THE ROLE OF MOLECULAR CHAPERONES IN MEDIATING CLUSTER TRANSFER FROM [2Fe-2S] CLUSTER-BOUND ISCU

by

PRIYANKA SHAKAMURI

(Under the Direction of Michael K. Johnson)

ABSTRACT

In bacteria, the ISC (Iron Sulfur Cluster) system assembles Fe-S clusters. In the ISC system, IscU acts as a scaffold protein and molecular chaperones HscA and HscB specifically interact with IscU and are proposed to facilitate ATP-driven cluster transfer. In this work, cluster transfer from [2Fe-2S]-IscU to apo-Grx5 with and without chaperones was monitored by CD spectroscopy. Grx5 is a monothiol glutaredoxin and proposed cluster carrier/storage protein. Our results indicate a ~700 fold enhancement in the rate of [2Fe-2S] cluster transfer in the presence of chaperones, yielding a rate constant of 20000 M^{-1} min^{-1} and >90% completion within 3 min. Thus, HscA and HscB are required for efficient ATP-dependent cluster transfer from [2Fe-2S]-IscU. This illustrates the severe limitations of interpreting in vitro cluster transfer studies involving [2Fe-2S]-IscU in the absence of the dedicated HscA/HscB co-chaperone system. Results also support the proposed role of monothiol glutaredoxins as [2Fe-2S] cluster carriers/storage proteins.

INDEX WORDS: Molecular chaperones, HscA, HscB, IscU, Monothiol glutaredoxin, Grx, Fe-S cluster biosynthesis, Cluster transfer, Cluster carrier
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Iron-sulfur proteins: Background

Iron-Sulfur (Fe-S) clusters constitute one of the most ancient, ubiquitous, and structurally and functionally diverse class of biological prosthetic groups (1;2). Initially Fe-S clusters were thought to be involved only in electron transport (3), but recent advances have shown that Fe-S clusters also function in gene regulation (4-10), regulation of enzyme activity (11), substrate binding and activation (12-15), disulfide reduction (16;17), iron and electron storage/buffering (18-20), repairing/locating damaged DNA (21;22) and as a sulfur donor (23;24). Development in biophysical techniques such as UV-visible absorption/Circular Dichroism (CD)/Magnetic Circular Dichroism (MCD), resonance Raman, Mössbauer, and Electron Paramagnetic Resonance (EPR) spectroscopies have led to major advances in the characterization and understanding of biological Fe-S clusters.

Diseases caused by defects in iron metabolism

Fe-S clusters are found in almost all organisms and Fe-S cluster biosynthesis proteins are conserved through all three domains of life. Thus, Fe-S cluster biosynthesis research involving prokaryotes and increased understanding of the mechanism of Fe-S cluster biosynthesis in bacteria provides insight into how these cofactors are made and repaired in higher organisms. Although Fe-S clusters can be assembled \textit{in vitro} in some simple Fe-S proteins by incubating apoproteins with Fe$^{3+/2+}$ and S$^{2-}$ under anaerobic conditions in a process termed spontaneous self-assembly (25), this mechanism is not possible \textit{in vivo} due to cytotoxicity of sulfide and free
intracellular ferrous iron that can generate hydroxyl radicals via Fenton chemistry (26;27). Defects in these genes encoding Fe-S cluster biosynthesis proteins leads to many neurodegenerative and hematological diseases. Friedreich’s ataxia (FRDA), which results from defects in the frataxin gene (28;29) is a spino/cerebellar ataxia resulting in muscle weakness, speech disorders, heart disease and progressive damage to the nervous system. X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A) is a disease associated with impairments in the Fe-S cluster transport or signaling pathway (30-32). Defects in the iscU gene, which encodes for a mitochondrial protein that serves as a primary scaffold for Fe-S cluster assembly (vide infra), results in myopathy with exercise intolerance, an inherited skeletal muscle disease (33). Microcytic anemia is a hematological disease characterized by deficient hemoglobin synthesis resulting in anemia (34). This disease is linked to defects in glutaredoxin Grx5 which is also involved in Fe-S cluster biosynthesis (35). Defects in Fe-S cluster biogenesis have also been linked to reduction of virulence in bacterial pathogens (36). Thus, a detailed understanding of the mechanism of Fe-S cluster biogenesis is necessary to fight against many human diseases.

**Structure and properties of biological Fe-S clusters**

There are four basic types of Fe-S centers classified by the number of Fe atoms: Fe(SCys)₄, Fe₂(µ₂-S)₂(SCys)₄, Fe₃(µ₃-S)(µ₂-S)₃(SCys)₃, and Fe₄(µ₃-S)₄(SCys)₄. Each Fe has approximately tetrahedral sulfur ligation and clusters are most commonly attached to the protein by cysteine thiolates, see Figure 1.1. These clusters are written as [2Fe-2S]ⁿ, [3Fe-4S]ⁿ and [4Fe-4S]ⁿ, respectively, where n indicates the oxidation state of the Fe-S core. The [2Fe-2S] center is approximately planar and can be considered to be the basic building block of all larger Fe-S clusters. The cubane [4Fe-4S] cluster can be visualized as two [2Fe-2S] rhombs fused together and the [3Fe-4S] cluster is best described as a cubane [4Fe-4S] cluster that is missing one Fe.
Similarly, the P cluster of the nitrogenase MoFe protein can be visualized as a fusion of two [4Fe-4S] clusters to form a double cubane [8Fe-7S] cluster with one \( \mu_6 \)-S and two \( \mu_2 \)-bridging cysteinates.

Most Fe-S clusters have all-cysteiny1 ligation but some have histidine, aspartate, serine, arginine, backbone amide nitrogen or water in place of one or two cysteine ligands. For example, the Rieske-type [2Fe-2S] centers shown in Figure 1.1 have one Fe ligated by two cysteine ligands and the other by two histidine ligands. The [2Fe-2S] clusters in some succinate dehydrogenases and sulfide dehydrogenases have one aspartate ligand \((37;38)\), whereas the [2Fe-2S] cluster in biotin synthase has one arginine ligand \((39)\). These alternate ligation environments change the properties of the clusters. For example, more electropositive histidine ligands result in a higher redox potential for Rieske proteins \(+380 \text{ to } -150 \text{ mV}\) compared to proteins containing all-cysteiny1 ligated \([2Fe-2S]^{2+} +\) clusters \(+100 \text{ to } -460 \text{ mV}\) \((40)\).

In many enzymes, non-cysteiny1 ligation at a unique Fe site of a [4Fe-4S] cluster creates a substrate binding site in order to facilitate catalysis. One of the best characterized examples are the radical S-adenosylmethionine (SAM) enzymes \((15;41;42)\). The binding of the SAM substrate at the unique Fe site of the reduced [4Fe-4S] cluster, induces a reductive cleavage reaction producing a 5’-deoxyadenosyl radical which initiates the radical reaction, see Figure 1.2. In other Fe-S enzymes, incorporation of a heterometal is used to create the substrate binding site. For example, carbon monoxide dehydrogenase (CODH), which catalyses the oxidation of CO to CO\(_2\), has a [Ni-4Fe-5S] cluster active site that can be visualized as a [3Fe-4S] cluster connected to a [Ni-(\( \mu_2 \)-S)-Fe] fragment via three (\( \mu_3 \)-S) sulfides to create a square-planar Ni that serves as the CO binding site, see Figure 1.2 \((43;44)\). Nitrogenase provides another example as it contains a complex mixed-metal double-cubane cluster. It catalyses the reduction of N\(_2\) to NH\(_3\) and H\(^+\) to
H₂ in an ATP dependent reaction (45). However, in this case, the role of the heterometal (Mo or V) in catalysis has yet to be fully defined.

**Biogenesis of biological iron-sulfur clusters**

Structure-functions studies involving bacterial proteins coupled with the elucidation of numerous bacterial genomes have revealed three distinct operons that encode for proteins involved in the assembly, delivery and repair of Fe-S centers, namely the **Nitrogen Fixation** (NIF), **Iron Sulfur Cluster** (ISC) and **Sulfur Utilization Factor** (SUF) systems for Fe-S cluster biogenesis, see Figure 1.3. All three systems have the same basic scheme for Fe-S cluster assembly involving a cysteine desulfurase that provides S and scaffold protein(s) on which clusters are assembled and transferred intact into acceptor apo-proteins. Specific iron donors have yet to be definitively identified for any of the three systems.

**NIF system**

Dean and co-workers were the first to discover the Nif system (46-48). This system participates in the maturation of Fe-S cluster proteins that are involved in nitrogen fixation in azotrophic organisms such as *Azotobacter vinelandii* (Av). The Av Nif operon encodes the *Nif*IscA, NifS and NifU proteins. Disruption of nif genes often results in defects in maturation of the Fe protein or the MoFe protein, which comprise the nitrogenase supercomplex. Deletion of *nifU* and *nifS*, individually or together, results in a dramatic loss in the activity of both the component proteins (49;50). This provides evidence that NifU and NifS are involved in production of Fe-S clusters for nitrogenase maturation.

NifS, a pyridoxal phosphate (PLP) containing homodimer, catalyses the conversion of L-cysteine to L-alanine with the concomitant formation of a cysteine persulfide on a flexible side arm of the enzyme (50). Enzyme-bound persulfides are then transferred to the active-site
cysteine residues on the NifU scaffold protein for in situ reduction to yield sulfide ions during the cluster assembly process. NifU is a modular homodimeric protein with a central domain that contains a redox active [2Fe-2S]$^{2+}$ cluster. The N-terminal domain initially assembles [2Fe-2S]$^{2+}$ clusters that undergo reductive coupling to generate [4Fe-4S]$^{2+}$ clusters, and the C-terminal domain directly incorporates [4Fe-4S]$^{2+}$ clusters (51). Both scaffold domains have been shown to be competent for in vitro activation of nitrogenase component proteins.

$^{\text{Nif}}$IscA can assemble both [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters in the presence of iron and NifS, and clusters can subsequently be transferred to suitable acceptor proteins (52). The homodimeric [2Fe-2S]$^{2+}$ cluster-bound form of $^{\text{Nif}}$IscA is converted to a homodimeric [4Fe-4S]$^{2+}$ cluster-bound form on addition of dithiothreitol under anaerobic conditions, in a process that appears to involve a dimer-to-monomer-to-dimer transition with reductive coupling of two [2Fe-2S]$^{2+}$ at the dimer interface. Moreover, upon exposure to O$_2$, the [4Fe-4S]$^{2+}$ cluster undergoes reversible oxidative cleavage to yield the original [2Fe-2S]$^{2+}$ cluster-bound $^{\text{Nif}}$IscA homodimer (53). Coupled with the observations that [4Fe-4S]$^{2+}$ cluster-bound $^{\text{Nif}}$IscA is competent for rapid maturation of the apo nitrogenase Fe protein and can accept clusters from holo NifU, these results suggest that A-type Fe-S cluster assembly proteins may function as alternative cluster scaffold or carrier proteins under oxidative stress conditions. In vitro iron binding studies carried by Mapolelo et.al (53) have also shown that $^{\text{Nif}}$IscA can also bind monomeric Fe$^{3+}$ or Fe$^{2+}$ that is selectively released free by L-cysteine and hence is capable of providing iron for Fe-S cluster assembly on NifU proteins in the presence of L-cysteine and a NifS. Hence it is possible that $^{\text{Nif}}$IscA has a dual role in providing Fe for cluster assembly on NifU and accepting preformed clusters from NifU for the maturation of [4Fe-4S]$^{2+}$ cluster-containing proteins under aerobic or oxidative stress conditions.
SUF system

The Suf system was first discovered in *E. coli*, where it is induced under conditions of oxidative stress and/or iron limitation (54-56). The *suf* operon shown in Figure 1.3 encodes 6 genes *Suf*ABCDSE whose protein products are involved in Fe-S cluster assembly. *SufS* is a cysteine desulfurase homologous to *IscS* and *NifS*. However, *SufS* works together with a sulfurtransferase protein *SufE* unlike *NifS* and *IscS*. Recent studies have shown that this *SufE*-dependent stimulation of *SufS* is further enhanced by interaction with the *SufBCD* complex (57-59). Moreover, the crystal structure of *SufE* has structural homology to *IscU* and *SufU* implying a similar sulfur transfer mechanism for *SufS* to *SufE* as with *NifS* to *NifU*, *IscS* to *IscU* and *SufS* to *SufU* (57;60). *SufA*, a homolog of *IscA* and *NifIscA*, and was originally proposed to function as an alternative scaffold protein for assembly of both [2Fe-2S] and [4Fe-4S] clusters under oxidative stress conditions (61;62). However, more recent *in vivo* and *in vitro* data indicate that *SufA* functions as a carrier protein that accepts clusters assembled on the *SufBCD* complex and delivers them to acceptor proteins (63-65). The other three gene products, *SufB*, *SufC*, and *SufD*, constitute the *SufBCD* complex and have no sequence homology to proteins in the ISC or NIF systems. *SufC* has ATPase activity and appears to be a soluble member of the family of ABC transporters (*ATP binding cassette*). A recent study by Saini *et al.* showed that *SufBCD* complex can assemble a [4Fe-4S] cluster *in vitro* and *in vivo*. Hence, *SufBCD* can serve as a primary system for *de novo* Fe-S cluster biogenesis in the SUF system, with *SufB* serving as the primary scaffold protein, and *SufC* and *SufD* being involved in recruiting Fe and facilitating cluster formation on *SufB* (66).

In accord with the ability of the SUF system to be functional under oxidative stress conditions, it serves as the primary machinery for Fe-S cluster assembly in the O$_2$-rich

6
environments encountered in cyanobacteria and plant chloroplasts (67). In addition, Gram-positive bacteria such as T. maritima and E. faecalis utilize a modified suf operon, sufBUSDC, which encodes for SufU rather than SufE and does not encode for SufA (see Figure 1.3), as their primary machinery for Fe-S cluster biogenesis (68). SufU shares three conserved cysteine residues with IscU, but does not have the LPPVK motif and hence does not utilize molecular chaperones for mediating transfer of Fe-S clusters, *vide infra*.

**ISC system**

The most common Fe-S cluster assembly machinery in bacteria, the ISC system, was initially characterized in *Azotobacter vinelandii* grown under non-nitrogen fixing conditions. The *isc* operon (see Figure 1.3) encodes *cysE2, iscR, iscS, iscU, iscA, hscB, hscA, fdx* and *iscX*. CysE2 is an *o*-acetylserine synthase which catalyzes the rate limiting step in cysteine biosynthesis and hence locally increases the production of cysteine which serves as the substrate for IscS. Expression of these genes, except *cysE2*, is regulated by IscR. Homologs of each of these proteins except IscR/IscX are involved in Fe-S biogenesis in eukaryotic mitochondria (69).

IscS shares high sequence homology with NifS and is a homodimeric pyridoxal phosphate (PLP) containing cysteine desulfurase that catalyzes the reduction of L-cysteine to L-alanine thereby providing the inorganic sulfur required for the biosynthesis of Fe-S clusters (70). Studies on *T. maritima* IscS reveal that IscS interacts at the N-terminal helix of IscU, which is conserved in all higher organisms (71). *In vitro* studies have also shown that the active-site cysteine of *A. vinelandii* IscS forms a heterodisulfide complex with one of the three conserved cysteines on *A. vinelandii* IscU (72). Moreover, the recent X-ray structure of the IscS/IscU complex from *E. coli* provides structural details of the interaction between flexible loop active-site cysteine on IscS and the three conserved cysteines on IscU (73).
IscA, an homolog of \textsuperscript{Nif}IscA, has three conserved cysteines and plays an important role in Fe-S cluster biosynthesis under aerobic and oxidative stress conditions (74). IscA can assemble both \([2\text{Fe}-2\text{S}]}^{2+}\) and \([4\text{Fe}-4\text{S}]}^{2+}\) clusters during \textit{in vitro} reconstitution using \(\text{Fe}^{2+}\), L-cysteine and cysteine desulfurase, and these clusters can be transferred to apo-Fdx and apo-BioB (61;75). In addition, there is preliminary evidence that \textit{E. coli} \([2\text{Fe}-2\text{S}]}^{2+}\) cluster-bound IscU will transfer its cluster to \textit{E. coli} apo-IscA, but not vice-versa, suggesting a role for IscA as a delivery system for clusters assembled on IscU (61). However, Ding and coworkers have shown that \textit{E. coli} IscA and human IscA (hIscA1) can bind \(\text{Fe}^{3+}\) which is released by L-cysteine and hence can function as a Fe donor for IscS-mediated cluster assembly on IscU (76;77). Therefore it seems likely that IscA may play a dual role in bringing Fe to and taking clusters away from IscU, as discussed above for \textsuperscript{Nif}IscA and NifU in the NIF system.

IscU is homologous to the N-terminal domain of NifU and functions as a primary scaffold protein for the assembly of Fe-S clusters (78). The importance of IscU in Fe metabolism in general and Fe-S cluster biosynthesis in particular can be recognized from the fact that it is one of the most widespread and conserved proteins in Nature, see Figure 1.4. IscU has three conserved cysteines, Cys\textsuperscript{37}, Cys\textsuperscript{63} and Cys\textsuperscript{106} (numbering refers to \textit{A. vinelandii}), that are all required for cluster incorporation. In the presence of excess \(\text{Fe}^{2+}\) and cysteine and catalytic IscS, IscU can sequentially assemble labile \([2\text{Fe}-2\text{S}]}^{2+}\) and \([4\text{Fe}-4\text{S}]}^{2+}\) clusters (79;80). Initially, the IscU homodimer assembles a single \([2\text{Fe}-2\text{S}]}^{2+}\) cluster that has been shown to be resistant to iron chelators. Subsequently, a second transient \([2\text{Fe}-2\text{S}]}^{2+}\) cluster is assembled on the homodimer and these two \([2\text{Fe}-2\text{S}]}^{2+}\) clusters can reductively couple to form a \([4\text{Fe}-4\text{S}]}^{2+}\) cluster at the subunit interface of the homodimer. Reductive coupling is a slow process without an exogenous one-electron donor, but the rate increases in the presence of dithionite or reduced Isc Fdx. Thus,
IscFdx could serve as the physiological electron donor for this cluster conversion (81). Upon exposure to O$_2$, the [4Fe-4S]$^{2+}$ cluster reverts back to the single [2Fe-2S]$^{2+}$ cluster with loss of two iron atoms. Moreover, both the [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters assembled on IscU can be transferred to the apo-forms of appropriate acceptor proteins (82). Thus, IscU has the ability to assemble either [2Fe-2S]$^{2+}$ or [4Fe-4S]$^{2+}$ clusters and transfer them to intermediate carrier or acceptor Fe-S proteins.

Resonance Raman and Mössbauer studies have shown that the clusters on IscU are coordinated by at least one non-cysteinyl ligand (80). A 2.3 Å resolution crystal structure of Zn-bound Streptococcus pyogenes SufU (83) and an NMR structure of Zn-bound Haemophilis influenzae IscU have been solved (84). The structural data reveal a compact globular core with flexibility in the N- and C-terminal regions. The crystal structure of S. pyogenes SufU shows that Zn is coordinated by the three conserved cysteine residues and a fully conserved aspartate residue (Asp$^{39}$ in A. vinelandii IscU), whereas in H. influenzae structure the Zn atom is coordinated by three conserved cysteine residues and a histidine (His$^{105}$ in A. vinelandii IscU), which is conserved in all IscUs and NifUs, but not in SufU. The only crystal structure of a [2Fe-2S] cluster-bound form of IscU is for the D39A variant of IscU (A. vinelandii numbering) from Aquifex aeolicus (Aa), see Figure 1.5 (85). The structure revealed a novel asymmetric trimer with a [2Fe-2S] cluster bound in only one subunit ligated by the three conserved cysteines and His$^{106}$. Despite this structural information, the fourth ligand to the cluster in WT IscU is still unknown, since spectroscopic studies indicate that a change in [2Fe-2S] cluster ligation is induced by the D39A mutation in both IscU and NifU (78).
Molecular Chaperones

Hsp70s (Heat Shock Protein) comprise a widespread family of molecular chaperones that play important roles in protein folding, cellular trafficking and translocation of proteins across membranes (86-88). Hsp70 chaperones contain two domains, i) an N-terminal nucleotide binding domain (NBD) (~45 kDa) with weak ATPase activity and ii) a C-terminal substrate binding domain (SBD) (~25 kDa). Initially, it was believed that prokaryotes contain only a single Hsp70 gene DnaK, which participates with the DnaJ-GrpE co-chaperone system (89). Later, a new homolog of Hsp70 was identified in E. coli by Vickery et al. (90). Expression of this gene was not induced by heat shock and hence named HscA (heat shock cognate). The HscA protein (Hsc66; 66 kDa) shows only 40% sequence identity to DnaK and the co-chaperone, designated HscB (Hsc20; 20 kDa) contains an N-terminal J-domain and a novel C-terminal domain. Biochemical studies on E. coli HscA/HscB by Silberg et al. showed that HscA exhibits low ATPase activity which was enhanced 6-fold higher in the presence of HscB. In contrast, the DnaJ-GrpE (co-chaperone for DnaK) at physiological concentrations was unable to stimulate HscA’s ATPase activity. Similarly, the ATPase activity of DnaK was unaffected by HscB. Thus, the HscA/HscB and DnaK/DnaJ/GrpE chaperone systems were shown to have distinct functions (91;92). The first indication that HscA/HscB and their eukaryotic homologs Ssq1/Jac1 in yeast function specifically in Fe-S cluster biogenesis came from the realization that they are encoded by the isc operon.

HscA is a specialized bacterial hsp70 class chaperone that only interacts with the apo- and cluster-loaded forms of IscU binding to the highly conserved LPPVK motif at positions 99-103, located close to the cluster binding site of IscU, see Figure 1.6 (93-95). Using site-directed fluorescence labeling and quenching strategies, Vickery et al. showed that the LPPVK motif (in
particular the central proline) plays a major role in stimulation of HscA ATPase activity (86). This motif is missing in the U-type proteins of the NIF and SUF systems that do not appear to have specific HscA/HscB molecular chaperones. In contrast, the LPPVK motif is conserved among IscU family members from both prokaryotes and eukaryotes indicating that their role in Fe-S cluster biogenesis has been conserved. Hence HscA and HscB function together as nucleotide-dependent co-chaperone system that is specific for IscU.

In vivo studies of yeast Ssq1/Jac1, suggest that the main function of chaperones lies in mediating transfer of Fe-S clusters from U-type scaffold proteins to apo-acceptor proteins (96;97). Subsequently, the role of bacterial HscA and HscB co-chaperones was greatly clarified by Chandramouli and Johnson (98), who monitored in vitro cluster transfer experiments from [2Fe-2S] cluster bound IscU to ferredoxin in the presence and absence of HscA, HscB, Mg-ATP and KCl, using CD and EPR spectroscopies. The results indicate that the molecular chaperones interact with [2Fe-2S] IscU and the rate of cluster transfer is increased more than ~20 fold in the presence of stoichiometric amounts of HscA/HscB and excess of Mg-ATP. The rate is further enhanced in the presence of physiological amounts of KCl which is known to increase HscA ATPase activity (99). This was the first evidence that [2Fe-2S] cluster transfer from IscU is an ATP-dependent reaction. Based on this work, and the ATPase catalytic cycle for HscA/HscB/IscU developed by Vickery et al. (100), a viable mechanism for ATP-dependent cluster release from IscU has been proposed, see Figure 1.7. Initially, ATP binds to HscA leading to a tense (T) state with decreased substrate binding affinity. HscB binds and escorts IscU and to HscA, which increases the HscA binding affinity for IscU in the ATP-bound T-state and greatly enhances HscA ATPase activity. The HscA/ATP/HscB/IscU complex then undergoes ATP hydrolysis and loss of HscB to yield an ADP-bound relaxed R-state of HscA.
with greatly increased affinity for IscU. This induces a conformational change in IscU that facilitates release of the [2Fe-2S] cluster in the presence of a suitable acceptor protein. Replacing ADP with ATP induces the R to T transition and hence the dissociation of apo-IscU. This mechanism only appears to apply to [2Fe-2S] cluster release from IscU, there is no enhancement on the rate of [4Fe-4S]^{2+} cluster transfer from IscU to apo-aconitase in the presence of HscA/HscB/MgATP (82).

No crystal structure of the HscA/HscB/IscU complex has been solved to date. However, a crystal structure of the substrate binding domain of HscA complexed with a small peptide with the LPPVK motif revealed that the peptide binds with the opposite orientation to that observed in DnaK peptide complexes, see Figure 1.8 (101). In addition, a 1.8 Å resolution crystal structure of full length co-chaperone HscB from E. coli was determined, see Figure 1.9 (102). The rigid structure of HscB suggests that HscB, not only enhances the ATPase activity of HscA, but also functions to facilitate positioning of the substrate protein on the chaperone. Recent isothermal titration calorimetry and nuclear magnetic resonance studies on HscB mutants by Fuzery et al. revealed that three hydrophobic residues (L92, L96, and F153) on HscB are important for formation of a stable complex with IscU, see Figure 1.9 (103). The substitution of these residues with alanine causes a ~15000 fold reduction in affinity of HscB for IscU. NMR studies also revealed that HscB binds at the N-terminal β-strands and the C-terminal α-helix of IscU, see Figure 1.6. However, IscU exits in two conformational states, a ordered state where IscU acts as Fe-S cluster scaffold, and a disordered state that serves to release the cluster. HscB binds to the ordered state of IscU and forms a HscB-IscU complex that exists in two or more distinct states that interconvert at the rate much faster than the rate of dissociation of the complex (104).
Role of monothiol Glutaredoxins (Grxs) in Fe-S cluster biogenesis

Glutaredoxins are small oxidoreductases that have thioredoxin fold architecture and use glutathione (GSH) as a cofactor. Grxs are divided into 6 classes based on their active site sequence and conserved motifs that are involved in GSH binding (105,106). One among them is monothiol Grxs with a CGFS active site sequence. These monothiol Grxs are widespread among prokaryotes and eukaryotes (107). The crystal structures of two monothiol Grxs namely, *E. coli* Grx4 (108) and human Grx2 (109) with bound [2Fe-2S]$_{2}^{2+}$ cluster have been solved. The crystal structure of *E. coli* [2Fe-2S]$_{2}^{2+}$ Grx4 showed that the holodimer is centered on a [2Fe-2S]$_{2}^{2+}$ cluster with tetrahedral coordination at each iron atom by two active-site cysteines, one from each Grx monomer, and the cysteines from two GSH molecules, see Figure 1.10. The involvement of glutathione and the complete cysteinyl ligation of the [2Fe-2S] cluster in homodimeric monothiol Grxs was initially proposed based on cysteine mutagenesis and spectroscopic (UV-visible absorption/circular dichroism, resonance Raman and Mössbauer) studies (110).

The role of monothiol Grxs are best understood in the yeast, *Saccharomyces cerevisiae* (Sc) that possess three such proteins: Grx3, Grx4 and Grx5. Studies carried out on ScGrx5 gave initial evidence that Grxs are involved in Fe-S cluster biogenesis (97,111). Deletion of the ScGrx5 gene resulted in a mutant that exhibited impaired Fe-S cluster biogenesis. Moreover, the yeast mutant was found to be more sensitive to oxidative stress as a result of increased accumulation of free iron. Later, $^{55}$Fe radiolabelled immunoprecipitation studies revealed build up of iron on Isu1p (human homologue of IscU), implying a role in Grx5 in facilitating the transfer of pre-assembled clusters on the U-type scaffold protein to apo-acceptor proteins (97,112). Prokaryotic and eukaryotic monothiol Grxs other than yeast were also shown to functionally substitute for Grx5 suggesting that the role of Grxs is conserved throughout
evolution (113). Furthermore, Herrero et al. (114) provided in vivo evidence that monothiol Grxs interact with A-type proteins (isa1p). More recently, in vitro cluster transfer studies showed that [2Fe-2S]$^{2+}$ clusters on monothiol Grxs were effectively transferred to A-type proteins as monitored by UV-visible absorption and CD spectroscopies (53). These results gave supporting evidence that monothiol Grxs participate in the iron-sulfur cluster assembly machinery by transfer/storage of assembled clusters.

In addition to Fe-S cluster biogenesis, monothiol Grxs also participate in the regulation of iron homeostasis. In yeast, Fra2 (Fe-repressor of activation-2 protein) is essential for iron regulation. Deletion of Fra2 results in constitutive activation of the iron regulon, which increases iron uptake into the cell. Spectroscopic, analytical and mutagenesis data revealed that Fra2 can displace one of the glutaredoxins in the homodimeric [2Fe-2S] cluster-bound forms of two cytosolic yeast monothiol glutaredoxins, Grx3 or Grx4, to form a Grx-Fra2 heterodimer with [2Fe-2S]$^{2+}$ cluster at the dimer interface. In the complex, the cluster is coordinated by the active-site cysteines of Grx and GSH, and a conserved histidine (His103) on Fra2. This complex is essential for iron regulation in yeast (115;116). In vivo studies showed that mutation of His103 to alanine in Fra2 results in inactivation of Fra2, i.e. constitutive activation of the iron regulon (116). Taken together, this indicates that Fra2 histidine ligation of the Fra2-Grx3/4 [2Fe-2S]$^{2+}$ cluster is the mechanism of cellular iron sensing.

In this work, the potential roles of HscA/HscB/ATP in mediating cluster transfer from [2Fe-2S] cluster-bound Av IscU to the potential intermediate carrier/storage proteins such as Av Grx5 are explored. The results provide further evidence that the molecular chaperones facilitate
[2Fe-2S] cluster transfer reactions from IscU. In addition, the results provide strong support for the proposal that monothiol Grxs function in storing and/or delivering [2Fe-2S] clusters assembled on IscU.

**Abbreviations:** Nitrogen fixation, *nif*; iron sulfur cluster assembly, *isc*; sulfur utilization factor, *suf*; pyridoxal phosphate, PLP; Heat shock cognate proteins, Hsc; Glutaredoxins, Grxs; Glutathione, GSH; *Azotobacter vinelandii*, Av; *Escherichia coli*, E. coli, Ec; *Aquifex aeolicus*, Aa; *Streptococcus pyogenes*, S. pyogenes, Sp; *Haemophilus influenzae*, H. influenzae, Hi; *Thermotoga maritima*, T. maritima, Tm; *Homo sapiens*, H. sapiens, Hs; *Saccharomyces cerevisiae*, S. cerevisiae, Sc; *Schizosaccharomyces pombe*, S. pombe, Sp.
References


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Figure 1.1: Structures of Fe-S clusters involved in electron transport as determined by X-ray crystallography. Structures are taken from the coordinates deposited in the protein Data bank: A. Fe Rd, PDB ID# 18RXN, rubredoxin from *Desulfovibrio vulgaris*; B. [2Fe-2S], PDB ID# 1FRD, Anabena pcc7120 Fd; C. Rieske [2Fe-2S] center in *Sulfolobus acidocaldarius*, PDB ID#1JM1; D. [3Fe-4S], PDB ID# 6FDR, *Azotobacter vinelandii* FdI; E. [4Fe-4S], PDB ID# 6FDR, *A. vinelandii* FdI; F. [8Fe-7S] PDB ID# 1MIN dithionite reduced *A.vinelandii* nitrogenase MoFe protein. Color code: pink, Fe; yellow, S; blue, N. Adapted from reference (3).
Figure 1.2: Crystallographically defined active-site structures of some Fe-S enzymes. A. S-Adenosyl Methionine bound [4Fe-4S] center in E.coli HemN, PDB ID# 1OLT; B. [Ni-4Fe-5S] center in reduced CO dehydrogenase II from *Carboxydotermus hydrogenoformans*, PDB ID# 1SU8; C. [Mo-7Fe-9S-X] FeMo cofactor in *Azotobacter vinelandii* nitrogenase, PDB ID# 1MIN. Color code: pink, Fe; yellow, S; grey, C; red, O; green, Ni; orange, Mo; black, unknown light atom X (N/O/C). Adapted from reference (3).
Figure 1.3: Schematic representation of the genes involved in Fe-S cluster assembly operons in 
*Azotobacter vinelandii* (Av), *Escherichia coli* (Ec), *Thermotoga maritima* (Tm), *Helicobacter pylori* (Hp). Color coding refers to genes from different organisms/different gene clusters having the same function with same color. NifU with different domains is represented by three different colors within the nifU gene. Adapted from reference (117).
Figure 1.4: Primary sequence comparison of IscU family of proteins. Cluster coordinating cysteine residues are shaded red and residues necessary for the specific binding with molecular chaperone HscA are shaded green and conserved aspartate and histidine residues are shown in blue. ‘*’ indicate invariant residues, ‘:’ indicate highly conserved residues and ‘.’ indicates semi conserved residues. Ec, *Escherichia coli*; Hi, *Haemophilus influenza*; At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Av, *Azotobacter vinelandii*; Aa, *Aquifex aeolicus*. 
Figure 1.5: A. Crystal structure of *Aquifex aeolicus* [2Fe-2S] D39A IscU having PDB entry 2Z7E (85), determined at a resolution of 2.3 Å. B. [2Fe-2S] cluster with ligands. Color code: brown, Fe; yellow, S; blue, N; grey, C; red, O.
Figure 1.6: Solution NMR structure of Zinc bound at the active site of *Haemophilus influenzae* IscU, PDB ID # 1R9P (84). Color code: green, Conserved LPPVK motif; Red, N-terminal β-sheets; blue, C-terminal α-helixes; orange, Zn atom.
Figure 1.7: Schematic representation of the proposed mechanism for the stimulation of IscU cluster transfer by the HscA/HscB ATPase cycle. Adapted from reference (98).
Figure 1.8: Crystal structure of *E. coli* HscA (SBD) peptide complex of PDB ID # 1U00 (101). Two views, rotated 90° relative to each other showing that petide (ELPPVKIHC) is completely buried in the β-subdomain. The α-sub domain and β-subdomain are shown in blue and red color respectively, whereas the bound peptide (ELPPVKIHC) is showed in green.
Figure 1.9: Crystal structure of *E. coli* HscB, PDB ID #1FPO (103). The J-domain is shown in orange, C-domain in green and the three hydrophobic aminoacids that make the greatest contribution to stability of HscB-IscU complex (L92, L96, and F153) are shown in red.
Figure 1.10: Crystal structure of *E. coli* Grx4, PDB ID# 2WCI. The [2Fe-2S] cluster is ligated by the active site cysteines of two Grx monomers and two glutathione (GSH) molecules. Color code: yellow, S; brown, Fe; grey, C; blue, N; red, O. Adapted from reference (118).
Introduction

Proteins containing Fe-S clusters are involved in various essential biological processes such as respiration, photosynthesis and electron transport \( (I) \). In bacteria, Fe-S clusters are assembled by one of the three different assembly systems, namely the \textit{Nitrogen Fixation} (NIF), \textit{Iron Sulfur Cluster} (ISC) and \textit{Sulfur Utilization Factor} (SUF) systems \( (2) \). The NIF system is involved in the maturation of Fe-S proteins associated specifically with nitrogen fixation in nitrogen-fixing bacteria. The SUF system functions primarily under iron-limitation or oxidative stress conditions and constitutes the sole system for Fe-S cluster biogenesis in many archaea as well as cyanobacteria and plant chloroplasts. The ISC system plays a general “housekeeping” role for general Fe-S cluster biogenesis in most bacteria and almost all the key components have been retained in mitochondria. The bacterial \textit{isc} operon of \textit{Azotobacter vinelandii} contains nine genes encoding for CysE2, IscR, IscS, IscU, IscA, HscB, HscA, Fdx and IscX \( (2) \). Of these components, IscU is central because it acts as a scaffold on which \([2Fe-2S]\) and \([4Fe-4S]\) clusters are assembled and subsequently transferred to recipient apo-acceptor proteins \( (3) \).

Biochemical and genetic experiments have demonstrated that HscA (\textit{heat shock cognate A}, 66 kDa), HscB (\textit{heat shock cognate B}, 20 kDa), and their eukaryotic mitochondrial homologues (Ssq1 and Jac1 in yeast) are associated with the maturation of Fe-S proteins in prokaryotes and eukaryotes \( (4-6) \). HscA and HscB are specialized types of Hsp70 molecular
chaperone and Hsp40 co-chaperone proteins, respectively, that specifically target IscU (7). The affinity of HscA for IscU depends upon the nature of nucleotide (ATP or ADP) bound to HscA. HscA alone has low ATPase activity but this activity is greatly enhanced by interaction with HscB and IscU (8). Furthermore, the affinity between HscA and IscU is increased when HscB binds and escorts IscU to HscA. Crystallography, mutagenesis and fluorescent-labeled oligopeptide studies show that HscA binds selectively to the conserved LPPVK motif of IscU (9-11), and NMR studies of Hemophilus influenzae show that the LPPVK motif is located within a solvent-exposed loop that is adjacent to the [Fe–S] cluster assembly site (12). Taken together these results implicated a role for the the ATPase activity of HscA in facilitating Fe-S cluster release from IscU.

Direct evidence in support of the proposed role of HscA/HscB in the biogenesis of Fe-S clusters remained elusive for several years. In vitro studies conducted on the general molecular chaperone system homologous to HscA/HscB (DnaK/DnaJ) in Thermotoga maritima suggested that molecular chaperones stabilize clusters assembled on SufU, which lacks the conserved LPPVK sequence (13). In contrast, in vivo $^{55}$Fe immunoprecipitation studies indicated that depletion of the HscA and HscB homologous molecular chaperones in yeast (Ssq1 and Jac1) resulted in accumulation of Fe on Isu1 (yeast homolog of IscU) (14), suggesting that the molecular chaperones are required to facilitate cluster transfer from Isu1. Definitive in vitro evidence for the role of HscA/HscB in ISC-mediated Fe-S cluster biogenesis was finally provided by Chandramouli and Johnson (15). Using purified Azotobacter vinelandii proteins, HscA and HscB were both shown to bind to the [2Fe-2S] cluster-bound IscU and the trimeric [2Fe-2S]-IscU/HscA/HscB complex was found to greatly enhance the rate of cluster transfer from IscU to apo-IscFdx in the presence of MgATP and KCl. However, there is a pressing need
to investigate the effect of HscA/HscB/MgATP on the rates of cluster transfer from IscU to other potential acceptor proteins to investigate the generality of this proposed role.

In this study, the ability of *A. vinelandii* IscU to transfer a [2Fe-2S] cluster to Grx5, a monothiol glutaredoxin that is not encoded by the *isc* operon, has been investigated in the presence and absence of HscA/HscB/MgATP. The initial evidence that monothiol Grxs play a role in Fe-S cluster biogenesis came from gene deletion and $^{55}$Fe-radiolabeled immunoprecipitation studies in yeast (14;16). Deletion of *S. cerevisiae* Grx5 (a mitochondrial monothiol Grx), resulted in impaired Fe-S cluster biogenesis and increased sensitivity to oxidative stress. Moreover, $^{55}$Fe-immunoprecipitation demonstrated that Grx5 deletion results in increased Fe content on Isu1 indicating that Grx5 is involved in facilitating cluster transfer from Isu1 to acceptor proteins. These results, coupled with the subsequent demonstration that monothiol Grxs bind [2Fe-2S] clusters in the presence of glutathione, that can be transferred to appropriate acceptor proteins (17), suggest a role for monothiol Grxs as intermediate Fe-S cluster carrier proteins. Since there are over 300 distinct types of Fe-S proteins, it is unlikely that IscU interacts specifically with each one to enable direct cluster transfer. Rather, carrier proteins (such as Grx5) are proposed to accept clusters from IscU and donate them to specific acceptor proteins. Hence if *A. vinelandii* Grx5 can be shown to accept [2Fe-2S] clusters from *A. vinelandii* IscU at a rate that is significantly enhanced by the addition of HscA/HscB/MgATP, this would confirm the role of Grx5, and likely other monothiol Grxs, as carrier proteins for [2Fe-2S] clusters assembled by U-type scaffold proteins and demonstrate a general role for HscA/HscB in facilitating ATP-dependent [2Fe-2S] cluster release from IscU.
Materials and Methods

All chemicals were purchased from Sigma-Aldrich, Fisher or Invitrogen, unless otherwise stated. The plasmids pDB 1303 and pDB 1036 overexpressing *Azotobacter vinelandii* HscA and HscB were provided by Dr. Dennis Dean (Virginia Polytechnic Institute and State University). Recombinant plasmid pDB1686 that contains Av grx5 gene with 347 bp fragment was cloned into NdeI-BamHI sites of pET-16b vector for production of AvGrx5 with N-terminal hisidine-tag. The cloning of gxr5 gene to pET-16b vector was performed by GenScript USA Inc. and nucleotide sequence was confirmed by DNA sequencing analyses performed by The Georgia Genomics Facility at the University of Georgia. Anaerobic experiments were conducted under Argon (Ar) in a Vacuum Atmospheres glove box at oxygen levels of <5 ppm. Protein concentrations were determined by the DC protein assay (Bio-Rad), using BSA as a standard. Fe concentrations were determined colorimetrically using bathophenanthroline under reducing conditions, after KMnO4/HCl protein digestion (18).

**Overexpression and Purification of A. vinelandii HscA and HscB:** The plasmids pDB 1303 and pDB 1036 were transformed into the *E. coli* host BL21(DE3) gold and 1 or 2 colonies were directly added to LB (Luria-Bertini) media containing 100 μg/mL ampicillin and allowed to grow at 37°C. When the cultures reached an OD600 between 0.8-1.0, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.8 mM, and the bacterial cultures were further cultivated at 28°C for 20 hours. The cells were harvested at 9000g for 5 min at 4°C and stored at -80°C until further use.

Av HscA and HscB were purified under aerobic conditions as described previously (15). Briefly, the cell paste (25g) was thawed and resuspended in 75 mL of 100 mM Tris-HCl buffer, pH 7.8 containing 1mM dithiotreitol (DTT) (buffer A). To this mixture Phenylmethanesulfonyl
fluoride (PMSF) (13mg), 10 µg/mL DNase I (Roche), and 10 µg/mL RNase A (Roche) were added. The cells were sonicated for 45 minutes by intermittent sonication, and the cell debris was removed by centrifugation at 39700g for 1 hour at 4°C. The cell free extract containing HscA or HscB was loaded onto a 110 mL Q-sepharose column previously equilibrated with Buffer A. Elution was achieved with a 0.0 to 1.0 M NaCl gradient using buffer A. Based on SDS gel analysis, the purest fractions were combined. These pure fractions were dialyzed into 100 mM Tris-HCl, pH 7.8, containing 1mM DTT and 1M ammonium sulfate by ultracentrifugation using YM 30 membrane for HscA or YM 10 membrane for HscB. The protein was further purified by loading into a 75 ml Phenyl Sepharose column and eluting with 1.0 to 0.0 M ammonium sulfate gradient using buffer A. The fractions were collected and were judged by the SDS-PAGE analysis, and the purest fractions were combined, concentrated using YM 30 or YM 10 and injected into size exclusion column, Superdex 200 in case of HscA and Superdex 75 for HscB previously equilibrated with 100 mM Tris-HCl, 200 mM NaCl, pH 7.8. The purified proteins were concentrated and exchanged anaerobically with buffer A and stored in liquid nitrogen until future use.

Overexpression and anaerobic purification of His-tagged Av Grx5: The E. coli expression strain C41(DE3) was transformed with Av Grx5 plasmid containing the helper vector pET-16b. LB cultures were grown at 37 °C and induced in exponential phase by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration of 0.8 mM) and the bacterial cultures were further cultivated at 34 °C for 4 hours. The reddish-brown cells were harvested by centrifugation at 5000g for 15 min at 4 °C and stored at -80 °C until further use.

8 g of reddish-brown cells were thawed and resuspended in 50 mM Tris-HCl buffer at pH 7.8 with 1 mM glutathione (GSH). 10 µg/mL phenylmethylsulfonyl fluoride (PMSF), 15 µg/mL
DNase and 5 μg/mL RNase were added to the mixture. The cells were lysed by sonication, and cell debris was removed by centrifugation at 39700g for 1 hr at 4 °C. The reddish-brown cell-free extract containing AvGrx5 was loaded onto a 3x5 mL His-Trap HP column (GE Healthcare) previously equilibrated with binding buffer (50 mM Tris-HCl, pH 7.8, containing 1 mM GSH, 0.5 M NaCl, 20 mM imidazole). The column was washed with 10 column volumes of binding buffer before the protein of interest was eluted with a 20-500 mM imidazole gradient. The purest fractions containing apo- and holo Av Grx5 were collected respectively and imidazole was removed by ultrafiltration using an YM-10 membrane.

Preparation of [2Fe-2S] Av IscU: IscU samples containing 0.9[2Fe-2S]2+ clusters/dimer were prepared as described previously (3). The [2Fe-2S]2+ cluster concentrations of Av IscU used in this work are based on the experimentally determined extinction coefficients (as assessed by Fe and protein determinations) are in agreement with the previously published values, ε_{456} = 9.2 mM\(^{-1}\)cm\(^{-1}\). The extinction coefficient of the [2Fe-2S]\(^{2+}\) center in Av Grx5 calculated based on iron and protein determinations is ε_{403} = 13.6 mM\(^{-1}\)cm\(^{-1}\).

ATPase activity assays: The HscA ATPase activity in the presence and absence of HscB and apo-IscU was determined by measuring the phosphate released in a coupled enzyme assay using an Enzchek phosphate assay kit at 23 °C.

Cluster transfer experiments from [2Fe-2S] IscU to apo-Grx5: The time course of cluster transfer from A. vinelandii [2Fe-2S] IscU to apo-Grx5 was monitored under anaerobic conditions at 23 °C using UV-visible and CD spectroscopy. Reactions were carried out in 100 mM Tris-HCl buffer, pH 7.8, with 3 mM glutathione (GSH). The reaction mixture (800 μL) was 0.15 mM in Grx5 monomer and 0.10 mM in IscU monomer with 0.9 [2Fe-2S] clusters/dimer to give a final IscU [2Fe-2S]\(^{2+}\) cluster concentration of 0.045 mM. The zero time point corresponds to the
addition of Grx5 to the reaction mixture. Reactions were also carried out in the presence of 0.10 mM Av HscA and HscB, 40 mM MgCl₂, 2 mM ATP, 150 mM KCl, and 3 mM GSH. The zero time point corresponds to the addition of ATP to the reaction mixture. Cluster transfer experiments were carried out in 1 cm cuvettes, and monitored by changes in CD spectra as a function of time. The time course of holo Grx5 formation was analyzed by fitting to second order kinetics using the Chemical Kinetics Simulator software package (IBM).

Spectroscopic measurements: UV-visible absorption and CD spectra were recorded using a Shimadzu UV-3101PC spectrophotometer and a Jasco J715 spectropolarimeter, respectively.

Results and Discussion

HscA ATPase activity: The ATPase activity of A. vinelandii HscA alone is low (0.042 mol of ATP hydrolysed (mol of HscA)⁻¹ min⁻¹). However, in the presence of 100-fold excess of HscB, the activity was enhanced 12-fold and in the presence of 100-fold excess of HscB and apo-IscU, the ATPase activity was enhanced 24-fold, See Figure 2.1. It has been previously shown that K⁺ ions are required for the optimal ATPase activity of Hsp70 molecular chaperones (19), thus we also tested activities in the presence of 150 mM KCl, the cytoplasmic concentration of K⁺, as well as 100-fold excess of HscB and IscU. Under these conditions, the ATPase activity of HscA was enhanced 85-fold. These results agree with previously published data (15), which reported 13-, 25-, and 100-fold increases in HscA ATPase activity on analogous additions of HscB, HscB/IscU, and HscB/IscU/KCl, respectively.

In vitro cluster transfer from [2Fe-2S]²⁺ cluster-bound IscU to apo-Grx5: Circular dichroism measures the difference in absorption of left and right circularly polarized light, and is sensitive to the chirality of the chromophore environment. Consequently, although [2Fe-2S] centers in different proteins generally have similar absorption spectra, they usually have quite
distinct CD spectra by virtue of differences in the chirality of the protein environment. Hence CD is the method of choice for monitoring the kinetics of interprotein [2Fe-2S]^{2+} cluster transfer. In these experiments UV-visible CD spectroscopy was used to measure the rate of [2Fe-2S] cluster transfer from Av IscU to Av Grx5.

The time course of cluster transfer from [2Fe-2S]^{2+} cluster-bound IscU to apo-Grx5 in the presence of a 1.7-fold excess of apo-Grx was monitored by CD as shown in Figure 2.2A. The reaction mixture was 45 μM in IscU [2Fe-2S] centers and 150 μM in monomeric Grx5, i.e. 75 μM in [2Fe-2S] cluster-binding dimeric form of Grx5. Simulated cluster transfer CD spectra were calculated based on a weighted average of the spectra for [2Fe-2S]^{2+} cluster-bound IscU and [2Fe-2S]^{2+} cluster-bound Grx5. The simulated spectra corresponding to 0-100% cluster transfer in 10% increments are shown in Figure 2.2B. The extent of cluster transfer as determined by comparing the time course of the reaction mixture CD spectra with the simulated data indicates that only 30% of cluster is transferred from [2Fe-2S] IscU to apo-Grx after 3 hours.

The IscU-to-Grx5 [2Fe-2S] cluster transfer was repeated under identical conditions except for the presence of HscA/HscB/MgATP and KCl. The CD spectra recorded as function of time during the cluster transfer reaction are shown in Figure 2.3A. The initial reaction mixture contains [2Fe-2S]^{2+} cluster-bound IscU, HscA and HscB. Grx5/Glutathione (GSH), KCl and MgCl\textsubscript{2} were added next, and the reaction was initiated by the addition of ATP. The data demonstrate that the [2Fe-2S] cluster was rapidly and quantitatively transferred to Av Grx5. On the basis of simulated CD spectra for a stoichiometric cluster transfer reaction, we estimate that the transfer is >90% complete after 3 min of reaction.
Quantitative assessments of the rates of cluster transfer were obtained by monitoring the difference in the CD intensity at 457 and 408 nm. These wavelengths were chosen in order to maximize the difference in CD intensity for the acceptor protein and minimize the difference in the CD intensity for the donor protein. For both sets of cluster transfer reactions, the data indicate direct cluster transfer and are fit to second-order kinetics based on the initial concentrations of [2Fe-2S] clusters on IscU and dimeric apo-Grx5. In the absence of HscA/HscB/MgATP the rate constant was found to be 30 M\(^{-1}\)min\(^{-1}\), see Figure 2.4A. However, in the presence of HscA, HscB, MgATP, and KCl the rate constant increased ~700-fold to 20000 M\(^{-1}\)min\(^{-1}\), see Figure 2.4B.

Prior to this work, ATP-dependent HscA/HscB enhancement of [2Fe-2S] cluster transfer from *A. vinelandii* and *E. coli* IscU has only been demonstrated using the corresponding Isc Fdxs as acceptor proteins ([15;20]). However, the rate constant for HscA/HscB/MgATP-dependent cluster transfer from IscU to IscFdx (800 M\(^{-1}\)min\(^{-1}\) ([15])) and the rate enhancement compared to non-chaperone-assisted cluster transfer (40-fold ([15])) are both substantially lower than those reported in this work for HscA/HscB/MgATP-dependent [2Fe-2S] cluster transfer from *A. vinelandii* IscU to Grx5 (rate constant = 20000 M\(^{-1}\)min\(^{-1}\) and rate enhancement = ~700-fold), see Figure 2.5. This suggests that Isc Fdx is unlikely to be a physiologically relevant acceptor protein for [2Fe-2S] clusters assembled on IscU. This conclusion is in accord with the observation that Isc Fdx is an essential electron donor for ISC-mediated cluster assembly in *A. vinelandii* ([21]). It does not seem likely that the ISC system is responsible for maturation of an essential component of the ISC machinery.

The *in vitro* results presented in this work clearly demonstrate that HscA/HscB are required for efficient ATP-dependent cluster transfer from [2Fe-2S] IscU to apo-Grx5 and
illustrate the severe limitations of interpreting *in vitro* cluster transfer studies involving [2Fe-2S] cluster-bound IscU in the absence of the dedicated HscA/HscB co-chaperone system. Moreover, the ability of IscU to rapidly transfer [2Fe-2S] clusters to Grx5 in *A. vinelandii* at physiologically relevant rates adds strong support to the proposed role for monothiol Grxs as storage or carrier proteins for [2Fe-2S] clusters assembled on U-type Fe-S cluster assembly proteins, see Figure 2.6 (17). There is clearly a pressing need to extend these studies to other potential carrier proteins such as A-type (22) or NfuA-type (23) proteins to assess if these proteins can also accept clusters from IscU in a HscA/HscB/MgATP-mediated reaction and to investigate the specificity of monothiol Grxs as cluster donors with a wide range of apo-acceptor proteins.

**Conclusion**

This study demonstrates that HscA and HscB are required for efficient cluster transfer from [2Fe-2S]-IscU in the presence of ATP. The rate of cluster transfer in the presence of chaperones from *Azotobacter vinelandii* [2Fe-2S]-IscU to apo-Grx5 *in vitro* is increased ~700-fold with a resulting rate constant of 20000 M$^{-1}$ min$^{-1}$, and the reaction proceeds to completion. This suggests that [2Fe-2S] clusters assembled on IscU are efficiently transferred to Grx5 supporting the role of monothiol Grxs as important storage or carrier proteins. Furthermore, *in vitro* [2Fe-2S] cluster transfers from IscU do not yield biologically meaningful rates unless HscA, HscB, Mg-ATP, and KCl are present, and those at low rates are unlikely to play any significant role due to the dominance of more efficient ones.
References


Figure 2.1: Effect of HscB, apo-IscU and KCl on the ATPase activity of *A. vinelandii* HscA. Time course of ATP hydrolysis at 23 °C by 1.0 µM HscA alone (cyan), in the presence of 100 µM HscB (blue), in the presence of 100 µM HscB and 100 µM apo-IscU (red), and in the presence of 100 µM HscB, 100 µM apo-IscU and 150 mM KCl (black).
Figure 2.2: Time course of cluster transfer from *A. vinelandii* [2Fe-2S] IscU to apo-Grx5 monitored by UV-visible CD spectroscopy at room temperature. A. CD spectra recorded 0, 7, 20, 40, 60, 80, 120, 180 minutes after adding [2Fe-2S] IscU to apo-Grx5 in the presence of 3 mM glutathione. The red and green spectra correspond to [2Fe-2S] cluster-bound IscU and [2Fe-2S] cluster-bound Grx5, respectively, whereas the time course of cluster transfer is shown as thin-line grey spectra. B. Simulated CD spectra corresponding to quantitative [2Fe-2S]^{2+} cluster transfer from [2Fe-2S] IscU to apo-Grx5 in 10% increments of cluster formation of holo-Grx5 for the reaction mixture used in (A). $\Delta \varepsilon$ values are based on the [2Fe-2S]^{2+} cluster concentration, and the path length was 1 cm.
Figure 2.3: Time course of cluster transfer from *A. vinelandii* [2Fe-2S] IscU to apo-Grx in the presence of HscA, HscB, Mg-ATP and KCl (and GSH) monitored by UV-visible CD spectroscopy at room temperature. CD spectra were recorded at 3, 6, 10, 14, 18, 22, 26, 30, 40, 50 and 60 min after the addition of Mg-ATP to the reaction mixture. The red spectra correspond to [2Fe-2S] IscU in the presence of HscA and HscB whereas the time course of cluster transfer is shown by thin-line grey spectra.
Figure 2.4: Kinetic analysis of [2Fe-2S] cluster transfer from IscU to apo-Grx5 monitored by CD spectroscopy. The conditions are given in Figures 2.2 and 2.3, and the data points correspond to the extent of [2Fe-2S] Grx5 formation as a function of time, as determined by the difference in the UV-visible CD intensity at 457 and 408 nm. Solid lines corresponding to the best fits to second-order kinetics based on the initial [2Fe-2S] cluster concentration on IscU = 0.045 mM and the initial concentration of dimeric apo-Grx5 = 0.075 mM. A. [2Fe-2S] cluster transfer from IscU to apo-Grx5, rate constant = 30 M⁻¹min⁻¹. B. [2Fe-2S] cluster transfer from IscU to apo-Grx5 in the presence of HscA, HscB, Mg-ATP and KCl, rate constant = 20000 M⁻¹min⁻¹.
A

Percent of Av [2Fe-2S] Grx5 formed

Time (min)

B

Percent of Av [2Fe-2S] Grx5 formed

Time (min)
Figure 2.5: Comparison of the rates of cluster transfer from [2Fe-2S] IscU to apo-Fdx and apo-Grx5 in the presence and absence of HscA/HscB/MgATP/KCl.
Rates of [2Fe-2S] cluster transfer from IscU

![Graph showing rates of [2Fe-2S] cluster transfer from IscU. The x-axis represents conditions (no chaperones, HscA/HscB/MgATP/KCl), and the y-axis represents rate constant (M⁻¹ min⁻¹). The graph compares apo-IscFdx and apo-Grx5, with apo-Grx5 showing a significantly higher rate constant compared to apo-IscFdx.](image-url)
Figure 2.6: Schematic representation of the [2Fe-2S] IscU to apo-Grx5 cluster transfer reaction mediated by the HscA/HscB co-chaperones and the role of Grx5 as a cluster storage or carrier protein in Fe-S cluster biosynthesis. Color code: pink, Fe; yellow, S.
holo- $Av$IscU

apo- $Av$IscU

HscA/HscB/Mg-ATP/KCl

holo- $Av$Grx5

apo- $Av$Grx5