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Function of IscA in biogenesis of Iron-sulfur clusters and repair of NO-modified iron-sulfur proteins

Juanjuan Yang

Louisiana State University and Agricultural and Mechanical College, juliaiudreams@hotmail.com

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FUNCTION OF ISCA IN BIOGENESIS OF IRON-SULFUR CLUSTERS AND REPAIR OF NO-MODIFIED IRON-SULFUR PROTEINS

A Dissertation
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by
Juanjuan Yang
B.S., Henan Normal University, 1999
M.S., South China Agricultural University, 2003
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Abstract

Iron-sulfur (Fe-S) clusters are ubiquitous prosthetic groups that function in diverse fundamental life processes. However, the biogenesis of iron-sulfur clusters *in vivo* is not a spontaneous process. Previous studies indicated that Fe-S clusters may be synthesized by three major systems: the Nif, the ISC, and the SUF systems. Among these three systems, IscU, NifU are the scaffold proteins for the Fe-S clusters assembly. IscA and its paralog, SufA are proposed as an alternative iron-sulfur cluster assembly scaffold proteins. The cysteine desulfurases: IscS, NifS and SufS catalyze desulfurization of the L-cysteine and provide sulfide for Fe-S clusters assembly. However, the iron donor for the Fe-S clusters assembly remains poorly understood.

In this research, we reported that IscA is a strong iron binding protein. Under physiological conditions, if only iron is available, iron will bind to IscA. The addition of L-cysteine to this iron-bound IscA mobilizes the iron center in IscA and transfer iron to IscU for the Fe-S cluster assembly. However, if both iron and sulfide are available, Fe-S clusters are preferred to be assembled in IscU. Under oxidative stress conditions, IscA fails to bind ferrous iron due to the oxidation of its iron binding thiolate groups. CyaY, an *E. coli* homology of Frataxin is able to bind iron under oxidative stress conditions and effectively alleviate the production of the deleterious hydroxyl free radicals. Nevertheless, unlike IscA, CyaY cannot function as an efficient iron donor for the Fe-S clusters assembly due to its weak iron binding property.

We also investigated the repair mechanism for the NO-modified aconitase B [4Fe-4S] clusters. We found that *E. coli* [4Fe-4S] aconitase B is readily converted to the protein-bound DNICs by NO *in vitro* and *in vivo*. L-cysteine and oxygen are required for decomposition of the protein-bound DNICs. We further demonstrated that a complete repair of the NO-modified aconitase B requires two sequential steps: decomposition of the protein-bound DNICs requires
both L-cysteine and oxygen, and the reassembly of Fe-S clusters, which requires Fe-S clusters assembly machinery.
Chapter I
Introduction

1.1 Diversity of Iron-Sulfur Proteins

Iron-sulfur [Fe-S] clusters are ubiquitous and ancient prosthetic groups that are essential for diverse fundamental biological life processes (Johnson et al., 2005). Iron-sulfur clusters contain nonheme iron and inorganic sulfide. When proteins contain [Fe-S] groups, they are referred to as iron-sulfur proteins (Johnson et al., 2005, Rees, 2002). In iron-sulfur proteins, Fe ions exhibit almost exclusively tetrahedral coordination to S ligands, which are provided either by the γ-sulfur of cysteine or by bridging ligand (Rees, 2002, Johnson et al., 2005). Iron-sulfur proteins are present in all living organisms and exhibit numerous functions involved in oxidative respiration, cofactor biosynthesis, ribosome biogenesis, regulation of gene expression, and DNA-RNA metabolism (Lill & Muhlenhoff, 2008, Rouault & Tong, 2005, Johnson et al., 2005, Rees, 2002). Iron-sulfur clusters are also sensitive to oxygen and reactive oxygen species. Oxidation of iron-sulfur clusters may cause the irreversible disassembly / rearrangement of the iron-sulfur clusters or reversible one-electron oxidation, depending on the prevalent redox potential or the specific oxidants (Beinert & Kiley, 1999). For example, the regulator of fumarate and nitrite reduction (FNR), which controls the expression of networks of Escherichia.coli genes in response to anaerobic growth (Lazazzera et al., 1993), only has its repressor activity when an intact [4Fe-4S] cluster is assembled under anaerobic conditions (Lazazzera et al., 1993, Khoroshilova et al., 1997). Mitochondria aconitase [4Fe-4S] cluster, an enzyme converting citrate to iso-citrate in the tricarboxylic acid cycle (Beinert et al., 1996, Beinert & Kiley, 1999), is highly sensitive to oxygen and reactive oxygen species, and requires an intact [4Fe-4S] cluster for its catalytic activity. In E.coli, the aconitaseB [4Fe-4S] cluster is also vulnerable to oxygen and reactive oxygen species, resulting in inactivation of the aconitase activity (Varghese et al.,
Another major function of iron-sulfur proteins is electron transfer process (Imlay, 2006). The [4Fe-4S] cluster found in ferredoxin I and ferredoxin III from *Azotobacter vinelandii* (Gao-Sheridan *et al.*, 1998, Chen *et al.*, 2002b, Chen *et al.*, 2002a), the [2Fe-2S] Rieske protein and Rieske like proteins (Hunsicker-Wang *et al.*, 2003), and other iron-sulfur proteins are the key components in electron transfer processes. Due to their ability on transfer electrons, iron-sulfur proteins are the essential members of the respiration, photosynthesis, and nitrogen fixation processes (Rouault & Tong, 2005). Nitrogenase, a metallocluster protein containing both [4Fe-4S] clusters and FeMo cofactors (Rees, 2002, Dean *et al.*, 1993), converts nitrogen gas into ammonium by the hydrolysis of ATP (Dean *et al.*, 1993, Rees & Howard, 2000), thus providing nitrogen sources for most organisms in the world (Dean *et al.*, 1993). Cytochrome *b*$_{5f}$, the center transmembrane protein complex in photosynthesis, and cytochrome *bc*$_{1}$, an analog of cytochrome *b*$_{6f}$ in respiration, both transfer electrons to convert light energy and oxidative energy to chemical energy, respectively (Smith, 2004). DinG, a DNA-damage inducible promoter (Lewis *et al.*, 1992, Lewis & Mount, 1992), is a member of the *E. coli* SOS regulon and exhibits increased expression in response to DNA damage or inhibition of DNA replication (Lewis & Mount, 1992). DinG and its human homolog XPD both contain a [4Fe-4S] cluster and function as DNA helicases (Ren *et al.*, 2009). The [4Fe-4S] cluster in DinG is very stable even in the presence of 100 fold excess of hydrogen peroxide and thus guarantees that DNA damage can be repaired by DinG helicase activity under oxidative stress conditions (Duan *et al.*, 2008). Another representative [4Fe-4S] protein is dihydroxyacid dehydratase (IlvD). Dihydroxyacid dehydratase is an enzyme required for the branched chain amino acids biosynthetic pathway by converting the 2, 3-dihydroxyisovaleric acid or 2’3-dihydroxy-3-methylvaleric acid to the corresponding 2-keto acids, and also involved in the biosynthesis of coenzyme A (Flint *et al.*, 1993a, Flint *et al.*, 1993b). The [4Fe-4S] cluster in dihydroxyacid
dehydratase is highly sensitive to oxygen or hydrogen peroxide in vitro (Flint et al., 1993b) and in vivo (Flint et al., 1993a). Comparing with the [4Fe-4S] cluster in DinG, the cluster in dihydroxyacid dehydratase is easily disrupted and the enzymatic activity readily inactivated (Flint et al., 1993a, Flint et al., 1993b). Repair of the damaged iron-sulfur cluster in aconitase B protein, in DinG protein (Duan et al., 2008), in dihydroxyacid dehydratase (Flint et al., 1993a) and other iron-sulfur proteins will restore the enzyme activity.

1.2 Biogenesis of Iron-Sulfur Proteins

It is now clear that the assembly of iron-sulfur clusters does not take place spontaneously. Instead it requires a complicated biosynthetic machinery (Johnson et al., 2005). Earlier studies showed that, when sufficient iron, sulfide are available, under reducing conditions, iron-sulfur clusters are assembled in proteins in vitro (Loiseau et al., 2005). At least four different types of iron-sulfur cluster biosynthesis machinery have been discovered (Loiseau et al., 2005). All of them contain a cysteine desulfurase and an iron-sulfur cluster assembly scaffold protein (Johnson et al., 2005). The first iron-sulfur cluster assembly machinery is NifSU system (Yuvaniyama et al., 2000, Zheng et al., 1993a). NifS protein from Azotobacter vinelandii was identified as the cysteine desulfurase in 1993 by Dean and coworkers (Zheng et al., 1993a) and was the first discovered member of the cysteine desulfurase family. Deletion of NifS gene resulted in the lower activity of nitrogenase in Azotobacter vinelandii (Zheng et al., 1993a). This invoked great interests to work on the property of NifS. Dean and co-workers revealed that NifS is a pyridoxal phosphate (PLP)-dependent enzyme that converts L-cysteine to inorganic sulfur and L-alanine (Zheng et al., 1993a). Because the cysteine desulfurase activity of NifS is sensitive to the thiol-specific alkylating reagents, they proposed that cysteinyl thiolate groups are involved in the active site (Zheng et al., 1993a). NifU is an iron-sulfur cluster assembly scaffold protein in Azotobacter vinelandii (Yuvaniyama et al., 2000). The NifS mediated [2Fe-2S] clusters can be
temporarily assembled in NifU and eventually transferred to nitrogenase (Yuvaniyama et al., 2000). Both NifS and NifU are required for full activation of nitrogenase activity in Azotobacter vinelandii (Yuvaniyama et al., 2000). IscA\textsuperscript{\textit{nif}} was also found in Azotobacter vinelandii, and its function was considered as an alternative scaffold protein.

The second iron-sulfur cluster assembly machinery discovered was the Isc\textit{SUA-HscBA-fdx} system (Zheng et al., 1998, Takahashi & Nakamura, 1999). In this system, IscS, a homolog of NifS, is also a PLP-dependent cysteine desulfurase and delivers sulfur for the iron-sulfur cluster assembly (Schwartz et al., 2000b); IscU, a homolog of the C-terminus of NifU, is considered as an iron-sulfur cluster assembly scaffold protein (Agar et al., 2000a); IscA, a homolog of IscA\textsuperscript{\textit{nif}} in Azotobacter vinelandii, is also believed as an alternative iron-sulfur cluster assembly scaffold protein (Krebs et al., 2001). This system is expresses under normal growth conditions and is so called the house-keeping iron-sulfur cluster assembly machinery. It exists not only in Azotobacter vinelandii but also in \textit{E. coli}, in yeast (Li et al., 1999c), plants and humans. All three genes encoding IscA, IscU and IscS are highly conserved from \textit{E.coli}, Azotobacter vinelandii, yeast, Haein. influenzae and human cells (Li et al., 1999c, Zheng et al., 1998, Takahashi & Nakamura, 1999, Zheng et al., 1993a, Lill & Muhlenhoff, 2006). Deletion of ISC system, especially IscS, has a dramatic effect on \textit{E.coli} cell growth (Schwartz et al., 2000b). Disruption of IscS in \textit{E. coli} cells also causes an auxotrophy for thiamine and nicotinic acid under aerobic conditions (Schwartz et al., 2000b). The IscR (iron-sulfur cluster regulator) is a transcription regulator for the expression of the ISC system (Schwartz et al., 2001). Deletion of IscR gene in \textit{E.coli} increases the gene expression of IscU, IscA, IscS and other ISC genes (Schwartz et al., 2001). The exact function of the two heat shock cognate proteins: HscB and HscA are not well understood. However, recent studies showed that IscU stimulates the ATPase activity of HscA, and the HscB promotes the interaction between IscU and HscA through
binding to IscU before IscU binds to HscA (Hoff et al., 2000). The ferredoxin [2Fe-2S] is a protein that may function as an electron donor or acceptor and provide reducing equivalent, for conversion from [2Fe-2S] clusters to [4Fe-4S] clusters on IscU (Fukuyama, 2004, Venegas-Caleron et al., 2009).

The third system is the SUF system which was first discovered in E. coli and in the related plant pathogen Erwinia Chrysanthemi and then in a parasite and Arabidopsis thaliana (Loiseau et al., 2005). SUF system contains SufABCDSE genes (Nachin et al., 2001). At first, it was thought that SUF system played a minor role in the iron-sulfur cluster assembly and potentially acted as back up for the deficiency of the ISC system. This view arose from the fact that mutation of the SUF system had no significant effect on E. coli cell growth under routine laboratory growth conditions (Loiseau et al., 2005). It is now clear that this system works as the iron-sulfur cluster assembly machinery under oxidative stress or iron limiting conditions. SufA, a homolog of IscA, was considered as an alternative iron-sulfur cluster assembly scaffold protein (Ollagnier-De Choudens et al., 2003). SufS, a homolog of IscS and NifS, is a cysteine desulfurase (Mihara et al., 1997, Outten et al., 2003). SufC is an ATPase subunit of ABC transporters and has been shown to exhibit ATPase activity (Rangachari et al., 2002). SufB and SufC proteins locate on the E. coli cell membranes (Rangachari et al., 2002). SufS and SufE interact with each other and form SufSE complex. SufB, SufC and SufD associate with each other and form SufBCD multi-protein complex (Layer et al., 2006). SufE and IscU share similar structure (Goldsmith-Fischman et al., 2004). SufE works as a sulfur acceptor protein in SUF system (Outten et al., 2003, Goldsmith-Fischman et al., 2004). The cysteine 51 residual in SufE directed the transfer of sulfur atom of cysteine persulfide from SufS to SufE (Ollagnier-de-Choudens et al., 2003). SufE promotes the sulfur transfer from SufS to SufB for the iron-sulfur clusters assembly (Layer et al., 2007). SufE also enhances cysteine desulfurase activity of SufS
up to 8 fold. Nevertheless, when both SufBCD complex and SufE are present, the cysteine desulfurase activity of SufS is increased up to 32 fold (Outten et al., 2003). The SUF genes are highly conserved from cyanobacteria to higher plants (Ellis et al., 2001, Outten et al., 2003).

The latest discovered iron-sulfur cluster assembly system is CSD system (Loiseau et al., 2005). This system contains CsdA-CsdE. CsdA is homolog of SufS and IscS, and has cysteine desulfurase activity that converts L-cysteine to L-alanine and sulfur. CsdE is a homolog of IscU, and works as a sulfur acceptor. Yeast two-hybrid system studies revealed that CsdA and CsdE tightly interact with each other during sulfur transfer process. CsdE stimulates the cysteine desulfurase activity of CsdA. The cysteine 61 residual in CsdE is essential in sulfur transferring process from CsdA to CsdE (Loiseau et al., 2005). Deletion of CsdA-CsdE system does not cause any defect in cell growth, auxotrophy, or colony morphology either under aerobic or anaerobic conditions. However, over-expressing of the CsdA-CsdE operon can repress the cell auxotrophy for nicotinic acid in the IscS deletion mutant (Loiseau et al., 2005).

In all these four iron-sulfur cluster assembly systems, NifS (Yuvaniyama et al., 2000, Zheng et al., 1993a) and its homologs, IscS (Schwartz et al., 2000b), SufS (Mihara et al., 1997, Outten et al., 2003) and CsdA (Loiseau et al., 2005) provide sulfur to form iron-sulfur clusters on scaffold proteins by converting L-cysteine to L-alanine and sulfur. Likewise, NifU (Yuvaniyama et al., 2000) and its homologs: IscU (Agar et al., 2000a), SufE (Outten et al., 2003, Goldsmith-Fischman et al., 2004, Ollagnier-de-Choudens et al., 2003, Layer et al., 2007) and CsdE (Loiseau et al., 2005) all function as sulfur acceptors and work as iron-sulfur cluster assembly proteins. Nevertheless, the iron donor for the iron-sulfur cluster assembly remains poorly understood. Intracellular free iron concentration is extremely low, as an elevated level of the intracellular iron content will promote the production of hydroxyl free radicals through Fenton Reactions (Keyer et al., 1995). Thus, there must be some proteins that can recruit iron and deliver iron for the
iron-sulfur cluster assembly. Although IscA (Krebs et al., 2001) and SufA (Ollagnier-De Choudens et al., 2003) were considered as alternative iron-sulfur cluster assembly scaffold proteins, recently studies show that IscA may function as an iron binding protein (Ding et al., 2005b, Ding et al., 2005a, Ding et al., 2004, Ding & Clark, 2004). It has been shown that in the yeast *Saccharomyces cerevisiae*, deletion of IscA homolog results in iron accumulation in mitochondria and inactivation of iron-sulfur enzymes involved in cell respiration chain (Jensen & Culotta, 2000). Furthermore, the X-ray crystallography of IscA (Bilder et al., 2004) and the NMR structure of IscU homologue from *Thermotoga maritima* (Bertini et al., 2003) suggested that these two proteins are structurally very different. The NMR data revealed that structure of IscU homologues are flexible with widely different conformations (Bertini et al., 2003, Ding & Clark, 2004). On the other hand, IscA has a highly conserved compact global domain and an apparently mobile C-terminal tail (Bilder et al., 2004). None of those IscU structural motifs appears to correspond directly to structural elements in IscA protein (Bilder et al., 2004). These structural studies suggested that IscA may have more unique function other than acting as an alternative iron-sulfur cluster assembly scaffold protein.

### 1.3 IscA in the Iron-Sulfur Cluster Assembly

Ding and his co-workers proposed that IscA may function as an iron donor for the iron-sulfur cluster assembly in 2004 (Ding & Clark, 2004). In their research, they found that IscA is an iron binding protein with an iron association constant of $3 \times 10^{19} \text{M}^{-1}$. IscA can further transfer iron for the iron-sulfur cluster assembly on the scaffold protein IscU in the presence of IscS, L-cysteine and dithiothreitol in vitro. IscA purified from *E. coli* cells has a redish color and an absorption peak at 315 nm, suggesting that IscA could be an iron binding protein (Ding & Clark, 2004). In extremely low intracellular iron concentration conditions, IscA is able to recruit iron and transfer iron for the IscS-mediated iron-sulfur cluster assembly in IscU (Ding et al., 2004).
When either of three conserved cysteines located in the central pocket of IscA dimers (Bilder et al., 2004) is mutated, IscA mutant fails to bind iron and transfer the iron for the iron-sulfur clusters assembly in IscU (Ding et al., 2004, Ding & Clark, 2004). It was further demonstrated that IscA is able to bind iron under physiological relevant conditions (Ding et al., 2005b), and that the iron center in IscA can be mobilized by L-cysteine and transferred to IscU for the iron-sulfur cluster assembly in the presence of IscS (Ding et al., 2005a).

To reconcile the two models for the IscA function, we have re-evaluated the iron-sulfur cluster binding affinity of IscA and IscU under physiological relevant conditions. We found that if equal amounts of IscA and IscU were incubated together in with ferrous iron and IscS but without L-cysteine under physiologically relevant conditions, ferrous iron was preferred to bind to IscA but not to IscU. However, when equal amount of IscA and IscU were incubated with ferrous iron, IscS and L-cysteine under physiologically relevant conditions, iron-sulfur clusters were assembled in IscU but not IscA. In another word, IscA is a preferred iron binding protein whereas IscU is a preferred iron-sulfur cluster assembly protein. In this context, we propose that IscA may bind iron and prevent the formation of inaccessible ferric hydroxide under aerobic conditions and that L-cysteine mobilizes the iron center in IscA for the iron-sulfur clusters assembly in IscU.

1.4 Role of CyaY and Frataxin in the Biogenesis of Iron-Sulfur Clusters

Besides IscA, *E. coli* frataxin/CyaY was also proposed as an iron donor for the iron-sulfur cluster assembly in different organisms (Layer et al., 2006, Babcock et al., 1997, Foury & Cazzalini, 1997). The human gene X25 encodes a 210-amino acid frataxin which is highly conserved from distant species, like *E. coli*, yeast and *Caenorhabditis elegans* (Campuzano et al., 1996). Friedreich Ataxia (FRDA), the most prevalent inherited ataxia in human, was identified as an autosomal recessive and degenerative disease resulting in dysfunction of the
central and peripheral nervous systems and heart (Patel & Isaya, 2001, Campuzano et al., 1996). FRDA occurred at a frequency of 1-2 per 50,000 and is clinically characterized by progressive gait and limb ataxia from early childhood and final death within several decades (Campuzano et al., 1996, Patel & Isaya, 2001). In the majority of cases (around 95% of the patients), the FRDA patients are caused by large expansions of a homozygous GAA triplet-repeat sequence (66-1800 triplets) located within the first intron of the gene X25. In minority cases (less than 5%), Friedreich Ataxia is resulted from the compound heterozygotes of the GAA expansion and the point mutations within the gene (Patel & Isaya, 2001).

Frataxin protein is a highly conserved from bacteria to humans (Gibson et al., 1996, Campuzano et al., 1996, Dhe-Paganon et al., 2000, Canizares et al., 2000). All frataxin orthologues have a highly conserved acidic block domain of 120 amino acids that folds into a globular compact domain (Adinolfi et al., 2002b). A conserved segment of frataxin has been detected, which is important for the function domain (Dhe-Paganon et al., 2000, Canizares et al., 2000). The crystal structure of human frataxin (Dhe-Paganon et al., 2000) showed that it is structurally similar to yeast frataxin (He et al., 2004) and the bacterial frataxin ortholog CyaY (Cho et al., 2000). Deletion of yeast frataxin homolog (YFH1) causes a severe growth phenotype in rich medium containing glycerol and ethanol as the carbon source. This phenotype indicated that the frataxin deletion mutant fails to perform oxidative phosphorylation (Babcock et al., 1997). Deletion of YFH1 not only results in mitochondria dysfunction but also deficiency of iron homeostasis (Babcock et al., 1997). Frataxin is essential for the embryonic development in mouse because deletion of frataxin gene (FRDA) in mouse causes the early embryonic lethality through cell apoptosis and necrosis but interestingly no iron accumulation (Cossee et al., 2000). RNAi-mediated suppression of frataxin homolog gene (Frh-1) prolonged the lifespan in Caenorhabditis elegans and made it resistant to oxidative stress. Frh-1RNAi animals are viable
and fertile yet are small and pale comparing with the control RNAi animals (Ventura et al., 2005). However, the specific function of frataxin/CyaY is still not fully understood. Recently, it was proposed that frataxin/CyaY may have an important function in iron-sulfur clusters biogenesis because FRDA patients have lower activities of the iron-sulfur cluster-containing respiratory complex I, complex II and complex III and aconitase and other iron-sulfur proteins (Rotig et al., 1997).

It has been reported that Frataxin/CyaY can bind ferrous and ferric iron with a fairly weak binding affinity (Adamec et al., 2000b). E. coli frataxin/CyaY and yeast frataxin (YFH1) can form similar iron-promoted aggregates (Adinolfi et al., 2002b). Iron-promoted aggregation in CyaY or Yfh1 can compete with chelating agents or affected by ionic strength (Adinolfi et al., 2002b). Frataxin/CyaY was also reported to bind iron and mediate the transfer of iron to IscU through protein-protein interaction in the early stage of the iron-sulfur clusters assembly in human (Yoon & Cowan, 2003), yeast (Ramazzotti et al., 2004, Gerber et al., 2003) and E. coli (Layer et al., 2006). However, only the holo-frataxin, but not apo-frataxin, can interact with IscU (Yoon & Cowan, 2003). Furthermore, the iron-loaded frataxin not only interact with IscU but also with the cysteine desulfurase IscS, (Gerber et al., 2003, Layer et al., 2006); the succinate dehydrogenase, other mitochondrial electron transfer complexes (Gonzalez-Cabo et al., 2005) and aconitase (Bulteau et al., 2004). Taken together, these results suggested that frataxin/CyaY could function as a physiological iron donor for the biogenesis of iron-sulfur clusters.

Interestingly, deletion of frataxin orthologues in E. coli (Li et al., 1999b) and S. cerevisiae (Duby et al., 2002) has no effect on the iron-sulfur cluster assembly, indicating that frataxin orthologues are not essential for the iron-sulfur clusters biogenesis. Instead, it was shown that deletion of frataxin orthologues in yeast cells led to a great sensitivity to reactive oxidative species, mitochondria-mediated apoptosis and DNA damage (Karthikeyan et al., 2002,
Karthikeyan et al., 2003). Furthermore, Condo et al reported that an extra–mitochondrial pool of frataxin can effectively prevent mitochondrial oxidative damage and apoptosis and replace mitochondrial frataxin in promoting survival of Friedreich ataxia cells (Condo et al., 2006). Thus, it was suggested that the primary function of frataxin/CyaY is to detoxify the redox active free iron in cells (Condo et al., 2006, Karthikeyan et al., 2003, Karthikeyan et al., 2002).

In search for the specific iron donors for the biogenesis of iron-sulfur clusters, we have shown that IscA, a key member of the iron-sulfur cluster assembly machinery conserved from bacteria (Zheng et al., 1998, Schwartz et al., 2001, Tokumoto & Takahashi, 2001), yeast (Jensen & Culotta, 2000) and human (Cozar-Castellano et al., 2004), is a novel iron binding protein with an iron association constant of $2.0 \times 10^{19} \text{M}^{-1}$ in the presence of dithiolthreitol or thioredoxin reductase system (Ding et al., 2004, Ding et al., 2005b). When IscA protein was purified from E. coli, it is partially iron-loaded and appears a redish color. L-cysteine can easily release the iron center of IscA and finally transfer the iron to iron-sulfur cluster assembly protein IscU.

To further elucidate the potential role of frataxin/CyaY in the biogenesis of iron-sulfur clusters, we compared the iron binding property of IscA and CyaY under physiological and oxidative stress conditions. The results showed that E.coli CyaY failed to bind ferrous iron under physiological condition even with 10-fold excess of iron in the incubation solution. On the other hand, IscA binds to iron with a high binding affinity under physiological conditions. However, in the presence of hydrogen peroxide, CyaY becomes a competent iron binding protein and dramatically alleviated the production of hydroxyl free radicals. However, IscA completely loses its iron binding activity in oxidative stress conditions. Thus, IscA and CyaY may work together for the iron-sulfur cluster assembly and iron metabolism in cells.
1.5 NO Cytotoxicity and Iron-Sulfur Proteins

Previous studies show that Iron-sulfur clusters are generally very sensitive to nitric oxide (Gardner, 1997, Gardner et al., 1997b). NO (nitric oxide) is a small lipophilic molecule and an unstable aqueous soluble gas with a very short half-life (around 5 seconds) in biological systems under aerobic conditions. NO is produced in aqueous solution by inducible nitric oxide synthase (iNOS) in activated macrophage cells and has the ability to diffuse across cell membranes and reach to adjacent cells. However, due to its short half-life time, NO can only reach adjacent cells or compartments. When diffusing to nearby cells, NO reacts with the iron atoms of the heme groups of the guanylate cyclase or other kind of hemoproteins to activate specific enzymes. This interaction represents a widespread mechanism that converts the extracellular stimulus to the biosynthesis of cyclic GMP in target cells (Ignarro, 1990). NO can also mediate the cell apoptosis through the chromatin condensation and internucleosomal fragmentation (Brune et al., 1999). Furthermore, it has been demonstrated that iNOS-generated NO can also protect mammalian cells from parasites, bacteria and several virus infections, promote tumor angiogenesis, and facilitate tissue destruction (Brune et al., 1999). At endogenous NO concentration levels, which are around 10nM to 1µM, NO modulates a signal transduction pathway in anti-apoptotic process (Brookes et al., 2000). However, if cells were exposed to NO at a concentration over 1.0 µM, NO will inactivate of ATPase and induce the necrotic cell death (Brookes et al., 2000). In rat mitochondria, it has also been shown that binding of NO to the binuclear heme α3/CuB center in the cytochrome c oxidase of the respiration chain reversibly inhibits the respiratory electron transfer (Brookes et al., 2003).

Besides the hemoproteins, the other major iron-containing proteins are iron-sulfur proteins (Beinert et al., 1997, Johnson et al., 2005). A number of studies showed that iron-sulfur proteins, particularly [4Fe-4S] cluster proteins (Gardner et al., 1997b, Kennedy et al., 1997), are
the major targets of NO (Drapier, 1997, Gardner et al., 1997b). Unlike the reversible binding of NO to the iron atoms in hemeproteins, NO converts the protein-bound iron-sulfur clusters to the protein-bound dinitrosyl iron complexes (DNICs). This specific structure of DNICs has a unique EPR (electron paramagnetic resonance) signal at g = 2.04 (Kennedy et al., 1997). The EPR signal of the protein-bound DNICs has been observed in cytotoxic activated macrophages where NO is produced by iNOS (inducible NO synthase) (Lancaster & Hibbs, 1990), in tumor cells co-cultured with activated macrophages (Drapier et al., 1991), in non-heme proteins of acute cardiac allograft injection (Pieper et al., 2003), in rat gastro-esophageal junction where NO was luminally generated from nitrite (Asanuma et al., 2007). Although small molecule thiols such as, L-cysteine, and N-acetyl-L-cysteine can also form the DNIC complex when ferrous iron and NO are available in vitro (Vanin et al., 1993, Boese et al., 1995b, Butler & Megson, 2002), existence of the small molecule thiols-bound DNICs have never been demonstrated in cells (Ueno & Yoshimura, 2000, Pedersen et al., 2007), likely due to the limitation of the short half-life of the small molecular thiol-bound DNICs in aqueous solution (Asanuma et al., 2007). When E.coli cells are exposed to NO at a rate comparable to that reported for the NO production in activated macrophages, neutrophils or other cells (Gobert et al., 2001, Kriegstein et al., 2001), a large number of iron-sulfur proteins are modified forming the protein-bound DNICs (Ren et al., 2008).

Because iron-sulfur proteins are involved in diverse physiological functions, inactivation of iron-sulfur protein by NO is expected to result in dysfunction of multiple cellular processes. Thus modification of iron-sulfur clusters by NO must be efficiently repaired if cells are to survive. It has been shown that in E. coli, the NO modified iron-sulfur clusters are quickly reared under aerobic growth condition (Ding & Demple, 2000, Hyduke et al., 2007). However, under anaerobic growth conditions, E. coli cells fail to repair any NO-modified iron-sulfur clusters (Ren et al., 2008). The repair mechanism for the NO-modified iron-sulfur clusters
remains largely elusive. To explore the repair mechanism for the NO-modified iron-sulfur clusters, here we reported that iron-sulfur clusters in *E. coli* aconitase B can be easily modified by NO forming the protein-bound dinitrosyl-iron complexes under both aerobic and anaerobic conditions. The EPR signal of DNICs in *E. coli* cells overexpressed with aconitase B further revealed that the interactions between the protein bound iron-sulfur clusters and NO are specific because oxygen does not significantly affect the NO-mediated modification of iron-sulfur clusters. We further found that repair of NO-modified iron-sulfur proteins in *E. coli* requires a combination of the iron-sulfur cluster assembly machinery, intracellular reduced thiol groups and oxygen. We also showed that the iron-sulfur assembly proteins: IscA/SufA are required for the repair of the NO-modified aconitase B but are not essential for the decomposition of DNICs. The results suggest that a complete repair of the protein-bound DNICs have two steps: decomposition of the protein-bound DNICs and reassembly of iron-sulfur clusters in protein.
Chapter II
Interplay of IscU and IscA in Biogenesis of Iron-sulfur Clusters

2.1 Introduction

Iron-sulfur [Fe-S] clusters are ubiquitous and one of the most ancient prosthetic groups that are required by different fundamental life processes including respiration, photosynthesis and nitrogen fixation which are central to the life on earth (Rouault & Tong, 2005, Johnson et al., 2005, Lill & Muhlenhoff, 2008). Iron-sulfur clusters also play an important role in the regulation of gene expression, amino acid metabolism, RNA modification and DNA repair. Iron-sulfur clusters consist of non-heme iron and inorganic sulfide. When proteins are bound to iron-sulfur clusters, they are referred to as iron-sulfur proteins (Johnson et al., 2005). Previous studies showed that when sufficient ferrous iron and sulfide are present under reducing conditions, iron-sulfur clusters are assembled in proteins (Loiseau et al., 2005). However, the biogenesis of iron-sulfur clusters is not a spontaneous process.

It has been reported that the sulfur in iron-sulfur clusters is derived from L-cysteine via cysteine desulfurases. There are at least four cysteine desulfurases that have been discovered in living cells so far: NifS, which is found in nitrogen fixation bacterium *Azotobacter vinelandii*; (Zheng *et al.*, 1993b) IscS, which is highly conserved from *E. coli* to Human cells; SufS (Outten *et al.*, 2003, Layer *et al.*, 2007), and the heteromeric CsdA-CsdE (Loiseau *et al.*, 2005). *E. coli* cells contain all these cysteine desulfurase except NifS in 1993. Dean’s lab first demonstrated that IscS contains a pyridoxal 5-phosphate, and can convert L-cysteine to sulfide and L-alanine (Zheng *et al.*, 1993b). Deletion of IscS gene greatly decreased the activity of iron-sulfur enzymes in *E. coli* cells and blocked the *E. coli* cell growth in minimum medium containing glucose (Schwartz *et al.*, 2000a).

IscS is a member of the *IscSU A* operon, which also encodes two other highly conserved
genes: IscA and IscU. It is now clear that IscS catalyzes desulfurization of L-cysteine and transfers sulfane sulfur for the iron-sulfur cluster assembly in the proposed scaffold protein IscU (Urbina et al., 2001, Agar et al., 2000a, Kato et al., 1966, Smith et al., 2005b). The assembled iron-sulfur clusters in IscU are finally transferred to target proteins (Agar et al., 2000a, Smith et al., 2005b, Mansy et al., 2002, Wu et al., 2002c, Wu et al., 2002b).

The function of IscA is still not very clear. Previous studies suggested that IscA is an alternative iron-sulfur cluster assembly scaffold protein (Ollagnier-de-Choudens et al., 2001, Krebs et al., 2001, Wu et al., 2002a). On the other hand, IscA is also shown to be a novel iron binding protein, with an iron association constant of 2.0-3.0×10^{19} \text{M}^{-1} in the presence of a thioredoxin/thioredoxin reductase system or dithiothreitol (Ding et al., 2005b, Ding et al., 2004, Ding & Clark, 2004). Thus it has been proposed that IscA may act as an iron donor for the iron-sulfur cluster assembly in IscU (Ding & Clark, 2004, Ding et al., 2004).

To reconcile the two models proposed for the function of IscA, we have studied the iron binding the iron sulfur cluster binding activity of IscA and iscU under physiologically relevant conditions. The results showed that in the presence of ferrous iron, L-cysteine and the cysteine desulfurase IscS, IscU is a preferred iron-sulfur cluster assembly scaffold protein. If L-cysteine is absent in the incubation solution, IscA but not IscU acts as an iron chaperon that binds ferrous iron. The iron binding in IscA appears to prevent the formation of the biologically inaccessible ferric hydroxide under aerobic conditions. Subsequent addition of L-cysteine mobilizes the iron center in IscA for the iron-sulfur cluster assembly in IscU even under aerobic conditions. The potential interplay between IscA and IscU for the biogenesis of iron-sulfur clusters will be discussed.
2.2 Methods and Materials

2.2.1 The Gene Cloning and Protein Purification of IscA/IscU/IscS

The coding regions of *E. coli* IscA, IscU and IscS were amplified from wild type *E. coli* genomic DNA by PCR using the PCR ready to go beads (Amersham Biosciences). Three pairs of primers were designed to contain an *NcoI* restriction site in one primer and a *HindIII* restriction site in the other primer. The sequences of the primers are as follows: IscA-1, 5’-AGGTTTGCCATGGCGATTACACT-3’; IscA-2, 5’-TGCGCATCAAAGCTTGAAAGCTTTTC-3’; IscU-1, 5’-GAATTTACCATGGCTTGACCGAA-3’; IscU-2, 5’-TTGCTTCAAGCTTGCTTTATAG-3’; IscS-1, 5’-GCATTGAGCCATGGACGGAGTTTA-3’; IscS-2, 5’-CCGATTAAGCTTAGCCATTCGA-3’. The *NcoI/HindIII*-digested PCR products were ligated to an expression vector pET28b+ to yield pTISCA (for IscA), pTISCU (for IscU) and pTISCS (for IscS). The plasmids were introduced into *E. coli* strain BL21 (DE3). The *E. coli* overnight culture for pTISCU or pTISCA or pTISCS was diluted (1:100) into fresh Luria-Bertani medium. After 3 hours growth in 37°C with aeration (250 rpm), 200 μM of isopropyl-B-D-thiogalactopyranoside was added to the culture. The cells were harvested after 2 hours incubation. The *E. coli* cells with overexpressed IscA or IscU or IscS were resuspended into 30 ml of prechilled buffer A containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.0. Cells were disrupted by passing them through a French press once and centrifuged at 20,000xg for 45 min to remove cell debris. The supernatants were run through a 2 ml superfine Ni-agarose column (Qiagen) attached to an AKTA-fast protein liquid chromatography system (Amersham Biosciences). The column was washed with 3 column volumes of buffer A (5mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.0) and 3 column volumes of Buffer B (15 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.0). The protein was then eluted with buffer C (250 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 8.0). The eluted protein was applied to a 5 ml Hitrap-desalting
column (Amersham Biosciences) equilibrated with buffer D (500 mM NaCl, 20 mM Tris, pH8.0) to remove imidazole from protein samples. The fast protein liquid chromatography system was controlled by UNICORN software (Amersham Biosciences) that allows a reproducible protein purification profile. The protein concentration was determined by the UV-visible Spectrometer according to their extinction coefficients. About 2 fold excess of the pyridoxal phosphate was added to purified IscS protein followed by another desalting process. The purity of each protein was greater than 95% judging from its electrophoresis on a 15% polyacrylamide gel containing SDS followed by staining with Comassie Blue (Ding & Clark, 2004, Yang et al., 2002).

2.2.2 The Protein Purification of Thioredoxin (TrxA) and Thioredoxin Reductase (TrxB)

The recombinant plasmid for expression TrxA and TrxB gene are named pDL59 and pTrR301 accordingly. They were kindly provided by Dr. Scott B. Mulrooney (University of Michigan).

2.2.2.1 Purification of Thioredoxin –TrxA

The E. coli cells containing the pDL59 vector were grown at 30 °C overnight with rapid shaking in 500 ml of 2 × YT rich medium (1.2% w/v of trypton, 2.4% w/v of yeast, 4 ml glycerol, 0.1 M K-phosphate pH 7.0) containing 100 μg/mL ampicillin. When the OD{sub 600} is around 1.0, the temperature was changed to 42°C to induce the protein expression. The cells were harvested by centrifugation when the OD{sub 600} reached to 10. The cells were broken by passing through the prechilled French press once in 20 mM Tris (pH 7.6). Then, 2% w/v of streptomycin sulfate was added to the solution with slowly stirring for 1 hour followed by 1 hour spin at 16,000 rpm. The supernant was equilibrated at 4°C in dialysis tubing with a cut off of 6-8 KDalton molecular weight against 20 mM Tris (pH 7.6) for three times. A Q-sephrose anion exchange column attached to the FPLC was applied by loading the dialyzed solution through it. The equilibration buffer contains 20 mM Tris (pH 7.6). The protein
was eluded by using over 8.4 column volumes of a linear gradient of 0-140 mM NaCl. The proteins started to elute out near the end of the gradient at ~2/3 of the way. All the fractions containing thioredoxin were pooled and precipitated with 80% ammonium sulfate. The pellet was resuspended in minimal amount of 20 mM phosphate (pH 7.6) and further loaded onto a Sephadex G-75 superfine sizing column equilibrated with 20 mM phosphate (pH 7.6). After gel filtration process, the second peak contained the thioredoxin. All the fractions with thioredoxin were pooled together and dialyzed against desalting buffer (20 mM Tris (pH 8.0), 0.5 M NaCl). The extinction coefficient at 280 nm = 13,700 M$^{-1}$ cm$^{-1}$ was used to quantitate the concentration of thioredoxin. The protein purity was over 90% judging form the 15% SDS PAGE gel (Veine et al., 1998a).

2.2.2.2 Purification of Thioredoxin Reductase –TrxB

Frozen cells containing pTrR301(TrxB) plasmid were streaked on 2×YT rich medium agar plate supplemented with 20 mM glucose, 100 µg/mL ampicillin, 25 µg/mL kanamycin and incubated at 37°C overnight. A single colony from the plate was inoculated to 500 ml of 2×YT liquid rich medium supplemented with 50 mM phosphate, pH 7.0, 20 mM glucose, 100 µg/mL ampicillin and 25 µg/mL kanamycin and grown for 24 hours with vigorous shaking. The cell culture was ice-chilled and centrifuged for 10 min at 4°C with a speed of 10,000 g. The cell pellet was resuspended in 2~3 volumes of 10mM phosphate buffer, 0.3 mM EDTA, pH 7.6 (bufferA), which contains 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µmol FAD (Sigma)(Mulrooney, 1997). The cells were disrupted by passing through the pre-chilled French press once and followed by adding streptomycin sulfate to a final concentration of 2% (w/v). The crude preparation was centrifuged at 27,000 g for 30 min at 4°C. The supernant was transferred to a new tube and 0.56 g/mL of ammonium sulfate was slowly added into it with a moderate stirring speed. Once the ammonium sulfate dissolved completely, the resulting
suspension was centrifuged at 12,000 g for 10 min at 4°C. The pellet showed a yellowish color and was dissolved in 10~15mL of bufferA and dialyzed overnight against large volume (4 liters) of bufferA with several changes. The dialyzed solution was then filtered through a 0.45 µM syringe filter.

A 2 cm diameter×19 cm long 2',5'-ADP Sepharose column (Pharmacia Biotech, Piscataway, NJ) pre-equilibrated with buffer A was applied for purification of the enzyme. Once the preparation was loaded, the column was washed with 3~5 column volumes of buffer A. The enzyme was eluted by using a 124 mL of 0 to 1M NaCl gradient in buffer A. 15% SDS PAGE gel was applied to check the purity of protein. The fractions containing the thioredoxin reductase were pooled together and the protein concentration was determined by the UV-Vis spectrometer using extinction coefficient of 17,700 M⁻¹cm⁻¹ at OD₂₈₀.

Both thioredoxin and thioredoxin reductase were purified as native form. The protein concentration of IscA and IscU were in reference to their dimeric species. BSA and other chemicals were purchased from Sigma.

### 2.2.3 Iron-Sulfur Cluster Assembly in IscA and IscU

For the iron-sulfur cluster assembly reactions, apo-IscA and/or apo-IscU were incubated with Fe(NH₄)₂(SO₄)₂ and IscS in the presence of the thioredoxin/thioredoxin reductase system, which contains 5 µM thioredoxin, 0.5 µM thioredoxin reductase and 500 µM NADPH or 2 mM dithiothreitol (DTT), in desalting buffer consisting of 0.5 M NaCl and 20 mM Tris, pH 8.0, anaerobically at 37 °C. NADPH has a 340 nm absorption peak which may introduce an unwanted complication to the absorption spectra of the iron-sulfur protein. In this study, dithiothreitol was generally used for the iron-sulfur cluster assembly reactions. For anaerobic conditions, all reaction solutions were purged with argon gas to get rid of oxygen. The iron-sulfur cluster assembly solution was preincubated at 37°C for 5 min before L-cysteine (1mM)
was added to initiate the iron sulfur clusters assembly reaction. To re-purify the IscA and IscU from incubation solutions, the samples were diluted 10 times by using 20 mM Tris pH 8.0. The proteins were loaded on a Mono-Q column and eluted with 0 to 1 M linear gradient of NaCl in 20 mM Tris (pH8.0). The eluted fractions were analyzed by a Beckman DU 640 UV-Visible absorption spectrometer and by the 15% SDS-polyacrylamide electrophoresis gel.

2.2.4 Iron and Sulfide Determination in Protein Samples

An iron indicator, ferrozine was used for determination of total iron content in protein samples. Protein samples were mixed with 2 mM L-cysteine and 500 uM Ferrozine and heated at 85°C for 20 min. The iron-ferrozine complex has an absorption peak at 564 nm and with an extinction coefficient of 27.9 mM⁻¹ cm⁻¹ (Cowart et al., 1993).

The total sulfide concentration in protein samples was determined according to the Siegel’s method (Siegel, 1965). N,N-dimethyl-p-phenylene-diamine sulfate (DPD) was dissolved in 7.2 M HCl and ferric chloride in 1.2 N HCl before they were added to the protein samples. In each reaction 20 µl of DPD (20 mM) and 20 µL of ferric chloride (30 mM) were added to 160 µL protein sample. The mix solution was incubated at room temperature for 20 min. When the incubation time was over, all samples were centrifuged at room temperature for 10 min to get rid of any precipitations. The amount of sulfide was then measured at an absorption peak at 669 nm. The E. coli ferredoxin [2Fe-2S] was used as a standard (Siegel, 1965, Rogers & Ding, 2001). The iron content in E. coli ferredoxin was calculated from the iron-ferrozine determination as previously described (Cowart et al., 1993). Because E. coli ferredoxin contains a [2Fe-2S] cluster, the protein should have equal amounts of iron and sulfur.

2.2.5 Iron Binding in IscA and IscU

To prepare apo-IscA, purified IscA was incubated with 2 mM L-cysteine at room temperature for 20 min followed by passing the samples through a HiTrap desalting column
attached to a FPLC (Amersham Biosciences) to remove extra L-cysteine and iron (Ding & Clark, 2004). To prepare apo-IscU, purified IscU was incubated with 2 mM EDTA and 2 mM dithiothreitol at 37°C for 60 min followed by passing the samples through a HiTrap desalting column (Ding & Clark, 2004).

For the iron binding, apo-IscA and apo-IscU were incubated with Fe(NH₄)₂(SO₄) and IscS in the presence of 2 mM dithiothreitol in buffer containing 0.5 M NaCl and 20 mM Tris pH 8.0 at 37°C for 30 min anaerobically. An anionic exchange column was used to repurify the IscA and IscU protein from the incubation solution. The UV-Visible absorption spectra of the protein samples and their total iron contents were then measured as described above.

2.2.6 Iron Binding Association Constant of IscA in the Presence or Absence of IscU

The iron-loaded IscA was incubated with 0-200 mM sodium citrate and 2 mM dithiothreitol in the presence or absence of 100 µM IscU in open-to-air microtubes at room temperature for 30 min before IscA was repurified by using a HiTrap desalting column as described above. The amplitudes of the absorption peaks at 315 nm of the protein samples were used for determining relative iron binding in IscA. The content of iron and sulfide in each protein sample were determined as described above.

2.2.7 EPR Measurements

The EPR spectra were recorded at X-band on a Bruker ESR-30 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 milliWatt; modulation frequency, 100 kHz, modulation amplitude, 2.0 mT; sample temperature, 4.5 K; receive gain, 1.0×10⁵.
2.3 Results

2.3.1 IscU Is a Preferred Iron-Sulfur Cluster Assembly Scaffold Protein

In previous studies, both IscU and IscA were proposed as an alternative iron-sulfur cluster assembly scaffold proteins (Urbina et al., 2001, Smith et al., 2001, Kato et al., 2002, Smith et al., 2005b, Agar et al., 2000b). However, the physiological relevance of IscA and IscU has never been demonstrated. Here we compare the relative iron-sulfur cluster binding activity of IscU and IscA under physiological relevant conditions.

For the iron-sulfur cluster assembly in IscA, 25 μM apo-IscA was incubated with 0.5 μM cysteine desulfurase IscS, 1 mM L-cysteine and 25 μM ferrous iron in the presence of 2 mM of dithiothreitol under anaerobic condition at 37°C for 30 min. When the incubation was over, an absorption peak appeared at 415 nm, which was similar as reported for the [2Fe-2S] cluster assembly in IscA (Ollagnier-de-Choudens et al., 2001, Krebs et al., 2001) (Figure 2.1A). However, when 25 μM apo-IscA was incubated with 0.5 μM IscS, 1 mM L-cysteine, 2 mM dithiothreitol, and 25 μM ferrous iron in the presence of 6.3 μM apo-IscU at 37°C for 30 min anaerobically, an absorption peak at 456 nm that reflects [2Fe-2S] cluster assembly in IscU (Agar et al., 2000a) appeared (Figure 2.1B). When the concentration of apo-IscU in the incubation solution was gradually increased from 0 to 25 μM, the absorption peak at 415 nm was progressively increased (Figure 2.1, B-D). At the same time the amount of iron-sulfur clusters assembled in IscA was gradually decreased. When same amount of apo-IscA and apo-IscU (25 μM) were incubated together under the described conditions, the absorption peak at 456 nm was essentially identical to that when only 25 μM IscU was used without apo-IscA in the incubation solution. The proteins in the incubation solution were then re-purified. As shown in Figure 2.2B, the repurified IscA had little or no iron-sulfur clusters. On the other hand, the repurified IscU showed a major peak at 456 nm and a minor peak at 420 nm, indicating that [2Fe-2S] clusters
were assembled on IscU.

When equal amount of apo-IscA and apo-IscU were incubated with IscS, L-cysteine, DTT in the absence of Fe(NH$_4$)$_2$(SO$_4$)$_2$, followed by repurification by using MonoQ ion exchange column, IscA and IscU proteins were well separated (Figure 2.2A). The UV-Vis spectra showed that there were no [2Fe-2S] clusters assembled on either IscU or IscA.

The total iron concentration of the IscA and IscU fractions in Figure 2.2B showed that there was 0.05±0.03 (n=3) per IscA dimer in fraction 8. And each IscU dimer in fraction 10 had 0.85±0.08 (n=3). In fraction 8 for IscA, the sulfide content was not detectable but in fraction 10 for IscU, it contains 0.78± 0.1(n=3). These data further proved that under physiologically relevant conditions, iron-sulfur clusters were assembled in IscU but not in IscA.

To test whether iron-sulfur clusters can be nonspecifically formed in thiol-containing proteins, different types of proteins were applied in incubation solutions as for the iron-sulfur cluster assembly. Figure 2.3d showed that there was no iron-sulfur cluster absorption peak in the range from 400 nm to 600 nm if protein was not included in the incubation solution. When 5 µM BSA (bovine serum albumin) which contains 35 cysteine residues per protein was included in the incubation solution, an absorption peak at 415 nm emerged (Figure 2.3c). Because the absorption spectra of IscA and BSA with iron-sulfur clusters were similar, it appears that the iron-sulfur cluster absorption peak in IscA (Figure 2.3b) at 415nm could be a nonspecific signal.

2.3.2 IscA Acts as an Iron Chaperon When L-cysteine Is not Present

To explore the role of L-cysteine in the formation of iron-sulfur clusters, we incubated apo-IscA, apo-IscU, and IscS, with dithiothreitol and ferrous iron without L-cysteine at 37°C for 30 min anaerobically. IscA and IscU proteins were then re-purified by using a Mono-Q column as described previously. As shown in Figure 2.4c, apo-IscA was changed to an iron-loaded IscA after incubation due to the major absorption peak at 315 nm and a shoulder peak at 435 nm as
described previously (Ding et al., 2005b, Ding & Clark, 2004, Ding et al., 2004). The amplitude of the absorption peak at 315 nm and 260 nm were almost same, indicating that IscA was almost fully saturated with iron. The iron content in IscA was measured and the ratio of iron to each IscA dimer was found to be 0.94±0.07 (n=3). On the other hand, the ratio of iron to IscU was only 0.07±0.04 (n=3). The sulfide content in both IscA and IscU was not detectable. Furthermore, the EPR measurement and the UV-Vis spectrum further confirmed that there was no iron binding in IscU (Figure 2.4e and 2.4f).

In contrast, a broad EPR signal at around the \( g = 4-6 \) region (Figure 2.4d), which represents a unique \( S = 3/2 \) ground spin state of the ferric iron center in IscA (Ding & Clark, 2004), was observed in the eluted IscA fraction. The observed EPR signal of IscA was identical to the EPR signal of purified IscA. There was no significant peak at the \( g = 4.3 \) position, indicating that there was very little non-specifically bound “junk” iron in IscA. All these data suggested that the iron center in IscA had a unique redox property.

We further analyzed the IscA iron binding association constant in the presence or absence of IscU using sodium citrate as a physiological iron binding competitor. The results showed that the iron association constant of IscA remained unchanged in the presence or absence of IscU (data not shown) (Ding et al., 2005b, Ding & Clark, 2004).

2.3.3 IscA Promotes the Iron-Sulfur Cluster Assembly in IscU under Aerobic Conditions

Figure 2.3 shows that in the presence of ferrous iron, IscS and cysteine, iron-sulfur clusters were assembled in IscU under anaerobic conditions. However, most organisms live under aerobic conditions. For example, in mitochondria where most of iron-sulfur clusters are assembled, there is a high content of oxygen (Imlay, 2006). Thus, iron-sulfur clusters have to be assembled under aerobic conditions. However, under aerobic conditions, ferrous iron is easily oxidized to form insoluble ferric hydroxide which is not a source for the iron-sulfur cluster
biogenesis. We postulate that IscA may stabilize iron and donate iron for the iron-sulfur cluster assembly under aerobic conditions.

To test this idea, apo-IscU was incubated with 0.5 µM IscS and 25 µM Fe(NH₄)₂(SO₄)₂ and the thioredoxin reductase system in the presence or absence of IscA under aerobically conditions at 37°C for 90 min. L-cysteine (1 mM) and NADPH (1mM) were then added into the incubation solution to initiate the iron-sulfur clusters assembly reaction. Figure 2.5A shows that without apo-IscA in the preincubation solution, there were very little iron-sulfur clusters assembled on IscU. When apo-IscA was added into the preincubation solution, iron-sulfur clusters were assembled on IscU (Figure 2.5B). IscA and IscU were then repurified from the incubation solution. The UV-visible spectra showed that iron-sulfur clusters were indeed assembled in IscU but not in IscA. To determine whether the function of IscA in promoting the iron-sulfur cluster assembly on IscU can be replaced with other iron chelators, either 100 µM EDTA (data not shown) or 100 µM sodium citrate (Figure 2.5C) was added into the preincubation solution instead of IscA. The results showed that sodium citrate and EDTA failed to promote the iron-sulfur cluster assembly on IscU. To determine whether the failure of iron-sulfur clusters assembled on IscU was due to the lack of iron source, freshly prepared ferrous iron (50 µM) was added into the solution together with L-cysteine and NADPH. As shown in Figure 2.5D, the iron-sulfur cluster assembly in IscU was partially restored. These results suggest that IscA can prevent iron from being oxidized and make iron accessible for the iron-sulfur cluster assembly under aerobic conditions.

To further explore the function of IscA under aerobic conditions, apo-IscA was incubated with IscU, IscS, ferrous iron and DTT in the presence or absence of L-cysteine under aerobic condition. The IscA and IscU proteins were again repurified. As shown in Figure 2.6, without L-cysteine, iron was mostly bound to IscA. However, in the presence of cysteine, iron-sulfur
cluster was assembled in IscU. ThuIscA is a preferred iron binding protein, whereas IscU is an iron-sulfur cluster assembly protein under both aerobic and anaerobic conditions.

2.4 Discussion

As key members of the iron-sulfur cluster assembly machinery family, both IscU and IscA proteins are highly conserved ranging from bacteria to human (Takahashi & Nakamura, 1999, Zheng et al., 1998). In previous studies, both IscU (Agar et al., 2000a, Urbina et al., 2001, Smith et al., 2001, Kato et al., 2002, Smith et al., 2005b) and IscA (Ollagnier-de-Choudens et al., 2001, Krebs et al., 2001, Morimoto et al., 2002, Wu et al., 2002a, Wollenberg et al., 2003) were proposed as the iron-sulfur scaffold assembly proteins. However, the physiological functions of IscU and IscA in the iron-sulfur cluster biogenesis are still not clear. In this study, we demonstrated that IscU is a preferred iron-sulfur protein while IscA is an iron binding protein under physiological relevant conditions. The x-ray crystal structure studies revealed that IscA exists as a tetramer with a central channel formed by the association of four IscA monomers. Three invariant cysteine residues (cys-35, cys-99, and cys-101) from each IscA monomer are projected to form a “cysteine pocket”, in which the mononuclear iron may bind (Ding et al., 2004, Bilder et al., 2004, Cupp-Vickery et al., 2004). The site-directed mutagenesis further showed that all conserved cysteine residues are required for the iron binding in IscA indicating that these cysteine residues are directly involved in iron binding in IscA (Ding et al., 2004).

Although IscA and its homologues have been characterized as the iron-sulfur cluster scaffold proteins (Ollagnier-de-Choudens et al., 2001, Krebs et al., 2001, Morimoto et al., 2002, Wu et al., 2002a), our data suggested that the iron-sulfur clusters assembled on IscA could be non-specific because other proteins containing free thiol groups such as BSA can also assemble iron-sulfur clusters (Figure 2.3). Furthermore the iron-sulfur cluster assembled in IscA does not require the conserved cys99 and cys101 residues, because in the IscA mutant C99S and C101S,
iron-sulfur clusters can still be formed (data not shown). It has been reported that in *Schizosaccharomyce pombe*, if anyone of the three invariant cysteine residues is replaced with alanine in the IscA homologue, the iron-sulfur clusters can still be formed in these mutant proteins (Wu & Cowan, 2003). All these data supported the notion that the iron-sulfur clusters assembly in IscA might not be specific. We also found that when equal amounts of IscA and IscU were incubated together in the presence of both ferrous iron and sulfide, iron-sulfur clusters were assembled in IscU but not IscA. On the other hand, if only iron was available, iron was bound to IscA but not IscU. Thus IscA is a specific iron binding protein whereas IscU is a preferred iron-sulfur cluster scaffold protein.

The NMR structure of IscU showed that it has a compact core globular structure containing a $\alpha$-$\beta$ architecture sandwich formed by 3 stranded anti-parallel $\beta$-sheets and 4 $\alpha$-helices (Ramelot et al., 2004). While the X-ray crystal structure studies revealed that IscA is structurally different from IscU (Bilder et al., 2004, Cupp-Vickery et al., 2004). It has been shown that IscS, a NifS like homodimeric L-cysteine desulfurase, can convert L-cysteine to L-alanine and form a IscS-persulfide intermediate on the conserved cysteine residue (Smith et al., 2005a, Zheng et al., 1998, Flint, 1996) and pass sulfur to IscU through the IscS-IscU protein interactions (Urbina et al., 2001) (Ramelot et al., 2004), indicating that IscA and IscU have different functions. HscA is a molecular chaperon who also interacts with IscU via the specific sequence LPPVK in the recognition center (Tapley & Vickery, 2004). HscA helps the iron-sulfur clusters assembled in IscU and promotes transfer of the assembled clusters from IscU to target proteins. HscB, a J-type cochaperon protein (Vickery et al., 1997), binds both IscU and HscA and promote the interaction between IscU and HscA (Hoff et al., 2000). Because IscS, IscU, HscA and HscB are all encoded by the *iscSUA-hscBA-fdx* operon, it may suggest that IscU is the dominating scaffold protein for the biogenesis of iron-sulfur clusters.
Almost all iron-sulfur cluster assembly reactions in proteins were carried out under anaerobic conditions, because under aerobic conditions, ferrous iron is readily oxidized to ferric iron, which will limit the iron source for the iron-sulfur clusters assembly (Figure 2.5A). On the other hand, iron-sulfur clusters must be assembled in aerobic condition or oxygen rich mitochondria or other organisms in eukaryotic cells (Muhlenhoff & Lill, 2000). Therefore there must be specific proteins involved in stabilizing the iron and facilitating the iron-sulfur clusters assembly under aerobic conditions. Frataxin, a small mitochondria protein relating to an autosomal recessive neurodegenerative human disease Friedreich’s ataxia, was proposed to function as an iron chaperon for the iron-sulfur cluster assembly (Campuzano et al., 1996).

However, when the homologue of frataxin in E. coli (Li et al., 1999a), and Saccharomyces cerevisiae (Duby et al., 2002) was deleted, there was no apparent effect on the iron-sulfur cluster biogenesis in these organisms. The weak iron binding ability of the human frataxin further suggested that the iron binding in frataxin is not the conserved part for all organisms. In contrast, the iron binding association constant in IscA is very consistent from E. coli to humans. In our studies, we reported that IscA can bind ferrous iron in both aerobic and anaerobic conditions and can effectively prevent the formation of ferric hydroxide, thus facilitating the iron-sulfur cluster assembly under oxygen rich aerobic conditions (Figure 2.5). The finding that the iron center in IscA can be readily released by L-cysteine and transferred to IscU to form iron-sulfur clusters further suggests that IscA could be the major iron chaperon for the iron-sulfur cluster assembly under aerobic conditions.

Because IscS, IscA and IscU are encoded by the iscSUA operon, it is tempting to suggest that they work in concert for the biogenesis of iron-sulfur clusters. We propose that IscU is an iron-sulfur cluster scaffold protein, IscA is an iron chaperon and IscS is a sulfur donor. They are the primary members in the iron sulfur clusters assembly. Figure 2.7 shows that under the
conditions when L-cysteine is limited, IscA binds iron in the presence of the thioredoxin reductase system and stabilizes ferrous iron from being oxidized. When L-cysteine is available, it will mobilize the iron center in IscA (Ding & Clark, 2004) and forms a transient L-cysteine-Fe complex. IscS will use this substrate and convert it to L-alanine and Fe-S element and transfer the Fe-S element to IscU to form iron-sulfur cluster via IscS-IscU specific interactions (Ramelot et al., 2004, Agar et al., 2000a, Urbina et al., 2001, Smith et al., 2005a).

Implication of this model is that L-cysteine is a major factor determining whether iron will bind to IscA or to be donated for the iron-sulfur cluster assembly in IscU when ferrous iron is sufficient in cells. Even though the L-cysteine biosynthesis has been well investigated, the genetic regulation of the L-cysteine biosynthesis is still not well understood. Regulation of intracellular L-cysteine content and related control of the iron-sulfur cluster assembly requires further investigated.
Figure 2.1 Competition of IscA and IscU for the iron-sulfur cluster assembly

Apo-IscA (25 μM) was incubated with different amounts of apo-IscU (0-25 μM) in the presence of 0.5 μM IscS, 25 μM Fe(NH₄)₂(SO₄)₂, and 2 mM of dithiothreitol at 37°C anaerobically. After 1 mM of L-cysteine was added for 0 min and 30 min, the UV-Vis absorption spectra were taken respectively. The think line represents the 0 min spectra and the thick line means 30 min. A, with 0 μM apo-IscU. B, with 6.3 μM apo-IscU. C, with 12.5 μM apo-IscU. D, with 25 μM apo-IscU.
Figure 2.2 **IscU is a preferred iron-sulfur cluster assembly scaffold protein.**
A, 25 µM of apo-IscA was incubated with an equal amount of apo-IscU (25 µM) in the presence of 0.5 µM IscS, 1 mM L-cysteine, 2 mM dithiothreitol anaerobically for 30 min at 37°C. The IscA and IscU were purified by using a Mono Q column as described in “Experimental Procedure” after incubation. Panel A a) shows the elution profile of IscA and IscU from a Mono Q column. Panel A b) shows the SDS PAGE gel analysis of the eluted fractions. The location of the IscA and IscU were labeled on the left side. Panel A c) and d) show the UV-visible absorption spectra of IscA (fraction 8) and IscU (fraction 10) respectively. Due to the high extinction coefficient of IscU on 280 nm (11.2 mM⁻¹ cm⁻¹), a full scale absorption spectrum of IscU (fraction 10) was inserted in incubation. Panel B, same as in solution A except 25 µM Fe(NH₄)₂(SO₄)₂ was included.
Figure 2.3 Iron-sulfur cluster assembly in IscU, IscA, and bovine serum albumin.

a, Apo-IscU (25 µM) was incubated with 0.5 µM IscS, 1 mM cysteine and 25 µM Fe(NH₄)₂(SO₄)₂ in the presence of 2 mM dithiothreitol at 37°C for 30 min anaerobically. The UV-Vis spectrum was taken right after the reaction was over. In b, c and d, everything was the same, except that 25 µm IscU was replaced with 25 µM IscA (b), 5 µM BSA in (c) or no protein (d).
Figure 2.4 IscA acts as an iron chaperon when L-cysteine is not present.
Apo-IscA 25 µM was incubated with an equal amount of apo-IscU in the presence of 0.5 µM IscS, 25 µM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 2 mM of dithiothreitol anaerobically at 37°C for 30 min. A Mono-Q column was used to repurify IscA and IscU as described in “Experimental Procedures”. Panel a shows the elution profile for IscA and IscU after Mono Q. Panel b shows the location of the eluted IscA and IscU from Mono Q on the SDS PAGE gel. Panel c and e show the UV-Vis spectra of eluted IscA and IscU, respectively. Panels d and f show the EPR spectra of the eluted IscA and IscU respectively.
Figure 2.5 IscA promotes the iron-sulfur cluster assembly in IscU under aerobic conditions
Panel A, apo-IscU (25 µM) was preincubated with 0.5 µM IscS and 25 µM Fe(NH₄)₂(SO₄)₂ in the presence of the thioredoxin/thioredoxin reductase aerobically. L-cysteine (1 mM) and NADPH (1 mM) were added after the solution was preincubated for 90 min. The UV-Vis spectra were taken every 2 min during the 20 min incubation. Panels B and C are same as panel A except apo-IscA (25 µM) (B) or sodium citrate (100 µM) (C0 was added to incubation solutions. Panel D is same as panelA except that freshly prepared ferrous iron (50 µM) was added right before L-cysteine and NADPH were added into the preincubation solution.
Figure 2.6 The iron-loaded IscA provides iron for the iron-sulfur cluster assembly in IscU under aerobic conditions.
Panel A, the iron-loaded IscA (25 µM) was incubated with apo-IscU in the presence of 0.5 µM IscS and 2 mM dithiothreitol aerobically at 37°C. The thin lines are the UV-Vis spectra taken at 0 min and the thick lines are the spectra after 30 min incubation. Panel B is same as in Panel A, except L-cysteine (1 mM) was added in the incubation solution. Panels C and D showed the UV-Vis spectra of IscA and IscU after MonoQ separation of incubation solution of A and B respectively.
Figure 2.7 A proposed USA model for biogenesis of iron-sulfur clusters.
During the iron-sulfur cluster biogenesis, ferrous iron will first bind to IscA. Addition of L-cysteine mobilizes the iron from IscA protein and through the function of IscS, sulfur will be released from L-cysteine to form sulfur-iron element which will eventually be transferred to IscU to form iron-sulfur clusters.
Chapter III
Distinct Iron Binding Activities of CyaY and IscA

3.1 Introduction

Friedreich ataxia (FRDA) is the most prevalent inherited ataxia, and is identified as an autosomal recessive disease. In most cases, FRDA results from the large expansions of a GAA triplet-repeat sequence in the first intron of the gene X25 (FRDA by the Human Genome Organization nonomenclature), which encodes the protein frataxin (Patel & Isaya, 2001, Campuzano et al., 1996). Only a small portion of the FRDA in patients is caused by the combination of the heterozygotes of the GAA expansion and the point mutation within the X25 gene (Pook et al., 2000, Patel & Isaya, 2001). FRDA occurs at a frequency of 1-2 per 50,000 and is clinically characterized by progressive gait and limb ataxia from early childhood and final death within a few decades (Patel & Isaya, 2001, Campuzano et al., 1996).

Frataxin is a highly conserved protein from bacteria to humans (Gibson et al., 1996). All frataxin orthologues have a highly conserved acidic block domain of 120 amino acids that fold into a globular compact domain (Dhe-Paganon et al., 2000, Canizares et al., 2000). The crystal structure of human frataxin (Dhe-Paganon et al., 2000) showed that it is similar to yeast frataxin (He et al., 2004) and the bacterial frataxin ortholog CyaY (Cho et al., 2000). Deletion of the frataxin homolog in yeast (YFH1) revealed that it plays an important role in iron homeostasis and mitochondrial function (Babcock et al., 1997, Canizares et al., 2000). Disruption of frataxin gene in mouse (Frda) causes the early embryonic lethality due to the iron accumulation (Cossee et al., 2000). RNAi-mediated suppression of frataxin homolog gene (frh-1) in Caenorhabditis elegans prolonged the lifespan of animal (Ventura et al., 2005). Because in FRDA patients, the respiratory complex I, complex II and complex III containing iron-sulfur proteins showed an lowered activity (Rotig et al., 1997), and the mitochondria aconitase which is a [4Fe-4S] enzyme
also exhibited a reduced activity in FRDA patients (Rotig et al., 1997), it was proposed that frataxin/CyaY may have a crucial function in the iron-sulfur cluster biogenesis. Frataxin or CyaY has been shown to bind both ferrous and ferric iron with a relatively weak binding affinity (Adamec et al., 2000b, Adinolfi et al., 2002b). In addition, frataxin were reported to have the ability to mediate the iron transfer for the iron-sulfur cluster assembly in IscU through the protein-protein interaction in human (Yoon & Cowan, 2003), yeast (Ramazzotti et al., 2004, Gerber et al., 2003) and E. coli cells (Layer et al., 2006). Iron-loaded frataxin were demonstrated not only to interact with IscU but also to interact with the cysteine desulfurase, IscS in yeast (Gerber et al., 2003, Layer et al., 2006), the succinate dehydrogenase and other mitochondrial electron transfer complex (Gonzalez-Cabo et al., 2005) and aconitase (Bulteau et al., 2004). Taken together, these results suggested that frataxin/CyaY may function as a physiological iron donor for the iron-sulfur cluster assembly.

Nevertheless mutation of frataxin orthologue in E. coli (Li et al., 1999b) and S. cerevisiae (Duby et al., 2002) has little effect on the iron-sulfur cluster biogenesis. Instead, deletion of frataxin orthologues in yeast cells increased sensitivity to reactive oxidative species, promoted mitochondria-mediated apoptosis and failed to prevent DNA damage (Karthikeyan et al., 2002, Karthikeyan et al., 2003). Furthermore, Condo et al (Condo et al., 2006) reported that an extra – mitochondrial pool of frataxin can markedly prevent mitochondrial oxidative damage and apoptosis in different cellular systems and replace mitochondrial frataxin in promoting survival of Friedreich ataxia cells (Condo et al., 2006). Thus, it was suggested that the primary function of frataxin/CyaY is to detoxify the redox active free iron in cells (Condo et al., 2006, Karthikeyan et al., 2002, Karthikeyan et al., 2003).

In search for the specific iron donors for the biogenesis of iron-sulfur clusters, we have shown previously that IscA, a key member of the iron-sulfur cluster assembly machinery
conserved from bacteria (Zheng et al., 1998, Tokumoto & Takahashi, 2001, Schwartz et al., 2001), yeast (Jensen & Culotta, 2000) and humans (Cozar-Castellano et al., 2004), is a novel iron-binding protein. *E. coli* IscA binds iron with an iron association constant of $2.0 \times 10^{19} \text{M}^{-1}$ in the presence of thioredoxin reductase system (Ding & Clark, 2004, Ding et al., 2005b). The iron center in the IscA protein can be easily released by L-cysteine (Ding et al., 2005a, Yang et al., 2006) and transferred to proposed scaffold IscU (Ding et al., 2005a, Ding et al., 2005b, Ding & Clark, 2004, Ding et al., 2004, Yang et al., 2006). The strong iron binding affinity (Ding & Clark, 2004, Ding et al., 2005b) and ease to mobilize the iron center in the protein by L-cysteine (Ding et al., 2005a, Yang et al., 2006) led us to hypothesize that the primary function of IscA is to recruit intracellular free iron and deliver the iron for the iron-sulfur clusters assembly (Ding et al., 2005a).

To further elucidate the roles of frataxin/CyaY and IscA in the biogenesis of iron-sulfur clusters, here we compared iron binding property of IscA and Cyay under physiological and oxidative stress conditions. The results showed that *E. coli* CyaY failed to bind ferrous iron under physiological condition even with excess 10-fold of iron in the incubation solution. On the other hand, IscA binds iron under same experimental conditions. However, in the presence of hydrogen peroxide, CyaY becomes a competent iron binding protein and dramatically decreases the iron-mediated production of hydroxyl free radicals, whereas IscA completely loses its iron binding activity under oxidative stress conditions. The possible physiological roles of IscA and CyaY in the biogenesis of iron-sulfur clusters and in the intracellular iron metabolism in response to oxidative stress will be discussed.

3.2 Methods and Materials

3.2.1 Protein Preparation

*E. coli* wide type genomic DNA containing the *cyay* gene sequence was used as the template
for the PCR. Two primers were used for *cyay* gene amplification. The sequences of the two primers were as following: CyaY-1, 5’-GATAACAACCATGGACGACAGTGAA-3’ and CyaY-2, 5’- CATGCAAAGCTTGCGAAACTGAC-3’. *Hind III* and *NcoI* restriction enzymes were used to cut the restriction site on the amplified PCR products and pET-28b+ as described in Chapter II. The digested PCR products and pET-28b+ were ligated together by using T4 DNA ligase. The cloned DNA fragment was confirmed by direct sequencing using the T7 primer. The recombinant CyaY was overproduced and purified as described for IscA, IscU and IscS in Chapter II. The precise molecular weight of purified CyaY protein was confirmed by using the electrospray ionization-mass spectrometry (Chemistry Department, Louisiana State University).

Apo-IscA and apo-CyaY were prepared by incubating proteins with 2 mM L-cysteine at 37°C for 30 min followed by using a HiTrap desalting column to remove released iron and L-cysteine. *E. coli* thioredoxin 1 and thioredoxin reductase were produced from the expression vectors pDL59 (Veine et al., 1998a) and pTrR301 (Mulrooney, 1997) which were kindly provided by Dr. Scott B. Mulrooney (University of Michigan). The purification of both thioredoxin 1 and thioredoxin reductase was as described in chapter II. The SDS-PAGE gel analyses indicated that both protein purities were higher than 95%. The concentration of apo-CyaY was determined by using an extinction coefficient at 280 nm of 30.0 M⁻¹ cm⁻¹. The concentration of apo-IscA, thioredoxin 1 and thioredoxin reductase were determined by using the same method as described in chapter II.

### 3.2.2 Iron Binding Assay

Apo-IscA and apo-CyaY were incubated with freshly prepared Fe(NH₄)₂(SO₄)₂ in the presence of 5 μM thioredoxin, 0.5 μM thioredoxin reductase and 500 μM NADPH in buffer containing 100 mM NaCl, 20 mM Tris (pH 8.0) in open-to-air microcentrifuge tubes at 37°C for 30 min. CyaY and IscA proteins were repurified by using a MonoQ column as described in
Chapter II. The repurification procedure did not significantly affect the iron binding in CyaY or IscA as after running the samples through the Mono-Q column, at least around 90% of the iron content in the iron-bound CyaY or the iron-bound IscA still remained. The proteins eluted from MonoQ column were analyzed by using the SDS-PAGE analysis. The total iron content in the eluted fractions was determined by using the inductively coupled plasma mass spectroscopy (Department of Geology and Geophysics, Louisiana State University) or an iron indicator ferrozine as described in Chapter II. The concentration of iron ferrozine complex was measured at 562 nm by using an extinction coefficient of 27.9 mM$^{-1}$ cm$^{-1}$ (Cowart et al., 1993).

3.2.3 Iron-Sulfur Cluster Assembly Assay

For the iron-sulfur cluster assembly assay, either IscA or CyaY was preincubated with apo-IscU and IscS in the presence of the thioredoxin reductase system in buffer containing 100 mM NaCl, 20 mM Tris pH 8.0 at 37°C. The incubation solution were flushed with argon gas to purge oxygen and preincubated at 37°C for 5 min before L-cysteine was added to initiate the iron-sulfur cluster assembly reaction. A Beckman DU 640 UV-visible absorption spectrometer equipped with a temperature controller was used to monitor the iron-sulfur clusters assembly on IscU.

3.2.4 Measurements of Hydroxyl Free Radicals

The iron-mediated generation of hydroxyl free radicals was measured according to the procedure described by Halliwell et al (Halliwell et al., 1987). Briefly, 2-deoxyribose is degraded by hydroxyl free radicals to form a malon-dialdehyde-like compound which can react with thiobarbituric acid to generate a chromogen. Through the measurement of the net generated chromogen, the amount of hydroxyl free radicals can be calculated. In the experiments, apo-IscA or apo-CyaY was preincubated with Fe(NH$_4$)$_2$(SO$_4$)$_2$ in buffer containing 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.4, 60 mM NaCl, 4 mM deoxyribose, and the thioredoxin reductase
systeme at 37°C for 10 min. Then 500 μM of hydrogen peroxide was added to initiate the Fenton Reaction. The reaction was continued at 37°C for another 25 min. A 400 ul developing solution containing 1% thiobarbituric acid and 2.8% trichloroacetic acid was mixed with 600 μL the above incubation solutions and then boiled for 15 min. After that, a 14,000 rpm centrifugation was applied to remove the precipitates from the solution. The relative amounts of the chromogen in the solutions were measured from the emission at a wavelength of 553 nm using an excitation wavelength of 532 nm in PerkinElmer LS-3 fluorescence spectrophotometer.

3.2.5 Measurements of the Total Free Thiol Contents

The total free thiol contents in the protein samples were analyzed by using 100 μM Ellman reagent (5,5’-dithiobis(2-nitrobenzoic acid) ) (Sigma). The protein samples were incubated with the Ellman reagent dissolved in methanol at room temperature for 20 min followed by centrifugation to remove the precipitates. The total free thiol content was calculated from the absorption at 412 nm using N-acetyl-L-cysteine as a standard.

3.2.6 EPR Measurements

The EPR measurements were performed as described in Chapter II.

3.3 Results

3.3.1 Iron Binding Activity of CyaY and IscA in the Presence of the Thioredoxin Reductase System

Previous studies showed that frataxin ortholog CyaY and IscA from E. coli bind iron with the iron association constants of 2.6×10^5 M^-1 (Adinolfi et al., 2002a, Bou-Abdallah et al., 2004) and 2.0×10^19 M^-1 (Ding & Clark, 2004, Ding et al., 2004) respectively. However, the iron binding studies for IscA and CyaY were carried out by different groups and under different experimental conditions. Because both IscA (Ding et al., 2004, Ding & Clark, 2004) and CyaY (Layer et al., 2006) were proposed as iron donors for the iron-sulfur cluster biogenesis, it is
necessary to re-evaluate the iron binding property of CyaY and IscA under the same physiologically relevant conditions.

The intracellular redox potential in cells is estimated to be in the range of -260 mV to -280 mV (Ding et al., 1996, Hwang et al., 1992) and this relatively low intracellular redox potential is mainly due to the function of the redundant thiol reducing systems (Aslund & Beckwith, 1999). Here we used the thioredoxin reductase system containing *E. coli* thioredoxin-1 (Veine et al., 1998b), thioredoxin reductase (Mulrooney, 1997), and NADPH as described in (Ding et al., 2005b). In this system, NADPH provides electrons to reduce thioredoxin-1 via thioredoxin reductase (Aslund & Beckwith, 1999).

To compare the iron binding activity of IscA and CyaY under physiologically relevant conditions, 100 μM apo-CyaY and 100 μM IscA were incubated with 50 μM freshly prepared ferrous iron in the presence of the thioredoxin reductase system in open-to-air micro-centrifuge tubes at 37°C for 30 min. IscA and CyaY protein were re-purified by using a Mono-Q column. The SDS-polyacrylamide gel electrophoresis analysis showed that IscA was eluted in fractions 8 and 9, while CyaY protein was eluted in fraction 12 and 13 (Figure 3.1B). The iron analysis of each fraction showed that around 94% of the total iron was bound to IscA and less than 4% of iron was bound to CyaY (Figure 3.1A). The UV-visible absorption measurements of the eluted IscA fractions revealed an iron-loaded IscA with a dominating absorption peak at 315 nm and a shoulder peak at 435 nm (Figure 3.2A). The 1.02 ratio of the amplitude at 315 nm to 260 nm indicated that IscA was almost completely saturated with iron (Ding et al., 2004). The EPR measurement further confirmed the specific iron binding in IscA (Figure 3.2C) (Ding et al., 2004). In contrast, the UV-visible absorption spectrum (Figure 3.2B) and EPR measurements (Figure 3.2C) indicated no iron binding in the CyaY.

To further elucidate the iron binding activity of CyaY under physiologically relevant
conditions, apo-CyaY was incubated with ferrous iron in the presence of the thioredoxin reductase system at 37°C for 30 min. After the incubation, CyaY was repurified by using Mono-Q column. The result of the UV-visible measurements and the iron binding analyses showed that there was no iron binding in re-purified CyaY (data not shown). On the other hand, regardless of the presence of CyaY, the iron binding in IscA was essentially the same under physiologically relevant conditions. All these results suggested that CyaY fails to bind iron, whereas IscA is a strong iron binding protein under physiologically relevant conditions.

Since IscA binds iron under physiological conditions (Figure 3.1A), it raised a question that whether the iron center in this repurifed IscA can be transferred to IscU to form iron-sulfur clusters. The iron-bound IscA was incubated with apo-IscU and IscS in the presence of thioredoxin reductase system at 37°C for 5 min. Then L-cysteine was added into the incubation solution to initiate the iron-sulfur clusters assembly reaction in IscU. The results showed that the iron in repurified IscA was efficiently transferred to IscU to form iron-sulfur clusters as previously reported (Ding et al., 2005b). In parallel, repurified CyaY could not bind iron and failed to promote the iron-sulfur cluster assembly in IscU. Collectively, all these results showed that IscA binds iron in the presence of the thioredoxin reductase system and functions as an iron donor, and that CyaY fails to do so under physiologically relevant conditions.

3.3.2 CyaY Does Not Form the Iron-Mediated Aggregate Complexes in the Presence of the Thioredoxin Reductase System

Previous studies showed that CyaY may bind iron and form the aggregate complexes in the presence of excessive amounts of iron and low concentrations of salts under aerobic conditions in vitro (Adinolfi et al., 2002b, Bou-Abdallah et al., 2004, Layer et al., 2006), and that the iron in the aggregated CyaY complexes can provide iron for the iron-sulfur cluster assembly in IscU (Layer et al., 2006). To test whether CyaY can bind iron in the presence of excessive amount of
iron in the presence of the thioredoxin reductase system, apo-CyaY was incubated with 10-fold excess of ferrous iron in the presence or absence of the thioredoxin/thioredoxin reductase system at 37°C for 30 min aerobically followed by repurification. When there is no thioredoxin reductase system in the incubation solution, the UV-visible spectrum of the repurified CyaY showed a broad absorption peak range from 300-400 nm, indicating the formation of the oxo/hydroxoferic iron species in the protein (Figure 3.3A). The iron content analyses revealed that the ratio of iron to the CyaY monomer was 4.5 to 1. The gel filtration elution profiles of the repurified CyaY also showed that CyaY formed aggregate complexes in the absence of thioredoxin reductase systems (data not shown). However, when apo-CyaY was incubated with 10-fold excess ferrous iron in the presence of thioredoxin reductase system, the UV-visible spectrum of repurified CyaY showed no indication of iron binding in CyaY (Figure 3.3B). The iron content analyses further showed that there was no iron binding in CyaY. The gel filtration elution profiles further indicated that no aggregate complexes were formed in the presence of thioredoxin reductase system. All these results demonstrated that CyaY fails to bind iron under physiological condition.

3.3.3 Hydrogen Peroxide Promotes the Iron Binding in CyaY and Blocks the Iron Binding in IscA

In the presence of hydrogen peroxide, ferrous iron will promote the production of hydroxyl free radicals, which will cause damages to DNA and other cellular components (Keyer & Imlay, 1997). As iron binding proteins, CyaY and IscA may have the ability to bind free iron and reduce the production of hydroxyl free radicals mediated by iron. To compare the iron binding ability of CyaY and IscA under oxidative stress conditions, apo-CyaY and apo-IscA were incubated with freshly prepared ferrous iron in the presence of the thioredoxin reductase system and 1 mM hydrogen peroxide at 37°C for 30 min in an open-to-air microcentrifuge tube,
followed by repurification of IscA and CyaY. The eluted fractions from the Mono-Q column were subjected to the SDS-PAGE gel (Figure 3.4B) and the iron content analyses (Figure 3.4A). The results showed that only 3% of the total iron was bound to IscA, and around 15-20% of total iron was bound to CyaY under oxidative stress conditions. These results suggested that under oxidative stress conditions, IscA completely loses its iron binding activity, while CyaY becomes a potent iron binding protein in the presence of hydrogen peroxide.

The iron-mediated production of hydroxyl free radicals in the presence of apo-IscA and CyaY was also measured to explore the iron binding activity of CyaY and IscA in the presence of hydrogen peroxide. In the Fenton reaction, ferrous iron reduces hydrogen peroxide to produce deleterious hydroxyl free radicals. If redox active ferrous iron was sequestrated by proteins, the production of the iron-mediated hydroxyl free radicals would be reduced. The measurement of the production of hydroxyl free radicals was performed by using the thiobarbituric acid and 2-deoxyribose as described in Halliwell et al (Halliwell et al., 1987).

Figure 3.5A shows the titration of apo-CyaY or apo-IscA with a fixed 5 μM concentration of ferrous iron in the presence of the thioredoxin reductase system and 0.5 mM hydrogen peroxide. As the protein concentration of CyaY was gradually increased from 0 μM to 50 μM, the production of hydroxyl free radicals was significantly decreased. When 10-fold excess of CyaY was added into the solution, the production of hydroxyl free radicals was almost completely eliminated. On the other hand, when the concentration of apo-IscA increased from 0 μM to 50 μM, the production of the hydroxyl free radicals remained the same, indicating that IscA could not prevent production of hydroxyl free radicals mediated by ferrous iron.

Figure 3.5B shows the production of hydroxyl free radicals as a function of the concentrations of the iron in the presence of the thioredoxin reductase system and 0.5 mM of hydrogen peroxide. The fixed amount (50 μM) of apo-CyaY or apo-IscA was included in the incubation solution.
Compared with no protein control (represented by the open triangles), apo-CyaY was able to attenuate the production of hydroxyl free radicals in the presence of iron (represented in filled circles). In contrast, apo-IscA had no effect on the production of hydroxyl free radicals. These data further indicated that CyaY but not IscA can prevent the production of hydroxyl free radicals through binding ferrous iron under oxidative stress conditions.

3.3.4 Hydrogen Peroxide Oxidizes the Free Thiol Groups in IscA and Blocks the Iron Binding

According to the published crystal structures, CyaY binds to iron via the carboxyl groups of the conserved aspartate and glutamate residues located on α1 and β1 sheet of the N-terminus of the protein (Nair et al., 2004, Adinolfi et al., 2002b), whereas IscA binds iron through the thiol groups of the three conserved cysteine residues (Ding et al., 2005b, Bilder et al., 2004, Cupp-Vickery et al., 2004). Hydrogen peroxide may oxidize the free thiol groups in IscA causing the failure of IscA to bind iron. On the other hand, hydrogen peroxide has no effect on the carboxyl groups on CyaY, thus allows CyaY to bind to iron in the presence of hydrogen peroxide and attenuate the production of hydroxyl free radicals.

To further explore why IscA fails to bind iron under oxidative stress conditions, we measured the total free thiol groups in IscA in the presence of hydrogen peroxide. Apo-IscA was incubated with ferrous iron and different amounts of hydrogen peroxide in the presence of the thioredoxin reductase system. In parallel, the iron binding activity of IscA was checked investigated. Figure 3.6A shows that as the concentration of hydrogen peroxide was increased, the iron binding ability of IscA was decreased. Figure 3.6B also shows that as the concentration hydrogen peroxide was increased, the total free thiol groups were progressively decreased. The correlation between the iron binding in IscA and the total free thiol groups in IscA suggested that the oxidization of free thiol groups in IscA may lead to the failure of iron binding in IscA in the
If the oxidization of thiol groups in IscA blocks the iron binding, the reduction of oxidized thiol groups in IscA should restore the iron binding activity in IscA. Figure 3.7 shows that when the hydrogen peroxide treated IscA was reincubated with the thioredoxin reductase system and ferrous iron, IscA regains its iron binding activity. These experiments substantiate the notion that oxidization of thiol groups in IscA is responsible for the failure to bind iron under oxidative stress conditions.

3.4 Discussion

Iron-sulfur clusters are ubiquitous prosthetic groups that are important in the fundamental life processes such as iron-sulfur clusters are required for electron transfer, amino acids metabolism, gene regulation, heme biosynthesis, etc (Johnson et al., 2005, Fontecave, 2006). It is now clear that sulfur in iron-sulfur clusters is derived from L-cysteine via the cysteine desulfurase (Zheng et al., 1993b, Urbina et al., 2001, Smith et al., 2001). However, the iron donor in the biogenesis of iron sulfur clusters remains unclear. Here we compared two putative iron donors, CyaY and IscA, for their iron binding activity under physiologically and oxidative stress conditions. CyaY is an orthologue of human frataxin in E. coli (Campuzano et al., 1996, Patel & Isaya, 2001), and IscA is a conserved key member in iron sulfur cluster assembly machinery which is responsible for the general iron-sulfur cluster biogenesis (Zheng et al., 1998, Takahashi & Nakamura, 1999, Schwartz et al., 2001, Jensen & Culotta, 2000, Cozar-Castellano et al., 2004). Our results showed that CyaY and IscA have very different iron binding property under physiological or oxidative stress condition. In the presence of thioredoxin reductase system which emulates the intracellular redox potential in physiological condition, CyaY fails to bind with iron. Even when a 10-fold excess ferrous iron was available under physiologically relevant conditions, CyaY fails to bind iron. In contrast, IscA binds iron with an iron association
constant of $2 \times 10^{19} \text{M}^{-1}$ under physiologically relevant conditions (Ding et al., 2004) and delivers iron for the iron-sulfur cluster assembly in a proposed scaffold IscU (Ding et al., 2004, Ding & Clark, 2004, Ding et al., 2005a, Ding et al., 2005b, Yang et al., 2006).

In the presence of hydrogen peroxide, IscA completely loses its iron binding activities but CyaY acts as an iron binding protein and attenuate the production of hydroxyl free radicals. The reason why IscA and CyaY has different iron binding properties is that hydrogen peroxide may oxidize the thiol groups in IscA, resulting in the failure of IscA iron binding activity. On the other hand, hydrogen peroxide did not affect the carboxyl groups in the iron binding site of CyaY which allows the iron binding in CyaY in the presence of hydrogen peroxide and alleviates the production of hydroxyl free radicals. All these results led us to propose that the primary function of IscA is to recruit the intracellular iron for the iron-sulfur cluster biogenesis under physiologically relevant condition, and that CyaY is to scavenge iron under the oxidative stress condition and prevent the production of the deleterious hydrogen peroxide.

Frataxin/CyaY and their orthologues have a well conserved three dimensional structure belonging to the $\alpha$-$\beta$ sandwich motif family. The highly conserved aspartic acid and glutamic acid residues located on $\alpha$-$\beta$ sandwich are proposed to involve in iron binding activity (Dhe-Paganon et al., 2000, He et al., 2004, Cho et al., 2000, Nair et al., 2004). The iron binding association constant of human frataxin is $1.8 \times 10^4$ (Yoon & Cowan, 2003) and the constant for *E. coli* frataxin/CyaY is $2.6 \times 10^5$ (Bou-Abdallah et al., 2004). In other words, compared with the iron binding affinity of IscA, both human frataxin and CyaY have a relatively weak iron binding affinity.

Figure 3.1A showed that CyaY fails to bind iron under physiologically revelant conditions. However, other data showed that in excessive amount of iron, CyaY binds to iron and forms iron-mediated aggregation complexes (Adamec et al., 2000a, Adinolfi et al., 2002b, Cavadini et
al., 2002, Bou-Abdallah et al., 2004). In yeast, it has been shown that frataxin orthologues is dispensable for the iron-sulfur cluster biogenesis (Aloria et al., 2004). In our studies, we found that even when 10-fold excess iron are available in the presence of the thioredoxin reductase system, CyaY still does not have the ability to bind iron. This result further supported that the iron-mediated aggregation complex of frataxin is not a physiological iron donor for the iron-sulfur clusters biogenesis. On the other hand, frataxin/CyaY can recruit ferrous iron under oxidative stress condition, and reduce the production of hydroxyl free radicals. The reason for the different iron binding property of IscA and CyaY is that the thiol groups responsible for iron binding in IscA can be oxidized, while the carboxyl groups for iron binding in CyaY can not (Figure 3.5). This is consistent with the observations that deficiency of frataxin in cells is associated with increase of the iron mediated production of reactive free radicals and cellular oxidative damage (Karthikeyan et al., 2002, Karthikeyan et al., 2003, Campanella et al., 2004, Condo et al., 2006).

Iron-sulfur clusters are considered as the primary target of reactive oxygen species in cells. Once iron-sulfur clusters are disrupted by superoxide or other reactive oxygen species, the iron will be released from iron-sulfur clusters and generate more hydroxyl free radicals, which is harmful to DNA or cell membranes (Keyer & Imlay, 1996). The iron binding of CyaY in the presence of hydrogen peroxide may reduce the production of hydroxyl free radicals generated in the Fenton reaction resulted in avoiding the harmful effect of hydroxyl free radicals.

On the other hand, losing iron binding activity of IscA under oxidative stress conditions would reduces the iron-sulfur clusters biogenesis, and further decreases the possibility of iron release from iron-sulfur clusters and production of hydroxyl free radicals. The oxidization of thiol groups in IscA is reversible (Figure 3.7). Once hydrogen peroxide-treated IscA was reincubated with the thioredoxin reductase system and ferrous iron, IscA regained its iron
binding property. This result suggests that it is a smart way for cells to utilize two proteins CyaY and IscA to regulate the intracellular iron in response to have oxidative stress conditions. Although the relative amounts of IscA and CyaY in cells have not been determined yet, we envisioned that the iron exchange between IscA and CyaY under different conditions could be important to avoid deleterious effect of intracellular iron in cells.
Figure 3.1 The iron binding activity of IscA and CyaY in the presence of the thioredoxin reductase system.

Apo-IscA (100μM) and apo-CyaY (100 μM) were incubated with freshly prepared Fe(NH$_4$)$_2$(SO$_4$)$_2$ (50 μM) in the presence of thioredoxin-1 (5 μM), thioredoxin reductase (0.5 μM) and NADPH (0.5 mM) at 37°C for 30 min. A Mono-Q column was applied to repurify the IscA and CyaY protein. Panel A, the total iron content analyses of the eluted fractions. Panel B, the SDS-polyacrylamide electrophoresis gel of the eluted fractions. The locations of IscA and CyaY on the SDS gel are indicated. The results represent three independent experiments.
Figure 3.2 Spectroscopic analyses of IscA and CyaY after incubation with ferrous iron in the presence of the thioredoxin reductase system.

Panel A, the UV-vis spectra of repurified IscA before and after incubated with ferrous iron in the presence of thioredoxin reductase system. Panel B, the UV-vis spectra before of CyaY and after incubated with ferrous iron in the presence of thioredoxin reductase system. Panel C, the EPR measurement of the repurified IscA and CyaY after incubation with ferrous iron in the presence of the thioredoxin reductase system. \(mT\) means millitesla.
Figure 3.3 CyaY does not form the iron-mediated aggregate complex in the presence of the thioredoxin reductase system.

Apo-CyaY (100 μM) was incubated with freshly prepared Fe(NH₄)₂(SO₄)₂ (1mM) in the presence or absence of the thioredoxin reductase system in buffer containing 100 mM NaCl and 20 mM Tris pH 8.0 at 37°C for 30 min. A MonoQ column was applied to repurify the CyaY protein from the incubation solutions. Spectrum a, the CyaY repurified from the incubation solution without the thioredoxin reductase system. Spectrum b, the CyaY repurified from the incubation solution with the thioredoxin reductase system.
Figure 3.4 The iron binding activity of IscA and CyaY in the presence of hydrogen peroxide.

Apo-IscA (100 μM) and apo-CyaY (100 μM) were incubated with freshly prepared Fe(NH₄)₂(SO₄)₂ (50 μM) in the presence of 2 mM of hydrogen peroxide and the thioredoxin reductase system at 37°C for 30 min in aerobic conditions. The solution was applied to a Mono-Q column to repurify CyaY and IscA protein as described in chapter I. Panel A, the total iron contents of the eluted fractions. Panel B, the SDS-PAGE gel of the eluted fractions. The location of IscA and CyaY on SDS gel was indicated. The results represent three independent experiments.
Figure 3.5 CyaY prevents the iron-mediated production of hydroxyl free radicals in the presence of hydrogen peroxide.

A, apo-CyaY or apo-IscA (0 μM to 50 μM) was preincubated with freshly prepared Fe(NH₄)₂(SO₄)₂ (5 μM) in the presence of the thioredoxin reductase system and 4 mM 2-deoxyribose at 37°C for 10 min before 0.5 mM hydrogen peroxide was added to initiate the Fenton reaction. The relative amount of hydroxyl free radicals produced in the incubation solution was plotted as a function of CyaY (filled circles) or IscA (open circles) concentration. B, apo-CyaY or apo-IscA (50 μM) was preincubated with 0 μM to 50 μM of freshly prepared Fe(NH₄)₂(SO₄)₂ in the presence of thioredoxin reductase and 4 mM 2-deoxy-ribose at 37°C for 10 min before 0.5 mM hydrogen peroxide was added into the solution to initiate the Fenton reaction. The relative amounts of hydroxyl free radicals produced in the solution was plotted as a function of the concentration of Fe(NH₄)₂(SO₄)₂ in the incubation solution. Open triangles represent no protein in the solution; filled circles represent apo-CyaY; Open circles represent apo-IscA. The samples without any ferrous iron were used as controls. The data are the representative of three independent experiments.
Figure 3.6 Hydrogen peroxide inhibits the iron binding in IscA and oxidizes the free thiol groups in the protein.

Apo-IscA (100 μM) was incubated with ferrous iron (50 μM) in the presence of 0 mM to 5 mM of hydrogen peroxide at 37°C for 30 min. The determination of the total free thiol groups and the iron binding activity in IscA were performed side by side. Panel A, hydrogen peroxide blocks the iron binding activity in IscA. The relative iron binding activity in IscA was plotted as the function of the concentration of hydrogen peroxide in the incubation solution. Panel B, hydrogen peroxide oxidized free thiol groups of IscA. The relative amount of free thiol groups in IscA was plotted as the function of the concentration of hydrogen peroxide in the incubation solution.
Figure 3.7 Recovery of the iron binding activity of the hydrogen peroxide-treated IscA by the thioredoxin reductase system.

Spectrum a, apo-IscA. Spectrum b, apo-IscA was incubated with an equivalent amount of freshly prepared Fe(NH$_4$)$_2$(SO$_4$)$_2$ in the presence of the thioredoxin reductase system at 37°C for 30 min. Spectrum c, same as spectrum b but has 2 mM of hydrogen peroxide in the incubation solution. Spectrum d, IscA in spectrum c was reincubated with ferrous iron and the thioredoxin reductase system at 37°C for 30 min. All protein samples were repurified from the solution by using a Mono-Q column. The absorption peak at 435 nm and 315 nm indicated the iron binding in IscA.
4.1 Introduction

Nitric Oxide (NO) is a small lipophilic molecule and an unstable aqueous soluble gas with a very short half-life (around 5 seconds). NO is generated by nitric oxide synthases in cells and can directly diffuse across cell membranes and reach to adjacent cells. When diffusing to nearby cells, nitric oxide reacts with the iron atoms of heme groups of the guanylate cyclase or other hemoproteins. This interaction represents a widespread mechanism that converts an extracellular stimulus to biosynthesis of cyclic GMP in target cells (Ignarro, 1990). In rat mitochondria, binding of NO to the binuclear heme α3/CuB center in the cytochrome c oxidase of the respiration chain also reversibly inhibits the respiratory electron transfer (Brookes et al., 2003). Besides hemoproteins, the other major iron-containing proteins in cells are iron-sulfur proteins (Johnson et al., 2005, Beinert et al., 1997). Previously studies indicated that iron-sulfur proteins, particularly [4Fe-4S] proteins (Gardner et al., 1997b, Kennedy et al., 1997, Drapier, 1997), are the major targets of NO and are readily inactivated through modification of iron-sulfur cluster by NO (Gardner et al., 1997a). Unlike the reversible binding of NO to the iron atoms in hemeproteins, the NO-mediated modification of iron-sulfur cluster forms a new structure: the protein-bound dinitrosyl iron complex (DNICs). This specific structure has a unique EPR (electron paramagnetic resonance) signal at g = 2.04 (Kennedy et al., 1997, Foster et al., 1999). The EPR signal of the protein bound DNICs has been observed in the activated macrophages where NO is produced by iNOS (inducible NO synthase) (Lancaster & Hibbs, 1990), in tumor cells co-cultured with activated macrophages (Drapier et al., 1991), in acute cardiac allograft injection (Pieper et al., 2003), in rat gastro-oesophageal junction where NO was luminally generated from nitrite (Asanuma et al., 2007). Thiol groups in BSA, other thiol containing
proteins, L-cysteine, and N-acetyl-L-cysteine can also form the DNIC complex with ferrous iron and NO in vitro (Vanin et al., 1993, Boese et al., 1995b, Butler & Megson, 2002). However, the existence of the small molecule thiols-bound DNICs has never been reported (Ueno & Yoshimura, 2000, Pedersen et al., 2007) likely because these small molecule thiol-bound DNIC are not stable in aqueous solution (Asanuma et al., 2007). When E.coli cells are exposed to NO at a rate comparable to that reported for the NO production in activated macrophages, neutrophils or other cells (Gobert et al., 2001, Krieglstein et al., 2001), a large number of iron-sulfur proteins are modified forming the protein-bound DNICs (Ren et al., 2008).

Because iron-sulfur proteins play important roles in amino acids biosynthesis, gene regulations, electron transfer, heme biosynthesis, RNA modification, DNA synthesis and repair, and other fundamental life processes in living organisms (Rouault & Tong, 2005, Johnson et al., 2005, Lill & Muhlenhoff, 2006), modification of iron-sulfur clusters by NO is expected to inactivate multiple cellular functions. In order for cells to survive, the NO-modified iron-sulfur proteins must be efficiently repaired. Experimental data showed that in E. coli, the NO-modified iron-sulfur clusters are indeed repaired under aerobic growth condition (Ding & Demple, 2000, Hyduke et al., 2007). However, under anaerobic growth conditions, E. coli cells fail to repair any NO-modified iron-sulfur clusters (Ren et al., 2008). The repair mechanism for the NO-modified iron-sulfur clusters remains elusive.

In this study, we report that the E. coli aconitase B [4Fe-4S] clusters are easily modified by NO forming the protein-bound dinitrosyl-iron complexes (DNICs) under both aerobic and anaerobic conditions and that oxygen does not affect the modification of iron-sulfur clusters by NO. Our data also showed that the E.coli cell respiratory electron transfer components remained unchange after NO exposure indicating that not all iron-sulfur proteins are sensitive to NO. We also found that repair of NO-modified iron-sulfur proteins in E. coli requires a combination of
the iron-sulfur cluster assembly machinery, intracellular reduced thiol groups and oxygen molecule. Deletion of the iron-sulfur cluster assembly proteins IscA and SufA gene effectively prevents re-activation of the NO-inactivated ironsulfur protein but has no effect on decomposition of the protein-bound DNICs. The results suggest that a complete repair of the protein-bound DNICs may have two steps: decomposition of DNICs and reassembly of iron-sulfur clusters in protein. And that IscA and SufA are involved in reassembly of iron-sulfur clusters but are not required for decomposition of DNICs signal.

Our results further showed that depletion of intracellular thiols inhibit the decomposition of the protein-bound DNICs in \textit{E. coli}. Furthermore, oxygen is required for L-cysteine mediated decomposition of the protein bound DNICs as L-cysteine has little or no effect on the protein-bound DNICs under anaerobic conditions. Additional studies reveal that L-cysteine can form the L-cysteine-bound DNICs through the thiol ligand-exchange equilibrium with the protein-bound DNICs. Unlike the protein-bound DNICs, the L-cysteine bound DNICs is easily decomposed by oxygen (Vanin et al., 1993, Vanin, 1993, Boese et al., 1995b, Butler & Megson, 2002).

This led us to propose that repair of NO-modified iron-sulfur protein comprises two sequential steps: the L-cysteine mediated decomposition of DNICs and reassembly of new iron-sulfur clusters in proteins, and that oxygen is required for the L-cysteine-mediated decomposition of the protein bound DNICs.

4.2 Methods and Materials

4.2.1 Protein Preparation

\textit{E. coli} genomic DNA was used as the template DNA for the PCR amplification of the gene encoding aconitase B (Varghese et al., 2003). Two primers for aconitase B amplification have the following sequences: AcnB-1, 5’-gaacggcatgtagtaaatcc-3’; AcnB-2, 5’-tgacattaaagcttagtctgga-3’. The \textit{Nco I} and \textit{Hind III} restriction enzymes were used to digest pET-
28b+ (purchased form Stratagene), pBAD plasmid (purchased from Invitrogen Company) and the aconitase B PCR products. The digested PCR product was ligated to digested pET-28b+ and pBAD to yield pTACN, pBACN, respectively. Both recombinant plasmids containing the aconitase B gene were transformed into BL21 (DE3) expression cell strain for aconitase B protein expression. IPTG (0.2 mM) or L-arabinose (0.02%) was used to induce aconitase B protein expression in pTACN and pBACN respectively. The protein purification processes are same as described in Chapter II. E. coli thioredoxin 1(TrxA) and thioredoxin reductase (TrxB) are produced from the expression vectors pDL59 and pTrR301 respectively and purified as described in Chapter II.

4.2.2 NO Exposure of E. coli Cells under Aerobic or Anerobic Conditions

Overnight E. coli cells containing pTACNB plasmid were inoculated to freshly prepared Luria-Bertani (LB) culture by 1:100 dilutions. The cell culture was incubated at 37°C with aeration at 250 rpm for 2 hours. IPTG (200 μM) was added to the cell culture to induce overexpress of recombinant aconitase B. After 1.5 hours induction, the cells were centrifuged at 4°C with a speed of 4000 rpm for 10 min. The cell pellet was then resuspended into M9 minimum medium to get an OD_{600} = 2. For anaerobic NO treatment, E. coli cells were purged with argon for 10 min to remove the oxygen before exposure to the Silastic tube (I.D. × O.D.; 0.025 × 0.047 in., Dow Corning co) of the NO delivery system. The NO gas was first passed through a soda-lime column to remove NO₂ and higher nitrogen oxides before flew through a Silastic tube for the NO treatment anaerobically. The length of the Silastic tube immersed into the cell culture was adjusted to keep the NO rate at 100 nM per second. For aerobic NO treatment, E. coli cells were directly exposure to Silastic tube NO delivery system in an open-to-air system with vigorous stirring. The sample at each time point was taken immediately for EPR samples and for enzyme activity analysis right after French press.
4.2.3 NO Exposure of E. coli Cells

*E. coli* cells containing pBACNB plasmid were grown in Luria-Bertani (LB) rich medium over night, and then further diluted 1:50 in freshly prepared LB medium with 100 µg/ml ampicillin. The cells were shaken at 250 rpm at 37°C for around 2 hours, when OD600 reached 0.8, L-arabinose (0.02%) was added into the culture to initiate the overexpression of the aconitaseB protein. After three hours induction, cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The cell pellet was washed with M9 minimal medium without glucose twice to remove the residual LB and further resuspended in the same minimal medium containing 34 µg/mL of chloramphenicol to OD at 600 nm of 4.0. The cell culture was then incubated at 37°C under aerobic conditions for 1h starvation followed by NO treatment. The cells were then purged with argon for 10 min to remove the oxygen before being treated with NO gas. For the NO exposure, pure NO gas (Air co) will be treated same as described in NO exposure of *E. coli* cells under aerobic or anaerobic conditions.

4.2.4 Enzyme Activity Assay for Aconitase

For aconitase activity assay, purified protein or the cell extract was added into pre-incubation solutions containing 90 mM Tris (pH 8.0) and 20 mM D,L-isocitrate at 25°C. The reaction was monitored following the formation of cis-aconitate at 240 nm using an extinction coefficient of 3.6 mM⁻¹cm⁻¹ (Henson & Cleland, 1967).

4.2.5 Preparation of the L-Cysteine-Bound DNICs

The L-cysteine-bound DNIC was synthesized according to the published methods (Boese *et al*., 1995a) with slight modifications. Pre-degassed Fe(NH₄)₂(SO₄)₂ (20 µM) was injected into a fully degassed solution containing 40 µM L-cysteine and 50 µM Tris (pH 8.0) in anaerobic condition. The solution was treated with pure NO for 2 to 4 seconds and then purged with argon for 3 min to get rid of residual NO. The newly synthesized L-cysteine-bound DNIC was
immediately transferred to an EPR tube and frozen in liquid nitrogen before EPR measurements. The rest of the sample was divided into two parts: one was incubated at 37°C under aerobic conditions and the other one was on anaerobic conditions.

4.2.6 Determination of Nitrite, Nitrate and Ferrous Iron

4.2.6.1 Nitrite Measurement

Nitrite in solutions was directly measured with the Griess reagents (Cayman Chemicals co) using sodium nitrite as a standard. In brief, 50 μl solution A and 100 μL cell extract or protein samples were mixed first, and then 50 μL solution B was added into the mixed solution followed by a vigorous vortex. The reaction solution was then left at the room temperature for over 15 min and centrifuged for 1 min to remove all precipitants. The samples were taken spectra the absorption peak at around 540 nm was used for measurement of nitrite. The nitrite concentration in different solution can be determined by using nitrite solution as standard.

4.2.6.2 Nitrate Measurement

Nitrate was determined after its reduction to nitrite by means of a cadmium reductor and then assessed by the Griess reagents as described above.

4.2.6.3 Ferrous Iron Content Measurement

Ferrous iron in protein samples was measured by using an iron indicator ferrozine. The procedure was same as described under in Chapter III.

4.2.7 EPR Measurements

A Bruker model ESR-300 EPR spectrometer equipped with an Oxford instruments 910 continuous flow cryostat were used for recording the X-band EPR spectra. Routine EPR conditions were: microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; sample temperature, 20 K; receive gain, 10^5. The amounts of the protein-bound DNICs were calculated from the amplitude of the EPR signal.
at \( g = 2.04 \) of the freshly prepared L-cysteine-bound DNICs as described above (Vanin et al., 1993, Boese et al., 1995b).

4.3 Results

4.3.1 The Iron-Sulfur Clusters in *E. coli* Aconitase B Are Sensitive to NO

To investigate the reactivity of NO with iron-sulfur clusters in proteins, we used a recombinant aconitase B [4Fe-4S] expressed in cells. Because aconitase B represents the major aconitase activity in the Tricarboxylic Citric Acid cycle in *E. coli* cells (Gruer & Guest, 1994), the aconitase [4Fe-4S] clusters were used as an example for the determination of NO modification on iron-sulfur proteins and subsequent repair in *E. coli* cells. The *E. coli* cells containing the aconitase B [4Fe-4S] cluster were treated with NO at a rate of 100 nM per second anaerobically. Figure 4.1A shows that 30 seconds NO exposure under anaerobic conditions was sufficient to inhibit 50% of the aconitase B activity in *E. coli* cells. The initial reaction rate was high and the reaction started to level off when cells were treated with NO for 1 min. A 2 min NO treatment of *E. coli* cells completely inactivated aconitase B activity. Figure 4.1C shows the EPR spectra of the *E. coli* cells treated with NO for different time under anaerobic conditions. The EPR signal at \( g = 2.04 \) of the protein-bound DNICs increased, closely followed the inactivation of the aconitase B activity (Figure 4.1A). Figure 4.1B shows the inhibition of the aconitase B activity by NO under aerobic conditions. Clearly, NO can also modify the aconitase B [4Fe-4S] cluster in *E. coli* cells under aerobic conditions, indicating that NO has a higher reactivity with the aconitase [4Fe-4S] clusters than with oxygen. Figure 4.1D shows that under aerobic conditions, the EPR signal at \( g = 2.04 \) of the protein-bound DNICs appeared when *E. coli* cells were exposed to NO. The results further confirmed that NO can readily modify the aconitase B [4Fe-4S] clusters in *E. coli* cells under aerobic conditions.

When *E. coli* cells overexpressing recombinant aconitase B were exposed to NO at a rate of
100 nM per second for 2 to 4 mins, the aconitase activity was completely inactivated (Figure 4.1A). This sample was measured by EPR spectroscopy side by side, a $g = 2.04$ EPR signal representing the protein-bound DNICs (Kennedy et al., 1997, Foster et al., 1999) showed up in the spectrum, indicating that iron-sulfur clusters were modified into dinitrosyl-iron complex by NO. The recombinant aconitase B protein has been purified from these NO treated cell extracts, and the enzyme activity analysis and the EPR measurement both demonstrated that the iron-sulfur clusters were modified, forming DNICs after NO treatment (data not shown).

### 4.3.2 Repair of the NO-Modified Iron-Sulfur Proteins in E. coli Cells Requires Glucose and Oxygen in Growth Medium

When 0.2% of glucose was supplied in the cell culture, around 70% of the aconitase activity was restored (Figure 4.2A) and 95% of the DNICs signal was decomposed (Figure 4.2B). These results supported the previous study that the protein-bound DNICs can be repaired in E. coli cells under aerobic growth condition (Ding & Demple, 2000). Addition of chloramphenical had no effect on the repair of the NO-modified aconitase B indicating that the newly synthesized proteins are not required for reactivation of aconitae B. When only oxygen or glucose was available in the culture, the NO treated E. coli cells failed to restore the aconitase activity (Figure 4.2A) and the EPR signal for DNICs were largely remained unchanged (Figure 4.2B). The results suggested that both glucose and oxygen are required for reactivation of NO modified aconitase [4Fe-4S] clusters.

### 4.3.3 Roles of the Iron-Sulfur Cluster Assembly Proteins IscA/SufA in Re-activation of the NO-Modified Iron-Sulfur Proteins in E. coli Cells under Aerobic Growth Conditions

There are two major gene clusters responsible for the iron-sulfur clusters assembly in E. coli: **iscRSUA-hscBA-fdx** (Zheng et al., 1998) and **sufABCDSE** (Takahashi & Tokumoto, 2002). It was reported that both gene clusters in E. coli cells are highly induced when cells are exposed to NO or NO derivatives (Mukhopadhyay et al., 2004, Justino et al., 2005). IscA and its paralog,
SufA, are proposed as the iron donor (Ding et al., 2005b, Ding et al., 2005a, Ding et al., 2004, Yang et al., 2006) or alternative iron-sulfur assembly proteins (Ollagnier-De-Choudens et al., 2004). Deletion of either IscA or SufA does not affect the cell growth of E. coli in M9 minimal medium under aerobic conditions. However, double deletion of IscA and SufA results in the null growth phenotype of the double mutant in M9 minimal medium under aerobic conditions (Lu et al., 2008). Recent studies further revealed that the \textit{iscA}/sufA double deletion mutant fails to assemble the \([4Fe-4S]\) clusters in proteins in \textit{E. coli} cells under aerobic growth conditions (Tan et al., 2009). Here we investigate the role of IscA and SufA in repair of the protein-bound DNICs in \textit{E. coli} cells.

Because IscA and SufA paralogs are essential for the \([4Fe-4S]\) cluster assembly under aerobic conditions (Tan et al., 2009), the aconitase \textit{B} \([4Fe-4S]\) clusters in the IscA/SufA double deletion mutant were overexpressed under anaerobic conditions. Figure 4.3A shows that when the IscA/SufA double deletion mutant with over-expressed aconitase \textit{B} was exposed to NO, the aconitase activity will be completely inactivated with concomitant formation of the protein-bound DNICs. However, when the NO-treated double-deletion mutant cells were repaired in M9 minimal medium in the presence of both oxygen and glucose, the aconitase activity was not restored (Figure 4.3A). The parallel EPR experiments showed that the aconitase-bound DNICs in the double deletion mutant \textit{E. coli} cells was completely decomposed (Figure 4.3B). The results demonstrated that IscA and SufA are required for reassembly of new iron-sulfur clusters but are not essential for decomposition of the protein-bound DNICs under aerobic conditions.

4.3.4 Depletion of Intracellular Reduced Thiols Prevents Decomposition of the Protein-Bound DNICs in \textit{E. coli} Cells

Our previously studies indicated that L-cysteine can specifically decompose the protein-bound DNICs \textit{in vitro} (Rogers & Ding, 2001, Ren et al., 2008). However, the physiological
roles of L-cysteine and other small molecule thiols in reactivation of the NO-modified iron-sulfur proteins remains not fully understood. Deletion of cysk and cysm genes encoding the cysteine biosynthesis enzymes in E. coli cells failed to completely inhibit the synthesis of L-cysteine in vivo, likely because L-cysteine is vital in different biological pathways. N-ethylmaleimide (NEM) was then used for modification of thiol groups (Ness et al., 1997).

In the experiments, NEM (50 μM) was added to the NO-treated cell culture. After 15 min, the incubation cells were returned to M9 minimum medium containing both glucose and oxygen at 37°C with aeration for 60 min. Figure 4.4A shows that without NEM in the M9 minimal medium, the protein-bound DNICs were completely decomposed. However, in the presence of 50 μM NEM, a significant amount of the protein-bound DNICs remained in E. coli. This result suggested that NEM may inhibit the decomposition of the protein-bound DNICs by depletion of intracellular reduced thiols.

Glutathione (GSH) is a predominant low molecular weight intracellular thiol in many organisms and plays an important role in preventing cells from oxidative damages. To test whether glutathione has the same function on decomposition of the protein-bound DNICs as L-cysteine does, glutathione was also added into cell extracts prepared from the NO treated E. coli cells. Figure 4.3B showed that addition of glutathione failed to decompose the protein-bound DNICs. The reduced thioredoxin represents the major reducing thiol system in E. coli (Aslund & Beckwith, 1999). Addition of the thioredoxin/ thioredoxin reductase system didn’t have any effect on the protein-bound DNICs either (Figure 4.4B). All these data indicated that only L-cysteine can decompose the protein-bound DNICs.

4.3.5 Oxygen Is Required for the L-Cysteine-Mediated Decomposition of the Protein-Bound DNICs

When E. coli cells were in exponentially growing stage, the intracellular cysteine
concentration was estimated to be 0.2 mM (Park & Imlay, 2003). To deplete intracellular L-cysteine content, the *E. coli* cells were starved in M9 minimal medium without glucose for 1 hour. This would presumably consume most of L-cysteine, thus decrease L-cysteine concentrations. To check the role of L-cysteine in decomposing the protein-bound DNICs, we supplemented L-cysteine (1mM) in the NO-exposed *E. coli* cell culture in minimal medium without glucose. The result showed that under aerobic conditions, L-cysteine can decompose the protein-bound DNICs *in vivo* (Figure 4.5A). However, under anaerobic conditions, L-cysteine did not have any effect on the protein-bound DNICs (Figure 4.5B). To exclude the possibility that L-cysteine was not transported into *E. coli* cells and incubated with L-cysteine under anaerobic conditions, cell extracts were prepared from the NO-treated cells under aerobic or anaerobic conditions. Figure 4.5C showed that under anaerobic conditions, L-cysteine did not have any effect on the protein-bound DNICs. However, under aerobic conditions, L-cysteine effectively decomposed the protein-bound DNICs.

Above results suggested that L-cysteine is essential in decomposition of the protein-bound DNICs in the cell extracts. To further explore the role of L-cysteine in the protein-bound DNICs, aconitase B-bound DNICs were purified and incubated with L-cysteine under aerobic or anaerobic conditions. Figure 4.6A shows that L-cysteine can readily decompose the protein-bound DNICs under aerobic conditions but not under anaerobic conditions. In another words, both L-cysteine and oxygen are required for decomposition of the protein-bound DNICs. Figure 4.6B shows that L-cysteine efficiently converted the DNIC-bound aconitaseB to apo-aconitase B and released ferrous iron and nitrite under aerobic conditions. Nitrate was not detectable at any time during the reaction process, suggesting that the L-cysteine-mediated decomposition of the protein-bound DNICs produced exclusively nitrite as a stable metabolite. The ratio of nitrite to iron released from the aconitase B-bound DNIC by L-cysteine was approximately 2:1 (Figure
4.5B), in good agreement with the expected decomposition of the aconitase B-bound DNICs.

4.3.6 The L-Cysteine-Bound DNIC Is Decomposed under Aerobic Conditions but Is Stable under Anaerobic Conditions

The earlier results showed that L-cysteine contributes to the decay of the protein-bound DNICs. However, it further calls for the reason why L-cysteine mediates decomposition of the protein-bound DNICs under aerobic conditions but not anaerobic conditions. It has been reported that a ligand-exchange equilibrium takes place between low mass thiols and the protein thiol ligands (Boese et al., 1995b). So, L-cysteine-bound DNICs could be transferred to the protein-thiol group through the ligand-exchange equilibrium to form the protein-bound DNICs. In this equilibrium, because protein-bound DNICs are stable for several hours at room temperature, the reaction is favored towards the protein-bound DNICs (Boese et al., 1995b). However, if excess amount of L-cysteine is available, the equilibrium will go toward to the L-cysteine-bound DNICs. If the cysteine-bound DNIC is unstable under aerobic conditions, the protein-bound DNICs will be transferred to the L-cysteine-bound DNICs and more and more protein-bound DNICs can be transferred to L-cysteine–bound DNICs and finally will be decomposed. Consistent with our hypothesis, we found that 50-100 fold excess of L-cysteine (0.5 mM to 1.0 mM) was required for efficiently decomposing the 10 μM aconitase B-bound DNIC under aerobic conditions (data not shown). If the amount of L-cysteine is less than five folds of the aconitase B-bound DNICs, it had little effect on the decomposition of the protein-bound DNICs. These results indicated that L-cysteine may establish the ligand-exchange equilibrium with the protein-bound DNICs. We then tested the stability of the L-cysteine-bound DNICs under aerobic and anaerobic conditions. The newly synthesized L-cysteine-bound DNICs were prepared under anaerobic condition as described in this chapter and then divided into two vials and incubated under aerobic and anaerobic conditions. Figure 4.7A shows that the L-
cysteine-bound DNICs were quickly decomposed under aerobic conditions but not under anaerobic conditions. The kinetics of oxygen mediated decomposition of the L-cysteine-bound DNICs under aerobic and anaerobic conditions are shown in Figure 4.7B. The half-life time of the cysteine-bound DNIC was about 5 min under aerobic conditions. On the other hand, the cysteine-bound DNIC remained essentially unchanged under anaerobic conditions.

**4.3.7 In vitro Repair of Aconitase B-Bound DNICs in *E. coli* Cell Extract**

To repair the NO-inactivated aconitase B in vitro, the freshly prepared NO-treated *E. coli* cell extract was incubated with IscA-Fe, IscS, thioredoxin reductase system, NADH and L-cysteine in buffer containing 20 mM Tris pH 8.0 at 37°C for 20 min under aerobic conditions. The cell extract was then taken for the aconitase activity assay. Figure 4.8 shows that comparing with the cell extracts without NO treatment, at least 60% of the aconitase activity was regained through the repair process with a complete system. When IscA-Fe (–IscA), IscS (–IscS), NADPH (–NADPH), NADH (–NADH) or L-cysteine was omitted in the incubation solution, the aconitase B activity was not detectable under aerobic conditions. This experiment further showed that L-cysteine, ISC assembly machinery and oxygen are required for completely repair of protein-bound DNICs and restore the activity of iron-sulfur enzymes. Thus a complete repair of the protein-bound DNICs requires two steps: removal of the DNICs from the protein; and reassembly of new iron-sulfur clusters in proteins.

**4.4 Discussion**

While Iron-sulfur clusters are ubiquitous and ancient prosthetic groups that are required for different fundamental life processes (Rouault & Tong, 2005, Johnson et al., 2005), they are also highly sensitive to reactive free radicals such as NO. The initial reaction between NO and the [4Fe-4S] cluster in aconitase B has an apparent rate constant of $7.0\pm2.0\times10^6 M^{-2} s^{-1}$ at 25°C (Duan et al., 2009), which is around 3 times faster than that of the NO autoxidation by oxygen in
aqueous solution (Liu et al., 1998, Ford et al., 1993). When *E. coli* cells are exposed to micromolar concentrations of NO under anaerobic or aerobic conditions, iron-sulfur proteins are modified forming the protein-bound DNICs (Ren et al., 2008). If cells are to survive, the NO-modified iron-sulfur proteins must be promptly repaired. In our study, we revealed that repair of the NO-modified iron-sulfur proteins requires a combination of the iron-sulfur cluster assembly machinery, intracellular reduced thiols, and molecular oxygen. Deletion of the key iron-sulfur cluster assembly proteins IscA/SufA causes the null-growth phenotype of *E. coli* cells in minimal medium under aerobic growth conditions (Lu et al., 2008). Deletion of IscA and SufA paralogs also blocks reactivation of the NO-modified iron-sulfur proteins (Figure 4.2A) but have no effect on the decomposition of the protein-bound DNICs (Figure 4.2B). On the other hand, depletion of the intracellular reduced thiols prevents the decomposition of the protein-bound DNICs (Figure 4.3A). Among the intracellular reduced thiols, L-cysteine appears to have a unique activity to decompose the protein-bound DNICs both in vivo and in vitro under aerobic conditions (Figure 4.4 and 4.5). This suggested that a completely repair of the NO modified iron-sulfur proteins requires two steps: Step 1: removal of DNICs from proteins. In this process, L-cysteine and oxygen are essential for decomposition of the protein-bound DNICs both *in vivo* and *in vitro*. Step 2: the reassembly of iron-sulfur cluster on iron-sulfur proteins. In this step, iron-sulfur cluster assembly machinery is required for the iron-sulfur clusters reassembly.

We also propose a model for the L-cysteine-mediated decomposition of the protein-bound DNICs. In the model, we postulate that the DNICs can be transferred between L-cysteine and reduced protein thiol groups through the ligand-exchange equilibrium (Figure 4.9). In a previously study, it was reported that the L-cysteine-bound DNICs can be transferred to the reduced thiol groups in BSA to form the BSA-bound DNICs (Boese et al., 1995b), so the ligand exchange equilibrium will largely shift towards the protein-bound DNICs. When an excess
amount of L-cysteine is available in the solution, the reaction equilibrium will go towards the L-cysteine-bound DNICs, which means that the protein-bound DNICs will be transferred to L-cysteine to form the L-cysteine-bound DNICs (Figure 4.9). Because the L-cysteine-bound DNICs are stable under anaerobic conditions, the DNICs signal can still be detected (Figure 4.7). Since L-cysteine-bound DNICs are readily decomposed under aerobic conditions, the protein-bound DNICs will shift to form the L-cysteine-bound DNICs resulting in decomposition of the protein-bound DNIC (Figure 4.7). So the net reaction will be that L-cysteine and oxygen convert the protein-bound DNICs to apo-proteins and release iron and nitrite (Figure 4.7). This explains why when both L-cysteine and oxygen are available, the protein-bound DNICs can be quickly decomposed in vivo and in vitro.

In E. coli cells, there are many reduced thiols. However, only L-cysteine can efficiently decompose the protein-bound DNICs under aerobic conditions. Although, glutathione is the predominant intracellular thiol (Greenberg & Demple, 1986), it doesn’t have any effect on the decomposition of the protein-bound DNICs (Figure 4.4B). This might be because that GSH can not establish the thiol ligand-exchange equilibrium with the protein-bound DNICs in solution due to its redox property. Although glutathione-bound DNIC was reported to be associated with the glutathione S-transferases in cells (Pedersen et al., 2007), it was suggested that the glutathione-bound DNIC is most likely produced directly from intracellular “free” iron and NO and stabilized by the glutathione S-transferases in cells (Pedersen et al., 2007). The thioredoxin reductase system is another major group of intracellular thiols (Aslund & Beckwith, 1999). However, reduced thioredoxin-1 also fails to decompose the protein-bound DNICs under aerobic conditions (Figure 4.4B). Interestingly, when thioredoxin-1 was incubated with the protein-bound DNICs, the repurified thioredoxin 1 shows a small amount of thioredoxin-bound DNICs indicating that thioredoxin-1 can extrude some DNIC from the protein through equilibrium.
Although glutathione and thioredoxins are not directly involved in the decomposition of the protein-bound DNICs, it is still possible that they have an essential role in maintaining reduced L-cysteine to decompose the protein-bound DNICs in cells.

Our research suggests that a complete repair of the NO modified iron-sulfur clusters requires two steps: decomposition of protein-bound DNICs and re-assembly of iron-sulfur clusters in the proteins. Although two paralogs of IscA and SufA are found to be required for the assembly of new iron-sulfur clusters (Figure 4.3), it is conceivable that other members in the iron-sulfur cluster assembly machinery system are also vital for reassembly of iron-sulfur clusters (Hyduke et al., 2007, Mukhopadhyay et al., 2004, Justino et al., 2005, Pullan et al., 2007, Djaman et al., 2004). Since reactivation of the NO-modified iron-sulfur proteins does not require newly synthesized proteins, pre-existing iron-sulfur cluster assembly proteins could be sufficient for the iron-sulfur clusters reassembly in the NO-modified proteins. Nevertheless both oxygen and glucose are required for reactivation of NO-modified iron-sulfur proteins (Figure 4.2). In addition to its role for decomposition of the L-cysteine-bound DNICs (Figure 4.9), oxygen may also promote the production of cellular energy. Glucose provides ATP and reducing powers such as NADH/NADPH to produce L-cysteine and reduced thioredoxins via the glycolysis and the pentose-phosphate pathway. Evidently if only glucose or oxygen is available, the protein-bound DNICs in *E. coli* can’t be fully repaired (Figure 4.2). The in vitro study further showed that ISC machinery, L-cysteine, NADH, thioredoxin reductase system are essential for repair of the protein-bound DNICs aerobically (Figure 4.8). Collectively our results suggest that a combination of oxygen, L-cysteine and energy supply are essential for a complete repair of the protein-bound DNICs in *E. coli* cells.
Figure 4.1 Modification of the aconitase B [4Fe-4S] cluster by NO in vivo under aerobic or anaerobic growth conditions.

*E. coli* cells containing overexpressed aconitase B [4Fe-4S] clusters were exposed to NO at a rate of 100 nM per second through the Silastic tube NO delivery system under aerobic or anaerobic conditions. Panel A, the relative enzyme activities of the recombinant aconitase B [4Fe-4S] cluster in cell extract prepared from the *E. coli* cells treated with NO at different time points under anaerobic conditions. Panel B, the relative enzyme activities of the recombinant aconitase B [4Fe-4S] cluster in cell extract prepared from the *E. coli* cells treated with NO at different time points under aerobic conditions. Panel C, the EPR spectra of the aconitase B protein purified from each sample of panel A. Panel D, the EPR spectra of the aconitase B protein purified from each sample of Panel B.
Figure 4.2 Reactivation of the NO-modified aconitase B in E. coli cells requires glucose and oxygen in growth medium.
Recombinant aconitase B was over-expressed in E. coli cells as described in Experimental Procedures. The cells were washed twice with minimum medium without glucose and resuspended in the same minimum medium to an OD of 4.0. Chloramphenicol (34 μg/mL) was added to block new protein synthesis before the cells were exposed to pure NO gas anaerobically. Control sample means untreated E. coli cells; +NO means the E. coli cells were treated with NO gas; The NO treated samples were divided into four flasks for recovery at 37°C for 60 min. sample a), NO treated cells were recovered without glucose anaerobically; b), NO treated cells were recovered without glucose aerobically; c), NO treated cells were recovered with glucose anaerobically; d) NO treated cells were recovered with glucose aerobically.
Panel A shows the relatively activity of aconitase B in each sample; Panel B shows the EPR spectra of each sample shown in Panel A.
Figure 4.3 The iron-sulfur cluster assembly proteins IscA/SufA are required for reactivation of the NO-modified aconitase B in *E. coli* cells.

Recombinant aconitase B protein was over-expressed in the *E. coli* iscA/sufA double mutant under anaerobic condition. The cells were washed twice with minimum medium without glucose and re-suspended in the same minimum medium to an OD of 4.0. Chloramphenicol (34 μg/mL) was added to block new protein synthesis before the cells were exposed to pure NO gas anaerobically. For recovery experiment, the NO treated *E. coli* cells were recovered in minimum medium containing 0.2% glucose at 37°C for 60 min aerobically. Panel A, the relative aconitase activity in the IscA/SufA double mutant cells before NO (1), after NO (2) and subsequent recovery (3). Panel B, the parallel EPR spectrum for each sample in Panel A. 1 is the EPR spectrum for *E. coli* IscA/SufA double mutant before NO treatment. Spectrum 2 is the EPR spectrum of the *E.coli* IscA/SufA double mutant after NO treatment. Spectrum 3 is the EPR spectrum for recovery of the NO-treated *E. coli* IscA/SufA double mutant.
Figure 4.4 Intracellular reduced thiols and decomposition of the protein-bound DNICs in E. coli cells.
Panel A, the depletion of intracellular reduced thiols inhibits the repair of the protein-bound DNICs in E. coli cells. The NO-treated E. coli cells were incubated with N-ethylnaleimide at final concentrations of 0 and 50 μM for 15 min before recovery in minimal medium containing 0.2% glucose at 37°C for 60 min aerobically. Cell cultures in each treated samples were directly taken out and frozen in liquid nitrogen for EPR measurement. Panel B, L-cysteine is unique in decomposition of the protein-bound DNICs. The NO-treated E. coli cell extract was incubated in minimal medium with addition of 1 mM reduced glutathione (GSH) (b), 1 mM L-cysteine (c) or thioredoxin reductase system (TrxA/TrxB, contained 20 μM of thioredoxin-1, 2 μM of thioredoxin reductase and 1 mM NADPH) (d) at 37°C for 60 min. After incubation, samples were directly frozen in liquid nitrogen for the EPR measurement.
**Figure 4.5** L-cysteine-mediated decomposition of the protein-bound DNICs in vivo.

Panel A, L-cysteine promotes decomposition of the protein-bound DNICs in *E. coli* under aerobic conditions. The NO exposed *E. coli* cells were recovered in minimal medium without glucose in the presence or absence of 1 mM L-cysteine at aerobic or anaerobic conditions. Panel B, L-cysteine has no effect on the decomposition of the protein-bound DNICs in the *E. coli* cells under anaerobic conditions. NO-exposed *E. coli* cells were recovered in minimal medium (without glucose) in the presence or absence of 1 mM L-cysteine at aerobic or anaerobic conditions. Panel C, the kinetics of L-cysteine mediated decomposition of the protein-bound DNICs in the NO-treated *E. coli* cell extracts. The cell extract was prepared from NO treated *E. coli* cells disrupted by passing through the French press once. The cell extract was then incubated with 1mM L-cysteine at 37°C under aerobic (open circle) or anerobic (open square) conditions. The amplitudes of the EPR signal at $g = 2.04$ resembles the protein-bound DNICs in the cell extracts were plotted as a function of incubation time.
Figure 4.6 Oxygen is required for the L-cysteine-mediated decomposition of the aconitase B-bound DNICs.

Panel A, L-cysteine mediated decomposition of the aconitase B-bound DNIC requires oxygen. Trace a) shows the EPR spectrum of 5 μM aconitase B-bound DNICs. Aconitase B-bound protein (same as in trace a) dissolved in buffer containing 20 mM Tris (pH 8.0) and 500 mM NaCl was incubated with 1 mM of L-cysteine under aerobic (c) or anaerobic (e) conditions. b) and d) represents the EPR spectrum of aconitase B-bound DNICs incubated with buffer in the absence of L-cysteine at aerobic or anaerobic conditions. All the EPR spectra were taken after 30 min incubation. Panel B, the kinetics of L-cysteine-mediated decomposition of the aconitase B-bound DNIC under aerobic conditions. 5 μM purified aconitase B-bound DNICs was incubated with 1 mM L-cysteine at 37°C under aerobic conditions. At each time point, the amplitude of the EPR signal at g = 2.04 in the incubation solution was measured as described in experimental procedures. The closed squares represent the protein-bound-DNICs in the incubation solution. The closed circles represent the ferrous iron content in the incubation solution. The open circles represent the nitrite concentration in the incubation solution.
Figure 4.7 The stability of L-cysteine-bound DNICs under aerobic and anaerobic conditions.

The L-cysteine-bound-DNICs was synthesized and divided into two vials: one was incubated at aerobic condition and another one was incubated under anaerobic conditions. At indicated time points, the samples were immediately transferred to EPR tubes and frozen in liquid nitrogen before EPR measurements. Panel A, the EPR spectra of the L-cysteine-bound DNICs incubated under aerobic or anaerobic conditions for 30min. Panel B, stability of the L-cysteine-bound DNIC under aerobic and anaerobic conditions. The closed circles represent aerobic incubation and the open circles represent anaerobic incubation. The amplitude of EPR signal at $g = 2.04$ was plotted as the function of incubation time.
Figure 4.8 Repair of aconitase B-bound DNICs in vitro.

_E. coli_ cells over-expressed with aconitase B protein were treated with NO gas. The cells were disrupted through passing the French press once. 40 μL cell extract prepared from the NO-treated _E. coli_ cells was incubated with 100 μM IscA-Fe, 4 μM IscS, 20 μM thioredoxin1, 5 μM thioredoxin reductase, 1 mM NADPH, 1 mM NADH, and 1 mM L-cysteine in buffer containing 20 mM Tris pH 8.0 to reach a total volume around 100 μL (complete system) at 37°C for 20 min aerobically. If all above reagents were added into the solution (complete system), at least 60% of the activity was restored. Control represents the aconitase activity for cell extract without NO treatment. When IscA-Fe (-IscA-Fe), IscS (-IscS), NADPH (-NADPH) or NADH (-NADH) was omitted in the incubation solution, the aconitase activity was not detectable or very low compared with control or a complete system.
Figure 4.9 A proposed model for the L-cysteine-mediated decomposition of the protein-bound DNICs under aerobic conditions.
Addition of excess L-cysteine will establish the ligand-exchange equilibrium with the protein-bound DNICs. Under anaerobic conditions, the equilibrium will be largely favored towards the protein-bound DNICs with a small amount of the L-cysteine-bound DNIC. Under aerobic conditions, the L-cysteine-bound DNIC is decomposed by oxygen to release ferrous iron and nitrite, thus driving the equilibrium towards the L-cysteine-bound DNIC.
Chapter V
Conclusions and Future Directions

Iron-sulfur clusters are essential prothetic groups involved in diverse fundamental life processes. Assembly of iron-sulfur clusters requires multiple proteins. It has been shown that IscS, a cysteine desulfurase containing a PLP, catalyzes the conversion of L-cysteine to L-alanine and sulfur and provides sulfide for the iron-sulfur clusters biogenesis. However, the iron source is still not well understood. Our studies showed that IscA is an iron-binding protein with an iron association constant of $2 \times 10^{19}$ M$^{-1}$, prevents formation of insoluble ferric hydroxide and delivers iron for the iron-sulfur cluster assembly under physiologically relevant conditions.

We further compared the iron binding activities of IscA and CyaY/bacterial frataxin in context of the iron-sulfur cluster assembly. CyaY/E. coli frataxin is shown to be associated with the iron metabolism in mitochondria in eukaryotic cells. It was proposed that CyaY may work as an iron donor for the iron-sulfur cluster assembly. Our results showed that under physiological conditions, IscA has a much stronger iron binding affinity than CyaY. The iron bound to IscA can be transferred to IscU to form iron-sulfur clusters. However, under oxidative stress conditions, IscA fails to bind iron due to the oxidation of the thiolate groups in its iron binding site. On the other hand, CyaY becomes a potent iron binding protein as the carboxyl groups in its iron binding site are not oxidized by hydrogen peroxide. Binding of iron in CyaY also effectively prevents the iron-mediated production of hydroxyl free radicals in the presence of hydrogen peroxide.

The iron-sulfur clusters are one of the primary targets of nitric oxide. When nitric oxide reacts with iron-sulfur clusters, iron-sulfur clusters are converted to the protein-bound dinitrosyl-iron complex. Because the modification of iron-sulfur clusters will affect the enzyme activity, the NO-modified iron-sulfur clusters must be repaired. Here, we showed that the aconitase [4Fe-
4S] cluster can be readily modified by NO. The reaction between NO and aconitase [4Fe-4S] cluster is very similar under aerobic or anaerobic conditions indicating that oxygen has little effect on the NO-mediated modification of iron-sulfur proteins. The complete reactivation of NO-modified aconitase B requires not only oxygen, L-cysteine but also the iron-sulfur cluster assembly machinery. Deletion of IscA and SufA results in failure to restore the aconitase enzyme activity, but does not have any effect on decomposition of the protein-bound DNICs in E. coli cells. The results suggested that reactivation of the NO-modified aconitase B requires two steps: decomposition of the protein-bound DNICs by oxygen and L-cysteine; and, re-assembly of new iron-sulfur clusters in proteins.

For the future research, it would be important to investigate the in vivo iron binding in IscA by using radioactive iron. All our data shown previously are in vitro iron binding affinity of IscA. The iron transfer from IscA to IscU in vivo will also need to be evaluated by applying of radio iron and sulfide. The hypothesis that the iron-sulfur clusters are formed by the Fe-S element but not by providing iron and sulfide individually also requires concrete evidence to further improve the USA model we proposed in JBC paper. For CyaY, we need to overexpress CyaY protein and determine whether the E. coli with CyaY will be more resistant to oxidative stress conditions comparing with the wide type E. coli cells. The in vivo role of CyaY will have to be further explored. For the repair mechanism of the NO-modified iron-sulfur proteins, more experiments are required to demonstrate the role of oxygen in decomposing the L- cysteine-bound DNICs.
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Vita

Juanjuan Yang was born in December 1977 in a small village close to Yellow River and at the root of Taihang Moutains in Henan Province, the People’s Republic of China. She got her bachelor degree in the Henan Normal University in biology education in 1999. She obtained her master degree in animal reproduction and physiology in the South China Agricultural University in 2003. Before she came to the Louisiana State University on January 10th 2005, she was a teacher in Beijing NO. 80 high school. She will get her Doctor of Philosophy degree in biochemistry major on August 2009 from the Louisiana State University.