required to remove 50% of the amplitude of the absorption peak at 315 nm of 100 μ M IscA was approx. 15 mM (Figure 3B). Using the iron association constant of sodium citrate (1.0×10^{17} M $^{-1}$ [39]), we estimated that the apparent iron association constant of IscA is approx. 3.0×10^{19} M $^{-1}$, which is slightly less than that of human transferrin (4.7×10^{20} M $^{-1}$) [44].

EPR measurements of the iron centre in IscA

The iron centre in IscA was also analysed using EPR. Figure 4(A) shows that iron-loaded IscA has a broad EPR signal around the

Figure 4 EPR spectra of IscA

(A) Iron-loaded IscA (250 μ M) was dissolved in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl. (B) Sample as in (A), except that freshly prepared sodium dithionite (4 mM) was added. (C) Apo-IscA (260 μ M). The spectra shown are the net spectra obtained by subtracting the baseline (containing buffer only). Three sets of independently prepared samples were measured by EPR, and similar spectra were obtained.

$g = 4$ –6 region, a typical signature of $S = 3/2$ spin. The broad EPR signal indicates substantial heterogeneity, which prevents the reliable assessment of spin Hamiltonian parameters. Purified IscA has the same EPR signal at $g = 4$ –6, but with a smaller amplitude, probably because the protein is not fully saturated with iron (results not shown). The lack of an EPR signal at $g = 4.3$ in the spectrum indicates that there is very little non-specifically-bound ‘junk’ iron in the protein sample. The EPR signal of the iron-loaded IscA differs significantly from that of the $S = 5/2$ spin iron centre observed in desulphoredoxin and rubredoxin [45], but is close to that of the $S = 3/2$ spin iron centre found in the nitrogenase Fe-protein from *Azotobacter vinelandii* [46]. As expected, no EPR signal was observed in the sample containing apo-IscA (Figure 4C). Remarkably, the broad $S = 3/2$ EPR signal of the iron-loaded IscA disappeared when sodium dithionite was added to

Figure 5 IscA-mediated assembly of transient iron–sulphur clusters in IscU

Purified IscU (200 μM) was incubated with IscS (1 μM) and dithiothreitol (2 mM) in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl anaerobically at 37 °C with no addition (**A**), 100 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (**B**) or 200 μM iron-loaded IscA (**C**). Iron-loaded IscA was prepared by passing it through a HiTrap desalting column twice after reconstitution with a 2-fold excess of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. Spectra were obtained every 2 min for 20 min after addition of L-cysteine (1 mM) to initiate the iron–sulphur cluster assembly reaction. The experiments were repeated at least three times, and similar results were observed.

the sample (Figure 4B), indicating that IscA contains a reducible (ferric) iron centre. Since ferrous iron was used for the reconstitution of IscA, it seems that ferrous iron is somehow oxidized when it binds to apo-IscA.

Iron-loaded IscA provides iron for the assembly of transient iron–sulphur clusters in IscU

The finding that IscA is an iron binding protein prompted us to speculate that IscA may provide iron for the assembly of transient iron–sulphur clusters in IscU, a scaffold for iron–sulphur cluster assembly [23–27].

When IscU (200 μM) was incubated with L-cysteine (1 mM), cysteine desulphurase (IscS; 1 μM) and dithiothreitol (2 mM) at 37 °C for 20 min, there was no significant change in the UV–visible absorption spectrum from 300 to 700 nm (Figure 5A). If ferrous iron (100 μM) was included in the reaction solution, an absorption peak at 456 nm, which has been attributed to transient iron–sulphur clusters in IscU [23], appeared (Figure 5B). Based on a molar absorption coefficient of 10.5 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ at 456 nm for transient iron–sulphur clusters in IscU [23], we calculated that approx. 20 μM iron–sulphur clusters were assembled in the reaction solution. In the control, no absorption peak at 456 nm was observed in the absence of IscS or L-cysteine in the reaction

solution (results not shown), consistent with the notion that the assembly of transient iron–sulphur clusters in IscU is mediated by IscS and L-cysteine [23].

When iron-loaded IscA was added instead of ferrous iron to the reaction solution, the same absorption peak at 456 nm, indicating transient iron–sulphur clusters of IscU, was apparent (Figure 5C). In the absence of IscU there was no absorption peak at 456 nm, indicating that transient iron–sulphur clusters are assembled in IscU, but not in IscA. Both L-cysteine and IscS were also required for the assembly of transient iron–sulphur clusters in IscU, as samples lacking L-cysteine or IscS failed to produce the typical absorption peak at 456 nm (results not shown). Considering that iron-loaded IscA contains approx. 0.45 mol of iron per mol of IscA, IscA-mediated iron delivery to iron–sulphur clusters in IscU seems to be more effective than the acquisition of free iron from solution (Figure 5B and 5C).

Figure 6 shows the kinetics of IscA-mediated iron–sulphur cluster assembly in IscU. The final yield of transient iron–sulphur clusters assembled in IscU, as judged from the change in the absorption amplitude at 456 nm, increased as the amount of the iron-loaded IscA added into the incubation solution increased. This result suggests that iron-loaded IscA could be a limiting factor for the assembly of transient iron–sulphur clusters in IscU under these experimental conditions.

Figure 6 Kinetics of the IscA-mediated assembly of transient iron-sulphur clusters in IscU

The IscA-mediated assembly of transient iron-sulphur clusters in IscU was performed as described in the legend to Figure 5. The concentrations of iron-loaded IscA used in the assembly reaction solution are indicated. The amplitudes of the absorption peak at 456 nm were used to monitor the formation of transient iron-sulphur clusters in IscU, and are plotted as a function of the time after addition of L-cysteine (1 mM). The data are means from three independent experiments.

DISCUSSION

It has been shown previously that sulphur in iron-sulphur clusters is provided by cysteine desulphurase (IscS) via removal of sulphur from L-cysteine [17–19]. However, the iron donor for iron-sulphur clusters is essentially unknown. The search for the iron donor for the assembly of iron-sulphur clusters has attracted much attention. Recently, it has been reported that human frataxin may act as the iron donor for iron-sulphur cluster assembly in ISU, a human IscU homologue [47]. However, frataxin homologues are not essential for iron-sulphur cluster assembly in *Saccharomyces cerevisiae* [48,49] or in *E. coli* [50], suggesting that the specific function of frataxin in the biogenesis of iron-sulphur proteins remains to be elucidated. Here we show that IscA, a highly conserved protein in organisms from bacteria to humans, binds iron with an apparent association constant of $3.0 \times 10^{19} \text{ M}^{-1}$, and that iron-loaded IscA can provide iron for the assembly of transient iron-sulphur clusters in IscU in the presence of IscS and L-cysteine *in vitro*. In light of these findings, we propose that IscA is capable of recruiting intracellular iron and delivering iron for iron-sulphur clusters. This notion is consistent with genetic studies in *Saccharomyces cerevisiae* which show that deletion of IscA homologues results in the accumulation of iron in mitochondria and in a deficiency of iron-sulphur proteins [28–30]. In *E. coli*, the *iscA* gene is also important for iron-sulphur cluster assembly [10], although the effect of inactivation of the *iscA* gene is not as dramatic as that of some other *isc* genes [11]. One possible explanation could be that there are two additional homologues of IscA in the *E. coli* genome: SufA [51] and a hypothetical protein YadR. SufA is a member of the gene cluster *sufABCDSE* that has been assigned as a redundant activity for the biogenesis of iron-sulphur proteins in bacteria [51]. The sequence alignments of IscA, SufA and YadR from *E. coli* are shown in Figure 7(A). To verify if SufA has a similar function to IscA, we have cloned and purified SufA from *E. coli* and found that SufA and IscA have essentially the same activities of iron binding and iron delivery for the assembly of transient iron-sulphur clusters in IscU (H. Ding and R. J. Clark, unpublished work).

Figure 7 IscA and its homologues in *E. coli*

(A) Alignment of the amino acid sequences of IscA, SufA and YadR of *E. coli*. Identical residues are highlighted. Three invariant cysteine residues are indicated by the star symbols above the sequence. (B) Space-filled rendering of the possible IscA tetramer, with the chains in blue, green, red and cyan. The conserved Cys-35 is shown in yellow. The last structured amino acid (Asp-97) of IscA is depicted in grey.

The finding that IscA is an iron binding protein is in contrast with previous biochemical studies showing that purified *E. coli* IscA [31] or homologues from other organisms [32–34] did not contain iron. It is most likely that different protein purification procedures may have led to the different results. Specifically, the inclusion by others of the metal chelator EDTA [31,33] or β -mercaptoethanol [32] in purification solutions might be the source of the discrepancy. β -Mercaptoethanol has been shown previously to destabilize $[2\text{Fe}-2\text{S}]$ clusters in the redox transcription factor SoxR [52]. We found that incubation of IscA with β -mercaptoethanol releases iron from the protein, indicating that β -mercaptoethanol can also destabilize the iron centre in IscA. It is worth pointing out that an IscA homologue (SLR1417) from the cyanobacterium *Synechocystis* PPC 6803 had a clear absorption peak at 315 nm even after the protein was dialysed for 24 h against 8 M urea and 5 mM dithiothreitol [33]. In light of the present study, we propose that the absorption peak at 315 nm of the SLR1417 protein reflects the firmly bound iron centre in this IscA homologue.

The recent X-ray crystallographic study of IscA revealed that IscA may exist in a tetrameric form [35], and that the three conserved cysteine residues from each IscA monomer are predicted to form a 'cysteine pocket' in a channel formed by association of the monomers (Figure 7B). The putative cysteine pocket may facilitate iron binding in IscA. On the other hand, the cysteine pocket may also be used for hosting transient $[2\text{Fe}-2\text{S}]$ clusters, as reported previously by others [31–34]. These two possibilities may not be mutually exclusive. Nevertheless, given that IscA has a high iron binding affinity and that it can provide iron for the assembly of transient iron-sulphur clusters in IscU, we postulate that the primary function of IscA is to recruit intracellular iron and deliver iron for the assembly of iron-sulphur clusters in proteins.

The stoichiometry of iron binding ($\text{Fe}/\text{IscA} \sim 0.45:1.0$) in iron-loaded IscA (Figure 2A) suggests that two IscA monomers form

one iron binding site. This would be consistent with either a dimeric or a tetrameric arrangement of IscA monomers [35]. The EPR measurements of iron-loaded IscA indicate that the iron centre in IscA is in an oxidized state and can be reduced by dithionite (Figure 4). If IscA is a tetramer, the two iron binding sites in the protein may be far apart and not magnetically coupled. Alternatively, the two iron atoms in a tetrameric IscA may be in different redox states. At present, these possibilities cannot be resolved, and additional characterization is required in order to elucidate the structure and redox properties of the iron centre in IscA. From the current X-ray structure model, the C-terminal 10 amino acid residues, which include two conserved cysteine residues (Cys-99 and Cys-101), are not visible in the electron density map, and therefore the structural details of the region cannot be defined (Figure 7B). It could be that the iron binding residues and any bound iron in IscA are flexible and are therefore 'silent' in the crystal structure. This notion is consistent with the unresolved broad EPR signal at $g = 4$ –6 observed in iron-loaded IscA (Figure 4A), which indicates structural heterogeneity of the iron centre in IscA. We envision that a flexible structure of the iron binding site may be imperative for IscA to recruit iron and deliver it for the assembly of transient iron–sulphur clusters in IscU.

If IscS provides sulphide and IscA delivers iron for the assembly of transient iron–sulphur clusters in IscU, IscA must act in concert with IscS and IscU. Co-ordination of IscA, IscS and IscU activities may be inferred from the fact that they are transcribed from the same *iscSUA* operon in *E. coli*. Expression of the *iscSUA* operon is repressed by a repressor, IscR, which contains a [2Fe–2S] cluster [53]. When the [2Fe–2S] cluster is removed from IscR, transcription of the *iscSUA* operon is switched on and cellular activity for assembly of iron–sulphur clusters is increased [53]. It may be speculated that co-ordination of IscA, IscS and IscU activities is achieved through protein–protein interactions to facilitate both sulphide transfer from IscS to IscU and iron transfer from IscA to IscU. While the IscU–IscS protein complex has been characterized [18–20,24], the interactions of IscA with IscS and IscU are yet to be investigated.

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