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Fe-S Cluster Assembly Pathways in Bacteria

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INTRODUCTION

Iron is an essential transition metal in biology due to its use as a cofactor for nitrogen fixation, photosynthesis, and respiration. Iron can bind proteins in mono- and di-iron reaction centers, can be incorporated into porphyrin rings to form heme, and can be combined with elemental sulfur to form iron-sulfur (Fe-S) centers. Iron is the fourth most abundant element by weight in the Earth's crust. The more soluble Fe^{2+} form was stabilized by the reducing atmosphere of the early Earth, ensuring that iron was bioavailable and that the Earth's primordial organisms incorporated iron as a cofactor for multiple biochemical reactions. In particular, Fe-S clusters are thought to be one of the earliest iron cofactors used in biology (125).

Iron-sulfur clusters, consisting of iron and elemental sulfur at various molar ratios, are often stable at multiple oxidation states and have physiologically relevant redox potentials (ranging from −500 to 150 mV) (12). Therefore, electron transfer is a primary role for Fe-S clusters. Fe-S clusters are also involved in substrate binding and activation in dehydratases and radical–*S*-adenosylmethionine enzymes (102). Finally, Fe-S clusters are used as "molecular switches" for gene regulation at both the transcriptional and translational levels due to their sensitivity to cellular redox conditions (52). The most common cluster forms, [2Fe-2S] and [4Fe-4S], are usually coordinated to proteins by Cys residues, although Asp, His, Ser, or backbone amides can coordinate clusters at single sites (82). In vitro, in the presence of $Fe^{2+/3+}$ and S^{2-}, Fe-S clusters can form spontaneously in proteins that have the correct number and arrangement of Cys ligands (71). However, as both Fe and S are highly reactive and toxic in vivo, Fe-S cluster assembly requires carefully coordinated biosynthetic pathways in living cells.

There are multiple Fe-S cluster assembly pathways throughout the three kingdoms of life. The three pathways identified to date are the Isc (*i*ron *s*ulfur *c*luster) system, the Suf (*su*lfur *f*ormation) system, and the Nif (*ni*trogen *f*ixation) system (reviewed recently in reference 46). The phylogenetic distribution of these three systems is complex. For example, in cyanobacteria the Suf pathway appears to be the major system for Fe-S cluster assembly compared to the Isc pathway, while in *Escherichia coli* the relative importance of Suf and Isc is reversed. Furthermore, organisms such as *Mycobacterium tuberculosis*, as well as some archaea, appear to possess only the Suf pathway for cluster assembly. In eukaryotes, the relative importance of each Fe-S cluster assembly pathway is further complicated by their differential localization to specific organelles. Homologues of the Isc pathway are found largely in the mitochondria, while Suf homologues have been conserved in the chloroplasts of some photosynthetic organisms.

However, biochemical and genetic analyses of the three pathways have revealed some differences in their typical physiological roles (see below). We suggest that the pathways can be subdivided loosely into those used for housekeeping cluster assembly (Isc), those used under stress conditions (Suf), and

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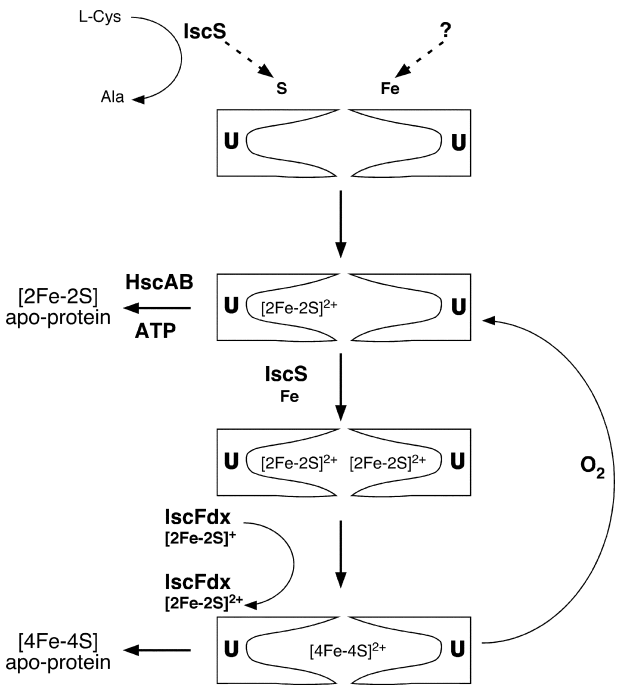

FIG. 1. Current model for Fe-S cluster assembly on the IscU scaffold. IscS donates sulfur liberated from L-cysteine, and iron is donated from an iron chaperone (such as CyaY) or by direct iron binding to IscU. Sequential iron and sulfur donations result in assembly of one [2Fe-2S] cluster, which is transferred to an apoprotein with the assistance of the HscAB molecular chaperone system. Further assembly of a second [2Fe-2S] cluster occurs, setting the stage for reductive coupling to form a [4Fe-4S] cluster. IscFdx may mediate reductive coupling in vivo through a one-electron reduction of each cluster (only one IscFdx molecule is shown for simplicity). The [4Fe-4S] cluster can be transferred to an apoprotein or can react with oxygen to degrade to a single [2Fe-2S] cluster.

those used to assemble complex or specialized clusters for a specific enzyme (Nif). One possible explanation for the diverse distribution of the three systems is that in certain organisms or organelles (within eukaryotes), the various biochemical attributes of each pathway may confer an advantage. For example, in some organisms high rates of cluster assembly to support rapid growth may be highly advantageous, while in other settings a carefully shielded, stress-resistant cluster assembly pathway would be selected. Clearly, this hypothesis remains to be confirmed by future experiments that carefully compare each pathway under a range of controlled conditions.

Several functional components are common in Fe-S cluster assembly. A cysteine desulfurase enzyme is required to liberate sulfur atoms from free cysteine for use in cluster assembly (Fig. 1) (138). The sulfur liberated by a cysteine desulfurase is donated to a second protein that acts as a "scaffold" for nascent Fe-S cluster assembly (Fig. 1). Following cluster assembly on the scaffold, the Fe-S cluster is then transferred to a target apoprotein (Fig. 1). In the nomenclature of the bacterial Fe-S cluster pathways, the cysteine desulfurase is designated with an "S" and the scaffold protein is signified with a "U" (as in IscS and IscU).

Despite the relative simplicity of this scheme, the mechanistic details of Fe-S cluster assembly are still being elucidated. A critical gap in our knowledge is the process of in vivo iron donation for cluster assembly. This is not a trivial question, since the basic details of intracellular iron trafficking in bacteria are still unresolved. Most cellular iron is sequestered into iron metalloenzymes and iron storage proteins, such as ferritin. It is generally accepted that only a fraction of total cellular iron, termed the labile iron pool, is available for iron cofactor biosynthesis. Efforts to define the exact concentration of the labile iron pool in bacteria have led to estimates of about 10 μM (as opposed to a total iron concentration of around 200 μM) (129). However, measurement of the labile iron pool often relies on chelation of "available" iron followed by detection of the metal-chelate complex. The concentration of chelatable iron in the cell may not be directly equivalent to the concentration of bioavailable iron in the labile iron pool used for iron cofactor biosynthesis, since the chelator itself might directly compete with cellular iron ligands or trafficking proteins. Furthermore, intracellular iron is not likely to be present in the "free" or "aquo" form but will be coordinated to cellular ligands. The identity of the ligands that bind iron in the labile iron pool is unknown, but they could be protein chaperones such as CyaY (see below), small metabolites such as citrate, nucleic acids, or other cellular components.

Any simplistic picture of Fe-S cluster assembly is further complicated by the involvement of a host of accessory proteins needed for cluster assembly in vivo. In many cases, the exact roles of these accessory proteins are not well characterized, and gene deletion mutants show a range of phenotypes for in vivo Fe-S cluster biosynthesis. Finally, many bacterial species contain multiple Fe-S cluster assembly pathways that are used under certain growth or stress conditions. In some cases, these pathways functionally overlap, while in other cases the cluster assembly systems are unique for a specific cellular condition.

This review is organized into sections focused on the biochemical characteristics of functional categories of Fe-S cluster assembly proteins. In addition, several sections are devoted to novel Fe-S cluster assembly proteins whose in vivo functions are still largely unknown. The review concludes with a thorough description of the in vivo regulation of Fe-S cluster assembly and a discussion of the functional cross talk between Fe-S cluster assembly pathways in vivo. Several recent reviews provide quite an extensive background on Fe-S cluster assembly (8, 34, 46, 63). Where appropriate, we cite these reviews and instead focus on recent advances in our understanding of Fe-S cluster biosynthesis.

CYSTEINE DESULFURASES

Sulfur donation by the NifS protein was the earliest step of Fe-S cluster assembly elucidated at the biochemical level (138). In most systems characterized to date, sulfur donation is catalyzed by a cysteine desulfurase, such as NifS, via donation of a persulfide to the scaffold protein. The reaction mechanism of cysteine desulfurases involves formation of a Schiff base between free cysteine and a pyridoxal phosphate (PLP) cofactor in the enzyme active site. Next, a cysteinyl persulfide is formed through nucleophilic attack by the active-site cysteine thiolate anion on the sulfur of the cysteine-PLP adduct, releasing alanine in the process. The enzyme-bound persulfide can then be

transferred to Cys residues on acceptor proteins, such as scaffolds, by another nucleophilic attack involving a reaction that is fundamentally similar to protein disulfide bond exchange (83). Trafficking of sulfur in this carefully controlled manner prevents release of the sulfur as toxic bisulfide (SH^-).

The cysteine desulfurases implicated in Fe-S cluster assembly can be subdivided into group I (NifS and IscS) and group II (SufS and CsdA) enzymes based on overall sequence homology (78). These groups are distinguished by two different consensus sequences around the active-site Cys residue in the C-terminal domain. Group I enzymes contain the sequence SSGSACTS, while the group II enzymes contain the sequence RXGHHCA. At present, the biochemical explanation for these differences is lacking, but clearly the different groups of desulfurase enzymes do have important structural and enzymatic differences. For this review, the salient fact is that deletion of the cysteine desulfurases impairs cluster assembly by their respective pathways.

IscS

Deletion of IscS is lethal in *Azotobacter vinelandii* and causes severe growth defects in *E. coli* (103, 114, 137). Many of these defects are directly linked to deficiencies in Fe-S enzymes. It has been shown that IscS directly interacts with and transfers sulfur to the IscU scaffold protein for Fe-S cluster assembly (2, 4). Sulfur transfer occurs from Cys328 on IscS to IscU. The exact site of sulfur deposition on IscU is somewhat unclear. Early work on the *E. coli* IscU protein identified a single acceptor, Cys63, that was also reported to form a heterodisulfide with IscS Cys328, followed by intramolecular transfer between Cys residues in IscU (51). However, recent studies seem to indicate that all three conserved Cys residues on *A. vinelandii* IscU can accept sulfur from IscS in vitro, and a heterodisulfide complex between IscS and IscU requires Cys37 rather than Cys63 (112). IscS has also been shown to interact with other proteins in the Isc system, namely, IscFdx and IscX (formerly YfhJ or ORF3) (120). The significance of these interactions is unclear.

In addition to a role in Isc-mediated Fe-S cluster assembly, IscS also functions as a sulfur donor for the synthesis of thiolated nucleosides 2-thiouridine and 4-thiouridine in tRNA and to the ThiI protein during thiamine biosynthesis (56, 57, 111, 121). Thiamine and biotin biosynthesis pathways also require an Fe-S protein (ThiH and BioB, respectively), and both processes are indirectly perturbed by loss of IscS due to diminished cluster content in the required enzymes. The synthesis pathways of the thiolated nucleosides dimethallyl-2-methylthioadenosine and 2-thiocytidine also require Fe-S enzymes for sulfur donation (MiaB and TtcA, respectively) and are deficient in an *iscS* deletion strain. Alanine scanning mutagenesis of the active-site loop of IscS in *E. coli* has identified specific point mutations that diminish the synthesis of Fe-S clusters in vivo but do not alter non-Fe-S-dependent synthesis of the 2-thiouridine and 4-thiouridine tRNAs (58). Thus, the multifunctional role of IscS in Fe-S cluster assembly and general sulfur trafficking may be facilitated by different regions of the IscS active-site loop.

SufS and SufE

SufS and SufE interact in a complex (SufS-SufE) (70). The cysteine desulfurase SufS mobilizes sulfur from free cysteine, resulting in formation of a persulfide on SufS Cys364 (79). The persulfide sulfur atom is then donated from SufS to the active-site Cys51 on the SufE protein (89, 93). Consequently, the presence of the SufE sulfur transfer shuttle stimulates the basal activity of SufS, and the two proteins together form a novel sulfur transfer system (70, 93). It was also demonstrated that the SufBCD complex enhances SufS-SufE sulfur transfer by an unknown mechanism (93). Recent work has established that a minimum complex of SufBC is responsible for stimulation of SufS-SufE activity and that SufE interacts directly with SufB to transfer sulfur to SufB for Fe-S cluster assembly on that protein (59). SufS or SufE also transfers sulfur to SufA in vitro. A mixture of SufS and SufE, precharged with persulfide, can deposit sulfur on Cys residues in SufA (105). The exact mechanism by which SufE enhances SufS activity is still a matter of debate and could occur by enhancing the initial cleavage of the C-S bond of the cysteine-PLP adduct rather than by accelerating subsequent breakage of the SufS persulfide (118).

The structure of the SufS cysteine desulfurase reveals that the active-site Cys364 of SufS is oriented into the protein interior and does not appear to be solvent accessible (36, 64). In contrast, the active-site Cys of the IscS desulfurase is highly exposed on a flexible loop structure (24). These differences in active-site orientation affect basal enzyme activity, since the specific activity of IscS is 20 times higher than that of SufS when the enzymes are assayed alone (77). However, the addition of the SufE sulfur transfer partner increases SufS activity so that it is comparable to that of IscS (70, 93). Interestingly, the active-site Cys51 of SufE is also oriented into the protein interior, as shown by the SufE structure (66). Interactions between SufS and SufE must somehow allow their solvent-inaccessible active-site Cys residues to contact each other to allow sulfur transfer from SufS to SufE.

SufS was first thought to be used explicitly for the acquisition and mobilization of selenium due to its high specific activity for L-selenocysteine compared to that for L-cysteine (77, 79). However, the presence of SufE results in an increase in SufS cysteine desulfurase activity to levels comparable to SufS selenocysteine lyase activity (70). Interestingly, SufE has no effect on SufS selenocysteine lyase activity, and the active-site Cys364 of SufS is not required for selenocysteine lyase activity. The in vivo relevance of SufS selenocysteine lyase activity remains to be elucidated.

The *E. coli* SufE crystal structure at 2.0-Å resolution showed surprising similarity to the nuclear magnetic resonance (NMR) structure of the Zn-bound form of *Haemophilus influenzae* IscU, despite no obvious sequence homology between them (Fig. 2) (38, 66, 98). The structural homology of IscU and SufE suggests that the two proteins will interact with their cognate cysteine desulfurase enzymes (IscS and SufS, respectively) in a similar geometry for sulfur transfer. In fact, overlaying the IscU and SufE structures shows that SufE Cys51 and IscU Cys37 are found in topologically equivalent locations (Fig. 2). However, the conserved Cys63 and Cys106 residues of IscU are not found in SufE. Moreover, all three invariant Cys residues in the IscU structure are either partially or fully solvent ex-

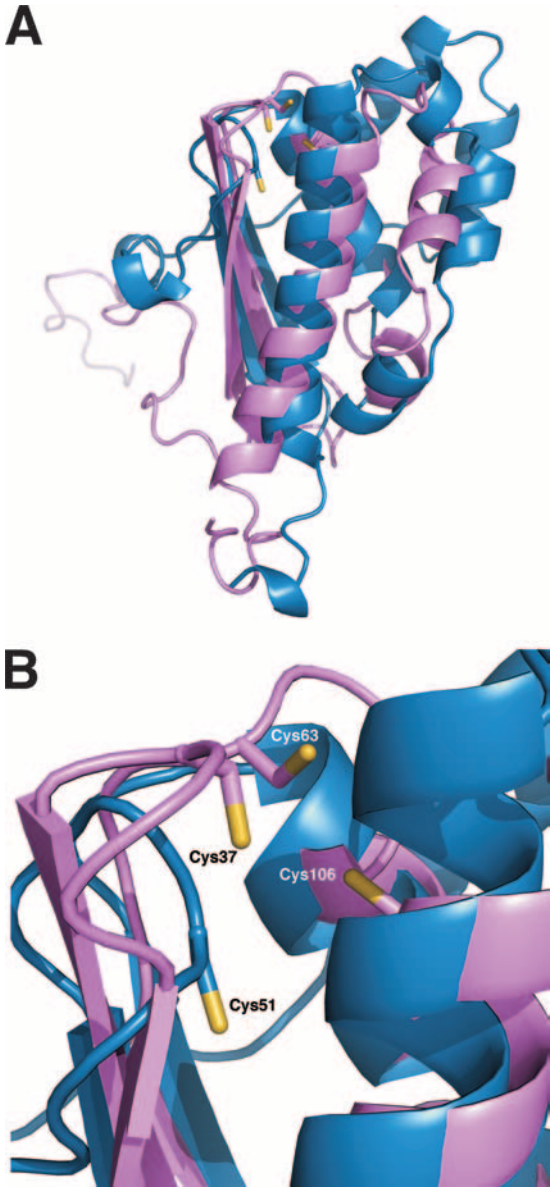

FIG. 2. Structural comparison of *E. coli* SufE (PDB accession no. 1MZG) with *H. influenza* IscU (PDB accession no. 1R9P). (A) Overlay of the carbon backbone of SufE (blue) with that of IscU (mauve), demonstrating the high degree of structural similarity. Relevant Cys side chains are shown for orientation. (B) Enlargement of the overlaid structures in the vicinity of SufE Cys51 and IscU Cys37, Cys63, and Cys106. SufE Cys51 is located in a similar region to that of the conserved IscU Cys37 residue but is oriented deeper into the protein interior and is not solvent exposed. Cys63 and Cys106 are not present in SufE. The superposition of SufE and IscU was performed using lsqkab from the CCP4 suite and DaliLite pairwise structure comparison. Figures were generated using PyMOL.

posed, in stark contrast to the solvent-inaccessible Cys51 of SufE. Thus far, there is no evidence that SufE itself can form an Fe-S cluster like IscU; rather, SufE appears to be a specialized shuttle for protected sulfur transfer among the Suf proteins.

CsdA and CsdE

E. coli contains a third desulfurase enzyme, known as CsdA (also referred to as CSD). In the genome, *csdA* is located next to *csdE* (formerly *ygdK*), which encodes a protein 35% identical to SufE. Structural characterization of both SufE and CsdE shows a high degree of similarity between the two proteins (66). Recent studies have shown that CsdE can enhance the cysteine desulfurase activity of CsdA by accepting persulfide sulfur on a conserved Cys residue, revealing a second SufS-SufE-like sulfur transfer pair in *E. coli* (69). Furthermore, the CsdA-CsdE pair could provide persulfide sulfur in vitro for cluster reconstitution on the [4Fe-4S] enzyme NadA, and overexpression of *csdA-csdE* from a plasmid can restore cluster assembly in the NadA and 6-phosphogluconate dehydratase enzymes in an *iscS* deletion strain. However, deletion of *csdA-csdE* did not show any phenotypes indicative of a defect in Fe-S cluster assembly. Therefore, it is still unclear if *csdA* and *-E* play a direct role in Fe-S cluster assembly under certain growth conditions in vivo or if they may be needed for sulfur trafficking to another pathway.

One cautionary note concerning the identification of sulfur acceptor proteins in vitro is that nonspecific sulfide transfer may occur under in vitro conditions. For example, all three cysteine desulfurases from *E. coli* can act directly as a sulfur source to reconstitute Fe-S ferredoxin in vitro without the need for a scaffold protein (55). In vitro, the presence of strong reductants, such as dithiothreitol, likely results in release of sulfide from the desulfurase or sulfur shuttle protein and uptake by the acceptor, but this probably does not reflect the true sulfur donation mechanism in vivo, where sulfide release would be toxic. Direct detection of protein-protein interactions may provide more conclusive evidence for specific sulfur transfer, and such interactions have been observed for well-characterized sulfur transfer partners (such as IscS-IscU, SufS-SufE, CsdA-CsdE, and SufE-SufBC) (59, 69, 70, 120).

SCAFFOLD PROTEINS

U-Type Scaffolds

NifU was the first scaffold protein to be characterized for Fe-S cluster assembly. NifU is a modular scaffold containing three distinct domains that can each form Fe-S clusters (3, 35, 135). However, the central, ferredoxin-like domain contains a stable [2Fe-2S] cluster that cannot be transferred to an apoenzyme and likely plays a role in redox processes during cluster assembly. The N-terminal domain of NifU is highly similar to IscU and forms both [2Fe-2S] and [4Fe-4S] clusters that can be transferred to apoenzymes (see further discussion of IscU below). The C-terminal Fe-S cluster-binding domain forms a [4Fe-4S] cluster that can also be transferred to an apoenzyme (113). Proteins similar to the C-terminal domain of NifU (referred to as Nfu) are found in cyanobacteria, plants, and higher eukaryotes, and they are thought to function as scaffolds in those organisms (86, 131). In vivo studies indicate that the two scaffold domains have somewhat redundant functions but that the N-terminal IscU-like domain is most important for nitrogenase Fe-S cluster assembly (31). Possibly NifU requires multiple scaffold domains for in vivo assembly of the complex, fused, double-cubane clusters found in nitrogenase (31).

In vitro reconstitution of *A. vinelandii* IscU revealed a dynamic cluster assembly process on this scaffold protein (2, 21). As reconstitution proceeds, the IscU homodimer first assembles a single [2Fe-2S] cluster. Then two [2Fe-2S] clusters form on each IscU homodimer. Over time, a slow conversion process occurs, resulting in one [4Fe-4S] cluster per IscU homodimer. The two [2Fe-2S]-to-one [4Fe-4S] cluster conversion was recently shown by UV-visible and Mössbauer spectroscopy to occur via a reductive coupling mechanism (21). The [2Fe-2S] clusters, which are thought to be located adjacent to each other at the dimer interface, each undergo a single electron reduction followed by rapid coupling to produce a single, bridging [4Fe-4S] cluster per IscU homodimer. In vitro reductive coupling can be initiated through the addition of a single reducing equivalent of dithionite or by addition of the reduced form of [2Fe-2S] IscFdx. IscFdx has been shown to be important for in vivo Fe-S cluster assembly and may be responsible for initiating the reductive coupling of IscU clusters in vivo. Conversion of the two [2Fe-2S] clusters to a [4Fe-4S] cluster by reductive coupling is not reversible, but the [4Fe-4S] IscU can be converted back to a [2Fe-2S] form (with the loss of one cluster equivalent of iron and sulfide) upon exposure to oxygen. The oxygen-catalyzed cluster degradation of [4Fe-4S] IscU might be involved in maintaining equilibrium between various cluster forms of IscU in vivo. At the very least, it indicates that the [4Fe-4S] form of IscU is unlikely to be maintained under conditions of oxidative stress. A similar reductive coupling mechanism has been proposed for cluster assembly on the N-terminal, IscU-like domain of NifU (113).

The discovery of multiple Fe-S cluster forms of U-type scaffolds also raised the question of which form was competent for cluster transfer to various [2Fe-2S] and [4Fe-4S] apoenzymes. Early characterization of the IscU proteins from various organisms showed that the [2Fe-2S] form could transfer its cluster to [2Fe-2S] acceptor proteins, such as ferredoxin (72, 130). However, it was unclear how a [4Fe-4S] cluster would be inserted in a [4Fe-4S] apoenzyme, as this could occur via two successive cycles of [2Fe-2S] transfer or from transfer of a single [4Fe-4S] cluster. Recently, this question was addressed for *A. vinelandii* IscU through the use of carefully controlled assembly and transfer reactions using specific Fe-S cluster forms of IscU, as monitored by Mössbauer spectroscopy (122). The studies clearly showed that the [4Fe-4S] form of IscU can transfer its cluster to the [4Fe-4S] enzyme aconitase, while the [2Fe-2S] form of IscU fails to transfer the cluster to this enzyme.

Despite the need for additional experiments that incorporate all of the Isc accessory proteins, the recent analysis of cluster assembly and transfer in IscU suggests the following model for Fe-S cluster biosynthesis (Fig. 1): one [2Fe-2S] cluster forms per homodimer of IscU, and with the aid of HscA and HscB, the [2Fe-2S] cluster can be transferred to [2Fe-2S] apoenzymes, such as ferredoxin. Through further cycles of sulfide and iron addition, the [2Fe-2S] form of IscU can be modified to contain two [2Fe-2S] clusters per dimer. Reduced [2Fe-2S] IscFdx may then initiate reductive coupling of the 2× [2Fe-2S] form of IscU to form the [4Fe-4S] form, which is capable of cluster transfer to [4Fe-4S] apoenzymes, such as aconitase. Although Fig. 1 shows this process occurring as a linear pathway for a single IscU dimer, in vivo the pool of IscU may exist as a mixed population with different cluster contents that is selectively drawn from depending on specific apoprotein demand. In this model, IscU exists in equilibrium as a mixture of these various forms in order to provide Fe-S clusters for both [2Fe-2S] and [4Fe-4S] enzymes. The equilibrium between [2Fe-2S] and [4Fe-4S] forms of IscU would be controlled by IscFdx activity, by cellular redox status, especially oxygen levels, and by apoprotein demand. The requirement for IscFdx for maturation of [4Fe-4S] clusters is not contradicted by IscFdx itself being an Fe-S protein, since apo-IscFdx could receive a [2Fe-2S] cluster from IscU or IscA before participating in subsequent assembly reactions. This model predicts several outcomes that could be tested in vivo. For instance, deletion of IscFdx should impair cluster assembly in [4Fe-4S] enzymes only, not in [2Fe-2S] enzymes. In contrast, deletion of HscA or HscB may impair cluster assembly in only [2Fe-2S] enzymes. All of these hypothetical predictions await testing at the experimental level.

A subclass of IscU proteins can be distinguished based on amino acid sequence analysis and phylogenetic distribution (46). It was proposed that this subclass of IscU be designated SufU because members can be found encoded with the Suf proteins in some organisms (46). While SufU and IscU are quite similar in sequence, SufU proteins contain an 18- to 21-amino-acid insertion between the second and third conserved Cys residues. In addition, SufU lacks the highly conserved LPPVK motif in IscU that is the recognition sequence for HscA binding to IscU. Genome comparison shows that organisms that contain SufU do not encode a genetically linked HscA and HscB homologue. This comparison indicates that SufU may function independently of the chaperones.

NMR structural analysis of the *Thermotoga maritima* SufU protein (referred to in the cited work as IscU) indicates that apo-SufU has a highly flexible tertiary structure, exists in a molten globule-like state, and does not have a well-defined cluster binding site (14). Comparison of apo-SufU to holo-SufU suggested that Fe-S cluster binding stabilizes the SufU protein fold (14). One drawback of this analysis was the use of an Asp40Ala mutant of SufU to help stabilize the Fe-S cluster, which may perturb the native structure to some degree. However, in support of this result, SufU from *Streptococcus pyogenes* could be crystallized with Zn bound to the three Cys residues that form the presumptive cluster binding site (67). The Zn-bound form of IscU from *H. influenzae* also appeared by NMR to be a stable, monomeric structure with a clear ternary structure and a well-defined metal binding site (98). The structural studies performed to date all suggest that the apoforms of U-type scaffolds are structurally dynamic and that cluster assembly will tend to stabilize the protein fold. The mechanistic role of this structural fluidity during Fe-S cluster assembly is not known.

Cyanobacteria, such as *Synechocystis* spp., contain a potential scaffold protein, encoded by *nfu*, that is similar to the C-terminal domain of NifU in *Azotobacter vinelandii* rather than to an IscU-like scaffold (86). In *Synechocystis*, *nfu* is essential, suggesting a prominent role for Nfu in Fe-S cluster assembly (7). Whether Nfu is integrated directly into the Suf pathway or the Isc pathway is unclear. If we group the cyanobacterial Fe-S assembly genes based on whether they are essential or not, then *nfu* clearly falls into the same category as

the *suf* genes, suggesting that Nfu might interact with the Suf pathway. This assignment is supported by the observation that a NifU homologue is colocated with the *suf* genes in cyanobacteria such as *Gloeobacter violaceus*. In contrast, the two *iscS* homologues, *iscS1* and *iscS2*, and the *iscA* gene are not essential in *Synechocystis*, so it is unlikely that they are the main sulfur donors for cluster assembly on Nfu (7, 11, 118). Nfu, encoded by ssl2667, can form a [2Fe-2S] cluster and transfer that cluster in vitro to apoferredoxin (86).

A-Type Scaffolds

The A-type proteins of Fe-S cluster biosynthesis pathways contain three highly conserved Cys residues (C-X_{42-44}-D-X_{20}-C-G-C) in their C-terminal regions. A-type proteins are found associated with the *isc*, *suf*, and *nif* pathways. The exact role of the A-type proteins in Fe-S cluster assembly is not clear. Two hypotheses concerning the biochemical function of the A-type proteins have recently emerged. The first states that the A-type proteins are alternative Fe-S scaffolds. In this model, the A-type scaffolds could be used for cluster donation to a subset of Fe-S apoproteins or could act as intermediates in cluster transfer from U-type scaffolds to apoproteins. The latter possibility has some support from in vitro studies showing that IscA can accept a cluster from IscU but cannot transfer a cluster to IscU (91). The second hypothesis is that the A-type proteins are actually iron chaperones that donate iron for Fe-S cluster assembly on U-type scaffolds.

$[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters can form on A-type proteins during reconstitution in vitro using sulfide or a cysteine desulfurase with cysteine and ferrous iron (54, 88, 90, 91). IscA from *Acidithiobacillus ferrooxidans* can be overexpressed and purified with an intact [4Fe-4S] cluster in *E. coli* (136). Mutagenesis and spectroscopic analysis of the A-type proteins suggest that all three conserved Cys residues are involved in cluster ligation (54, 136). The Fe-S clusters in the A-type proteins are reductively labile, just like those in the U-type scaffold proteins. In vitro studies have shown that the holoforms of A-type proteins can transfer Fe-S clusters to target apoproteins, leading to the hypothesis that they act as scaffolds (54, 88, 90). Protein-protein interactions between A-type scaffolds and apoproteins have also been reported for IscA and IscFdx and for SufA and BioB (90, 91).

It was shown in vitro that both IscU and IscA were able to mediate multiple cycles of [2Fe-2S] cluster formation and catalytic transfer to apoferredoxin (16). Analysis of kinetic data suggested that the formation of IscFdx-[2Fe-2S] in the presence of IscU or IscA followed Michaelis-Menten behavior. An apparent K_m of $\approx$27 μM was observed for $IscU_2$-[2Fe-2S] transfer to apoferredoxin, while the K_m of $\approx$210 μM observed for $IscA_2$-[2Fe-2S] seemed to be indicative of a weaker interaction between apoferredoxin and $IscA_2$-[2Fe-2S] during cluster transfer. In addition, the low cluster turnover number for IscA ($\sim$7-fold lower than that for IscU) may reflect a difference in the mechanism of [Fe-S] cluster transfer to apoferredoxin or a difference in their preferences for apoproteins. Genetic studies showed that deletion of IscU was lethal to *A. vinelandii* and that IscA cannot substitute for the functions of IscU in the biogenesis of [Fe-S] clusters, consistent with the in vitro biochemical differences between them (47). Depletion of the *iscA* gene shows severe effects on the growth of *A. vinelandii* only at elevated oxygen levels, which led to the proposal that IscA functions in the maturation of Fe-S proteins at elevated oxygen levels.

In addition to its ability to coordinate Fe-S clusters, IscA has a remarkable iron binding association constant of 3.0×10^{19} M^{-1}. In contrast, IscU failed to bind iron under similar experimental conditions (26). More importantly, IscA could bind iron and deliver it to IscU for biogenesis of [Fe-S] clusters in the presence of IscS and the iron chelator sodium citrate, suggesting a role for IscA in iron recruitment (27). The inability of C99S and C101S IscA mutants to bind iron showed that a cysteine pocket may form the binding site for a monoiron center (see below) (28). Recent studies also indicated that L-cysteine could mobilize the iron center in IscA for Fe-S cluster assembly on IscU, whereas biologically related thiols such as *N*-acetyl-L-cysteine or reduced glutathione failed to mobilize the iron center (25). Based on these experiments, IscA was proposed to act as an iron chaperone that binds free iron for donation to IscU (132).

However, one possible interpretation of these reports is that iron release by cysteine represents an aborted intermediate step in cluster assembly on IscA. Possibly, iron nucleation on IscA occurs first, followed by subsequent sulfur addition to form the Fe-S cluster. The addition of L-cysteine in vitro may initiate disulfide bond formation with the iron-nucleating IscA Cys residues, thereby releasing the iron rather than incorporating it into a nascent cluster, as would occur upon sulfide donation. Interestingly, competition experiments indicate that IscU rather than IscA preferentially forms an Fe-S cluster when both proteins are mixed simultaneously with IscS, iron, and cysteine. In contrast, IscA appears to bind iron much more tightly than IscU does if cysteine is omitted (132). A similar "iron-first" stepwise model of cluster assembly has been proposed for SufU (referred to as IscU in the cited work), based on its ability to bind iron with a high affinity (87). However, tight iron binding by other U-type proteins has not been confirmed by analytical or spectroscopic techniques. Potential differences in iron binding ability between SufU and IscU remain to be tested.

The recent crystal structure of holoform *Thermosynechococcus elongatus* IscA (2.5-Å resolution) is consistent with the view that IscA could be a scaffold for Fe-S clusters and suggests that IscA exists as a dimer and could ligate a partially exposed [2Fe-2S] cluster between two conformationally different IscA monomers (α and β) (81). The asymmetric cysteinyl S ligation by Cys37, Cys101, and Cys103 from the α monomer and by Cys37 from the β monomer of *T. elongatus* IscA is in agreement with proposed cysteinyl ligation for $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters in *A. vinelandii* $IscA^{Nif}$ and the $[2Fe\text{-}2S]^{2+}$ cluster in *Synechocystis* Isa1, although cluster binding has not been characterized structurally for these A-type proteins (54, 128). In contrast, the two other crystal structures of the apoform of *E. coli* IscA suggest that IscA can exist as a dimer, tetramer, or oligomer and do not provide information about potential [Fe-S] cluster ligation because the Cys-X-Cys motif in the C-terminal domain is not visible due to its highly flexible conformation (15, 23). Unfortunately, the *E. coli* IscA crystal structure does not provide any additional information to confirm if IscA is solely an iron binding protein or an Fe-S scaffold.

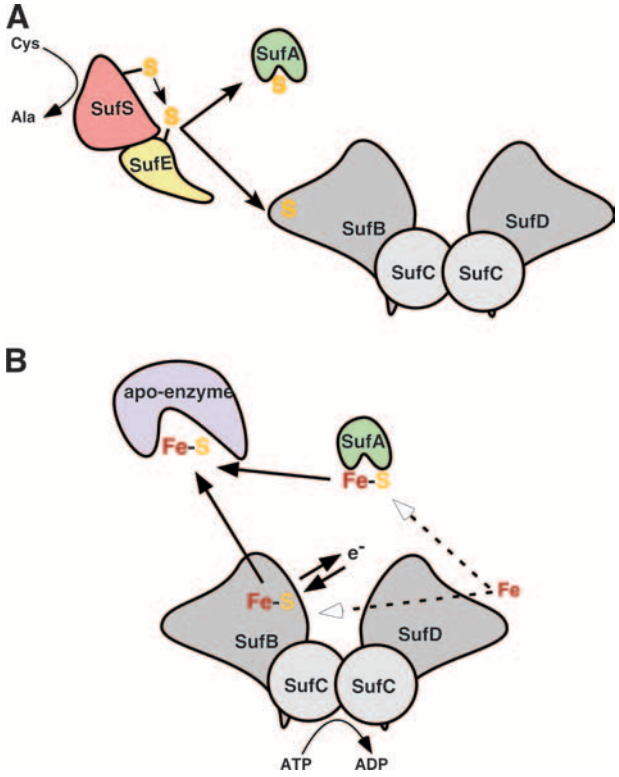

FIG. 3. Current model for Fe-S cluster assembly by the Suf system. (A) SufS and SufE mobilize sulfur for donation to SufB (as part of SufBCD) and SufA. (B) An Fe-S cluster can form on both SufA and SufB. The Fe-S cluster on SufA can be transferred to an apoprotein. The SufB Fe-S cluster could be transferred to an apoprotein or could participate in redox processes during cluster assembly (e^-). Dotted arrows indicate possible donation of iron by SufD or by direct binding to SufA. The exact role of SufC ATPase activity is unknown.

E. coli SufA was shown to transfer Fe-S clusters to the [2Fe-2S] protein ferredoxin as well as the [4Fe-4S] enzyme biotin synthase (BioB) (88, 90, 91). Fe-S cluster transfer from SufA to BioB was more efficient than simple reconstitution with free iron and sulfide. The cluster transfer process was also resistant to disruption by iron chelators. SufA from cyanobacteria has also been characterized at the biochemical level. SufA, encoded by slr1417 in *Synechocystis* sp. strain PCC 6803, can be reconstituted with a [2Fe-2S] cluster and can transfer that cluster to both [2Fe-2S] and [4Fe-4S] proteins (128). These extensive studies suggest that SufA is an Fe-S assembly scaffold. Since the *suf* operon is not always found with a SufU-type scaffold protein, it is logical to assume that SufA may function as the main scaffold for the Suf pathway in some organisms, such as *E. coli* (Fig. 3). However, genetic analysis of *suf* mutant phenotypes of *E. coli* does not support this model, as deletion of *sufA* results in a much less severe phenotype than does deletion of the other *suf* genes (92, 115). Possibly the *suf* operon encodes an atypical Fe-S scaffold that functionally replaces the U-type scaffold, relegating SufA to an alternate scaffold. Alternatively, IscA may compensate for the loss of SufA in a *sufA* deletion strain. This hypothesis has not been tested directly.

The crystal structure of SufA has been determined at 2.7-Å resolution (126). SufA crystallized as an asymmetric homodimer, and the three invariant Cys residues were resolved in only one of the SufA monomers. A computer model of the SufA dimer interface shows two of the invariant Cys residues (Cys114 and Cys116) from each monomer positioned at the dimer interface in an orientation that could allow coordination of iron or an Fe-S cluster between monomers. Interestingly, the Glu118 residue from each SufA monomer is also predicted to be near Cys114 and Cys116 in the dimer interface, possibly to provide carboxylate ligands for direct iron binding. The crystal structure of SufA has some similarities with that of IscA, except that SufA is dimeric while IscA is tetrameric. Unfortunately, the SufA crystal structure is consistent with a role for SufA as either an Fe-S scaffold or an iron chaperone and does not convincingly refute either hypothesis.

Recently, a third A-type protein was identified in *E. coli* (68). The gene *yadR* encodes a protein that is 40% identical to IscA and 34% identical to SufA and contains the three Cys residues that are critical for the in vivo function of A-type scaffolds. In contrast to deletion of *iscA* and *sufA*, deletion of *yadR* is lethal under aerobic conditions or if anaerobic respiration is required. However, the *yadR* deletion strain grows normally under fermentative conditions. Loiseau et al. (68) demonstrated that the deletion of *yadR* reduces quinone production, presumably from the loss of the [4Fe-4S] enzymes IspG and IspH. IspG and IspH are required for synthesis of the quinone precursor isopentenyl diphosphate. In support of this hypothesis, addition of the eukaryotic mevalonate-dependent pathway for isopentenyl diphosphate synthesis, which does not require Fe-S enzymes, was able to partially rescue the *yadR* growth defects. Based on these phenotypes, it was proposed that *yadR* be renamed *erpA*, for *e*ssential *r*espiratory *p*rotein *A*. Purified apo-ErpA could be reconstituted in vitro and contained a mixture of 40 to 50% [2Fe-2S] and 15 to 25% [4Fe-4S] clusters, as determined by Mössbauer spectroscopy. Furthermore, holo-ErpA was able to transfer clusters to IspG, resulting in formation of a [4Fe-4S] cluster on IspG.

The differential phenotypes that result from stepwise deletion of the A-type scaffold genes in a single organism, such as *E. coli* or *Synechocystis*, suggest that each may be adapted for cluster assembly by a specific biosynthesis pathway, for a subset of target enzymes, or under certain growth conditions. Clearly, the A-type scaffolds play an important role in Fe-S cluster biosynthesis, but their exact function is still uncertain. Further characterization of the A-type proteins in vivo is required to answer this question.

MOLECLUAR CHAPERONES FOR FE-S CLUSTER ASSEMBLY

The Isc pathway contains HscA and HscB proteins that are homologues of the DnaJ and DnaK molecular chaperones. Deletion of *hscA* or *hscB* perturbs Fe-S cluster assembly in vivo (114, 121). Careful biochemical experiments clearly show that HscA interacts with apo-IscU and cluster-loaded forms of IscU (22, 40–42, 109). This interaction is enhanced by HscB, which can bind to both IscU and HscA, leading to a strong enhancement of the intrinsic HscA ATPase activity. HscA binds to a conserved stretch of amino acids (LPPVK) in IscU (22, 40, 109, 116, 117). This motif is missing in SufU-type and NifU-type

scaffolds, which are not usually encoded with HscA or HscB, suggesting that SufU and NifU either do not require molecular chaperones or utilize a completely separate chaperone system (46).

Recent work has shown that *A. vinelandii* HscA and HscB can enhance transfer of [2Fe-2S] clusters from IscU to apoproteins in an ATP-dependent manner (20). Deletion of *hscA* or *hscB* does diminish the activity of succinate dehydrogenase (SDH or complex II) in *E. coli* (121). However, SDH contains [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters, and perturbation of any of these clusters would presumably decrease SDH activity in vivo. The exact mechanism by which HscA and HscB enhance transfer is unclear but may involve partial IscU unfolding to destabilize or expose the cluster in order for apoprotein ligands to access the cluster. The LPPVK motif is located near a highly conserved Cys (Cys106) residue in IscU, so IscU binding to HscAB and subsequent ATP hydrolysis might alter the interaction of this cysteine with clusters on IscU. The exact reaction cycle for this process, incorporating binding, ATP hydrolysis, and cluster transfer, is currently under intense scrutiny (123).

THE SufBCD COMPLEX

The SufB and SufD proteins are homologous to each other throughout their C-terminal regions, while their sequences diverge in the N-terminal halves of the proteins. The SufB amino acid sequence includes 13 Cys residues, with 4 highly conserved Cys residues. The N terminus of SufB contains a putative Fe-S binding motif, C-X_2-C-X_3-C, although this motif is not strictly conserved in more divergent SufB homologues. Recent studies have shown that SufB can coordinate $[4Fe\text{-}4S]^{+/2+}$ clusters in vitro and that SufB accepts sulfane sulfur from SufE (Fig. 3) (60). This result has raised the possibility that SufB is a novel Fe-S scaffold for the Suf pathway. However, the [4Fe-4S] cluster in SufB is not reductively labile, as typically observed with the A-type and U-type scaffold proteins, indicating that SufB could be an unusual type of scaffold or that it may instead perform a redox function during Fe-S cluster assembly. The *suf* operon does not encode a direct homologue of Fdx, and the [4Fe-4S] form of SufB may function in a similar capacity (Fig. 3).

SufD contains only three Cys, and only one is highly conserved, arguing against Fe-S cluster formation on SufD. However, it is possible that SufD could donate an Fe-S ligand (such as Cys or His) when bound in a complex with SufB and SufC. SufD does contain 23 His residues (compared to 12 His residues in the SufB sequence). Eight of the His residues in SufD are arranged in two repeating patterns of H-$X_{11\text{ or }13}$-H-X_2-H-X_{13}-H. Since the imidazole moiety of His residues is an excellent ligand for ferrous iron coordination, these motifs may be used for iron binding. The crystal structure (1.75 Å) of SufD shows that SufD consists of a flattened, right-handed beta helix of nine turns, with two strands per turn, and helical subdomains at the carboxyl and amino termini (6). Some highly conserved His and Cys residues are located at the dimer interface between two SufD monomers, but their importance is not clear in the absence of a specific biochemical function for SufD.

Both SufB and SufD interact directly with the SufC protein, forming the SufBCD complex (85, 93, 99). These interactions presumably occur between the C-terminal regions of SufB and SufD and the SufC protein. SufC is a canonical ABC transporter ATPase subunit with conserved Walker A and Walker B motifs as well as the Q loop and D loop motifs. In many cases, these ATPase subunits are bound in a multiprotein complex to form a transmembrane transporter. However, in the Suf pathway, SufC and the SufBCD complex are soluble and appear to be localized largely to the cytoplasm (85, 93). While SufB, SufC, and SufD may associate with the inner membrane of *E. coli* at low levels, these studies were carried out with tagged or nonnative Suf proteins expressed from a multicopy plasmid, and thus the significance for in vivo Suf function is not clear.

The crystal structure of SufC reveals that it is an atypical member of the ABC-ATPase superfamily due to the unusual structural position of Glu171, a highly conserved residue that functions as a catalytic base in the conversion of ATP to ADP and P_i. In the SufC structure, Glu171 is flipped out of its usual orientation, resulting in low basal ATPase activity for SufC (53). However, the addition of SufB stimulates the rate of ATP hydrolysis by SufC up to 100-fold, possibly by providing a catalytic residue or by causing reorientation of SufC Glu171 (32). The role of the SufC ATPase activity is not known. Since ATP is not required for the SufBCD complex to enhance SufS-SufE sulfur transfer, SufC ATPase activity must be involved in some other step of cluster assembly, such as iron acquisition or cluster transfer to apoprotein (32, 93). Interestingly, in order for SufB to interact with SufE for sulfur transfer, SufB must be in a complex with SufC (60). Therefore, SufB stimulation of SufC ATPase activity logically occurs simultaneously with SufE sulfur transfer to SufB, providing coordinated regulation of these two processes during Fe-S cluster assembly.

The requirement for the accessory proteins SufE and SufB to stimulate low SufS and SufC basal activity is supported by the structures of SufS and SufC. Structural and biochemical analyses suggest that the entire Suf pathway is carefully regulated by protein-protein interactions. Unfortunately, there have been no published structures of the SufS-SufE or SufBCD protein complexes that represent the truly functional units of the Suf system.

POSSIBLE IRON DONORS AND OTHER ACCESSORY PROTEINS

As mentioned in the introduction, the in vivo iron donation process has not been elucidated fully. Current models tend to assume that a metallochaperone acquires iron and directly donates the iron to the assembly pathway by interacting with one of the scaffold proteins. Such a hypothetical pathway would protect iron from chelation by other cellular components and limit the reaction of iron with oxygen and reactive oxygen species (ROS). Metallochaperones have been identified for other essential transition metals, such as copper. For example, in eukaryotes the Atx1 copper chaperone is needed to traffic copper to the Ccc2 copper transporter for incorporation into the multicopper oxidase Fet3 (65, 97). The Atx1 metallochaperone is matched to its cognate acceptor, and protein-protein interactions mediate the transfer of copper (44).

Presumably, the iron metallochaperone would have similar characteristics.

To date, frataxin is the main candidate as the iron metallochaperone for Fe-S cluster assembly (13). While frataxin has clearly been implicated in Fe-S cluster assembly and homoeostasis in eukaryotes, the connection is less clear for bacteria. CyaY is the bacterial homologue of the eukaryotic protein frataxin. Gene deletion of *cyaY* does not lead to Fe-S deficiencies comparable to those observed when frataxin is deleted in higher organisms (62, 124). The only reported phenotype of a *cyaY* deletion is a reduction in the levels of Fe-S cluster-containing respiratory complexes, i.e., NADH:ubiquinone oxidoreductase and succinate dehydrogenase (complexes I and II), in *E. coli* (96). Similar reduction in NADH:ubiquinone oxidoreductase activity was reported for a *cyaY* deletion in *Salmonella enterica* as well (124). Importantly, the phenotype of a *cyaY* deletion is aggravated when combined with the *yggX* gene deletion in *S. enterica*, and nearly all of these defects are linked to iron and Fe-S cluster metabolism (124). Thus, the lack of a strong phenotype for the *cyaY* deletion in bacteria may reflect multiple redundant systems (such as YggX) that are not present in eukaryotes rather than the lack of a role in Fe-S cluster assembly. In support of this interpretation, the bacterial CyaY protein is capable of partially rescuing a frataxin (Yfh1) deletion in yeast if it is expressed with a mitochondrial targeting sequence (10).

In vitro, CyaY can bind iron and forms various oligomeric mixtures in the iron-bound state (13, 17). Iron-to-protein ratios of 2.5 Fe^{2+} ions/polypeptide and 8 Fe^{3+} ions/polypeptide were recently reported and are consistent with other published studies for CyaY iron binding (60). Ferric iron binding seems to promote the formation of large oligomers of CyaY, while ferrous iron binding produces mostly monomeric CyaY (60). Ferrous iron binding by CyaY was shown to be relatively sensitive to dilution or chelation, and the ferrous iron association constant for CyaY was measured at $2.6 \times 10^5\ M^{-1}$. In contrast, CyaY bound to ferric iron with an iron association constant greater than that of ferric citrate ($1.0 \times 10^{17}\ M^{-1}$).

Recently, it was shown that the apoform of *E. coli* CyaY interacts strongly with IscS and can form a heterotrimeric complex with IscS and IscU (60). CyaY prepared with ferric iron can donate iron for Fe-S cluster assembly in IscU in the presence of IscS and cysteine, although it was not determined if ferric iron CyaY can still bind IscS or IscU. The same study reported that cysteine is a potent reducing agent for ferric CyaY, and it was suggested that reduction of ferric iron in CyaY by cysteine could provide a mechanism to release ferrous iron for Fe-S cluster assembly. Presumably, the CyaY interaction with IscS could facilitate this process, as cysteine is a substrate for IscS cysteine desulfurase activity. This model is consistent with other work that shows that iron binding by CyaY is facilitated by hydrogen peroxide, possibly by oxidizing the ferrous iron used for those experiments to ferric iron, which would bind CyaY more tightly (29). The iron binding properties of CyaY and its ability to interact with IscS suggest that CyaY may be an iron donor for Fe-S cluster assembly in vivo.

It was suggested that frataxin may play a role in modulating the activity of aconitase by donating iron to convert a damaged $[3Fe\text{-}4S]^+$ cluster back to the active $[4Fe\text{-}4S]^{2+}$ form (18). It remains to be seen if CyaY might function to donate Fe during cluster repair, as opposed to de novo synthesis, or if it might be able to carry out both functions in bacteria. CyaY could play additional roles as a general ferric iron buffering system that complements the ferritin storage protein and maintains a more available pool of iron for Fe-S and heme biosynthesis. However, the in vivo regulation of *cyaY* and the in vivo abundance of CyaY need to be elaborated further before these possible functions can be assigned confidently. It is also unclear how CyaY could interact with multiple proteins, including U-type and A-type scaffolds, for iron delivery when other characterized metallochaperones, such as Atx1, are highly specific for their partner proteins (45).

A second iron binding protein was recently shown to play a role in Fe-S cluster repair after stress in vivo (48, 49). YtfE was found to be induced under nitrosative stress and iron starvation, and deletion of *ytfE* resulted in defects in Fe-S enzyme activity in *E. coli* (50). Purified YtfE contains a di-iron center ligated by carboxylates and His residues. Careful in vivo studies showed that Fe-S cluster repair of the [4Fe-4S] enzymes fumarase and aconitase was diminished in a *ytfE* deletion strain after hydrogen peroxide or nitrosative stress (49). The Fe-S cluster repair defect could be overcome by the addition of holo-YtfE to cell lysates, but the addition of iron or apo-YtfE had no effect. These results suggest that YtfE may directly donate Fe to damaged [4Fe-4S] clusters as part of the repair process during stress. Interestingly, *ytfE* mutants also show pronounced defects during anaerobic growth, although it is unclear why there would be a high demand for Fe-S cluster repair in the absence of oxygen (50).

Other genes that play a more tangential role in Fe-S cluster metabolism have been characterized for *S. enterica*. Both *apbC* and *apbE* were identified in a genetic screen for genes involved in thiamine biosynthesis (9, 95). Interestingly, these mutants showed phenotypes similar to that observed for *isc* mutants identified in the same screen (111). Both *apbC* and *apbE* deletion strains had reduced activities of the Fe-S enzymes aconitase and succinate dehydrogenase (110). The same work demonstrated that an *apbC isc* double mutant had a more severe phenotype than did either of the two parent strains, suggesting that ApbC may work independently of *isc* to perform a redundant or separate function in Fe-S cluster metabolism. In contrast to the case for *isc* mutants, the addition of exogenous $FeCl_3$ was able to rescue the growth defects and Fe-S enzyme defects of both the *apbC* and *apbE* mutants. These results led to the hypothesis that ApbC and ApbE are involved in iron donation for in vivo Fe-S cluster assembly or Fe-S cluster repair (110). Sequence analysis of ApbC reveals that it belongs to the Mrp/Nbp35 ATP-binding protein family. Provocatively, Nbp35 is a P-loop NTPase that contains a [4Fe-4S] cluster and has been shown to be important for Fe-S cluster assembly in the cytosol and nucleus of eukaryotes (39). ApbC was shown to have ATPase activity in vitro (110). ApbE is a periplasmic lipoprotein, but its role in Fe-S cluster metabolism is unclear.

Several other genes in *Synechocystis* have been implicated in Fe-S cluster biogenesis. The *rubA* gene encodes a rubredoxin protein with a single transmembrane domain. Deletion of *rubA* results in the specific loss of the F_x Fe-S cluster of photosystem I (PSI) in cyanobacteria. The exact role of RubA in F_x cluster

assembly is not clear, but it has been proposed to act as an electron shunt to prevent overreduction of iron or the nascent Fe-S cluster during cluster assembly or transfer to the F_x cluster site (106, 108). In other cyanobacteria, such as *Gloeobacter* and *Rhodopseudomonas*, the *suf* genes are located next to a small open reading frame (ORF), glr1375, that encodes a homologue of Mrp and Nbp35. However, Glr1375, while sharing some sequence features with Nbp35, lacks the N-terminal Fe-S cluster binding motif present in Nbp35. Instead, Glr1375 is more similar to other members of the Mrp/Nbp35 family, such as PaaD, a component of a multiprotein complex involved in ring hydroxylation of phenylacetate-coenzyme A in *E. coli* and other bacteria (7). The importance of glr1375 is not known, as Fe-S cluster assembly has not been studied in the more divergent cyanobacteria.

REGULATION OF FE-S CLUSTER ASSEMBLY

IscR

Since Fe-S clusters are required under a wide variety of growth conditions, the regulation of Fe-S cluster assembly genes is quite complex. The regulatory pathways that control Fe-S cluster biosynthesis in bacteria have been studied most well for *E. coli*, although the regulatory features are widely conserved in other bacteria. In *E. coli*, the *isc* gene locus is under the transcriptional control of the IscR repressor, which is encoded as part of the *iscRSUA* locus and autoregulates its own expression as well as that of *iscSUA* (37, 104). IscR is a member of the Rrf2 family (PF02082) of transcriptional regulators and possesses a winged helix-turn-helix DNA binding domain. IscR contains three Cys residues and was shown to be an Fe-S protein that purifies with a $[2Fe\text{-}2S]^{+}$ cluster, suggesting that those Cys residues are used for Fe-S cluster ligation (104). The [2Fe-2S] cluster in IscR can interconvert from $[2Fe\text{-}2S]^{+}$ to $[2Fe\text{-}2S]^{2+}$ in a stable manner in response to oxidizing or reducing conditions (104). Fe-S clusters are used by numerous transcription factors, such as SoxR and FNR, to sense cellular redox status (reviewed in reference 52). The redox conversion of the IscR Fe-S cluster may be similar to that of the SoxR superoxide-sensing transcription factor, which is regulated by the oxidation/reduction of its stable [2Fe-2S] cluster. However, the effect of a similar redox conversion on IscR DNA binding activity is unknown.

Holo-IscR was able to repress transcription of the *iscRSUA* promoter in vitro, and an *iscR* deletion strain showed elevated, constitutive expression of the *isc* genes in vivo. Repression of the *isc* locus by IscR is disrupted when other components of the Fe-S cluster machinery, such as IscS or HscA, are deleted, leading to elevated transcription from the *isc* promoter (104). These results led to a model where [2Fe-2S] IscR is the active repressor of the *isc* locus while apo-IscR cannot repress *isc*. In this model, the Fe-S cluster in IscR is a sensor for the Fe-S cluster status of the cell (Fig. 4). When Fe-S cluster assembly is adequate, holo-IscR represses the *isc* locus, but as the demand for Fe-S clusters increases, apo-IscR predominates and *isc* transcription increases to meet the demand. IscR cluster status also may regulate different aspects of IscR activity. It was recently shown that apo-IscR actually activates *suf* transcription in response to oxidative stress, and an IscR-specific binding site was mapped in the *suf* promoter (133). Thus, a loss of the IscR Fe-S cluster may lead to increased levels of both *isc* and *suf* transcription under some conditions.

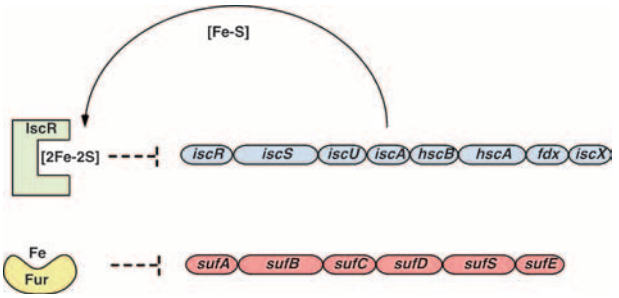

FIG. 4. Regulation of Fe-S cluster assembly pathways in *E. coli* under normal growth conditions. Holo-IscR and apo-IscR will be present in an equilibrium that is dependent on the amount of Isc proteins available for cluster synthesis. Holo-IscR will repress *isc* transcription when there is sufficient cluster assembly capacity (i.e., when the Isc proteins are not titrated away for cluster assembly in other proteins). Under normal growth conditions, *suf* transcription will be low due to repression by Fe^{2+}-Fur.

A recent DNA microarray analysis of IscR-regulated genes in *E. coli* also identified a host of new genes that together form an IscR regulon (37). Among the genes regulated by IscR are *erpA* and *yhgI*, which both encode a homologue of IscA. The role of *yhgI* in Fe-S cluster assembly has not been established. The *yfhJ-pepB-sseB* locus, just downstream of the *iscRSU-iscA-hscBA-fdx* region, was also shown to be repressed by IscR, raising the possibility that the gene products might be involved in Fe-S biogenesis. Previous studies have shown that YfhJ (IscX) interacts with IscS and that PepB interacts with HscA, providing additional support to this hypothesis (19, 120). Furthermore, a number of genes that encode Fe-S proteins were repressed by IscR, including the *hyaABCDEF* and *hybO-hybABC DEFG* operons, encoding hydrogenases 1 and 2, respectively, as well as *napFDAGHBC*, encoding periplasmic nitrate reductase. These studies also identified two distinct target DNA sequences for IscR binding. The sum of the microarray experiments points to IscR as a broad regulator of Fe-S cluster assembly and Fe-S proteins in *E. coli*.

Oxygen and Oxidative Stress

Recent studies have shown that *isc* and *suf* are repressed under anaerobic conditions but highly induced in response to ROS, such as H_2O_2. Clearly, the redox status of the cell plays an important role in Fe-S biosynthesis and metabolism. In *E. coli*, exposure to hydrogen peroxide (H_2O_2) leads to a strong increase in *suf* transcription (61, 139). Induction of the *suf* pathway by H_2O_2 is mediated through the OxyR oxidative stress response transcription factor, although recent work has shown that IscR also plays a role in *suf* induction during oxidative stress (Fig. 5) (133, 139). H_2O_2 is known to directly oxidize exposed [4Fe-4S] clusters in dehydratase enzymes, with an in vitro rate constant of 10^2 to 10^3 M^{-1} s^{-1} (33). H_2O_2 also can oxidize protein cysteinyl residues to form sulfenic and sulfinic acid. Persulfide sulfur is sensitive to H_2O_2 as well.

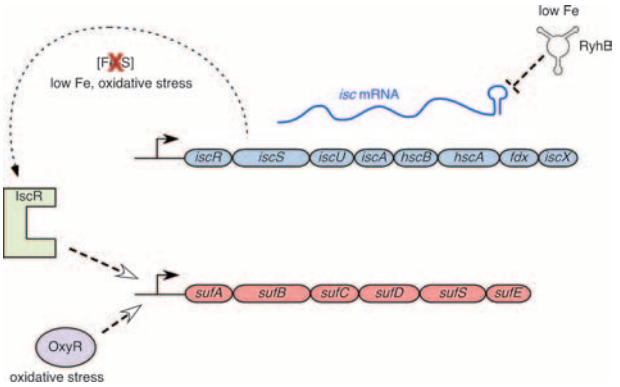

FIG. 5. Regulation of Fe-S cluster assembly pathways in *E. coli* under stress conditions. During oxidative stress or iron starvation, apo-IscR will predominate as the Isc proteins are titrated away by increased demand for cluster assembly. This will relieve *isc* repression and induce the operon. Simultaneously, apo-IscR will activate *suf* transcription as Fur repression is relieved. Under hydrogen peroxide stress, OxyR will also activate *suf* transcription in an integration host factor-dependent manner. Under iron starvation conditions, induction of the RyhB small RNA will lead to posttranscriptional repression of the Isc system so that Suf becomes the predominant Fe-S cluster pathway.

Oxidation by H_2O_2 would perturb sulfur transfer during Fe-S cluster assembly, since those steps consist of donation of persulfide sulfur by protein cysteinyls. Given the consequences of H_2O_2 exposure, it seems likely that *suf* is activated to help meet an increased demand for de novo Fe-S assembly. In addition, *suf* regulation suggests that the Suf pathway is resistant to H_2O_2, an agent that directly attacks both Fe and S atoms at multiple steps throughout cluster assembly.

The fact that *suf* transcription is activated in addition to *isc* upregulation also suggests that the Isc pathway may not function efficiently under oxidative stress compared to the Suf pathway. This hypothesis was supported by careful plasmid complementation studies of an *iscSUA-hscBA sufABCDSE* double mutant strain, which is unviable unless a functional Fe-S cluster assembly pathway is provided in *trans*. When the *isc* operon was provided in *trans* to this Fe-S assembly-deficient strain, it showed severe growth defects during H_2O_2 exposure, whereas when the same strain was provided with the *suf* operon in *trans*, it grew as well as wild-type *E. coli* during H_2O_2 exposure (119). Therefore, while the *isc* pathway is obviously important as the housekeeping Fe-S cluster assembly system of *E. coli*, the *suf* pathway plays a critical role in maintaining Fe-S cluster biosynthesis under oxidative stress conditions.

DNA microarray analysis of the IscR regulon mentioned above also revealed differential regulation of Fe-S proteins and Fe-S cluster assembly in response to oxygen (37). Expression of both the *isc* and *suf* operons was reduced under anaerobic growth conditions, despite the large number of Fe-S enzymes used for anaerobic respiration. This suggests that damage to and subsequent turnover of Fe-S clusters during oxygenic growth is responsible for the bulk of the demand for de novo Fe-S cluster biosynthesis. Switching to anaerobic growth conditions decreases ROS production, Fe-S cluster damage, and consequently, Fe-S cluster demand.

Iron

Iron limitation is a common environmental stress for most organisms due to the need for iron in heme and Fe-S clusters and the low solubility of iron in aqueous environments. Iron status may indirectly influence *isc* regulation through a loss of the [2Fe-2S] cluster in IscR. However, both *isc* and *suf* are directly regulated in response to cellular iron status via the Fur iron metalloregulatory protein and its associated regulatory small RNA RyhB (Fig. 4 and 5) (75, 92, 94). Fur binds ferrous iron and represses genes involved in iron acquisition under iron-replete conditions. As available iron levels drop, Fur repression is lost and transcription increases for genes involved in adaptation to iron starvation. Fur directly binds the *suf* promoter over the −10 and −35 RNA polymerase binding sites and represses *suf* transcription under iron-replete conditions (92). As iron levels drop, Fur repression is lost and *suf* transcription increases. Presumably, *suf* transcription is also further activated by apo-IscR under iron-limited conditions, but this has not been tested directly.

Fe^{2+}-Fur also represses the small RNA RyhB (74). RyhB promotes degradation of target mRNAs that encode iron-containing proteins (73). RyhB expression under iron starvation conditions leads to down-regulation of iron-containing proteins in order to spare iron for essential processes. By repressing RyhB under iron-replete conditions, Fe^{2+}-Fur causes de facto increases of RhyB target mRNAs. Recent DNA microarray studies analyzed the role of RyhB in remodeling global iron use in *E. coli* (75). Transcripts from the *iscRSUA-hscBA-fdx-yfhJ-pepB-sseB* locus were all among the target mRNAs identified through microarray studies when RyhB was overexpressed. The *suf* genes were not found to be direct targets of RyhB overexpression, although their transcription was influenced indirectly by RhyB levels due to the change in the pool of available cellular iron sensed by Fur. It appears that *isc*, but not *suf*, is directly down-regulated if RyhB is overexpressed (Fig. 5). One caveat to these microarray studies is that under more physiological conditions (when RyhB is not overexpressed), *isc* transcription is actually seen to increase as iron levels decrease, suggesting that a loss of IscR repression may override more subtle regulation by RyhB (92).

The *sufR* gene is adjacent to and divergently transcribed from the *sufBCDS* locus in *Synechocystis* and is found in a similar orientation in many other cyanobacterial species. The *sufR* gene, also known as sll0088 in *Synechocystis* sp. strain PCC 6803, encodes a transcription factor similar to the DeoR family members, with an N-terminal helix-loop-helix DNA binding domain and a C-terminal $C\text{-}X_{12}\text{-}C\text{-}X_{13}\text{-}C\text{-}X_{14}\text{-}C$ metal binding motif. Purified SufR can be reconstituted with a [4Fe-4S] cluster in vitro, although it is unclear if SufR contains an Fe-S cluster in vivo (127). Deletion of *sufR* results in constitutive expression of the *sufBCDS* operon in *Synechocystis*, indicating that SufR functions as a transcriptional repressor of *suf*. A *sufR* mutant strain was able to restore photoauxotrophic growth and to cause an increase in PSI reaction centers in a PsaC C14S mutant (127). This suppression likely occurred because the *sufBCDS* genes were constitutively active in the *sufR* mutant and increased levels of the Suf system led to increased Fe-S assembly in PSI. The *sufBCDS* genes are essential in *Synecho-*

cystis for other Fe-S clusters as well, since *suf* is required for heterotrophic growth in the dark.

It was proposed that SufR contains an Fe-S cluster in vivo and that this cluster is the sensor that controls SufR DNA binding (127). In the proposed model, holo-SufR would bind DNA and repress transcription of the *sufBCDS* genes. As cellular Fe-S requirements increased, apo-SufR would predominate, leading to a loss of DNA binding and activation of *sufBCDS* transcription. Recently, this proposed model was confirmed (107). A SufR homodimer can be reconstituted with two $[4Fe\text{-}4S]^{+/2+}$ clusters. Holo-SufR could bind to specific inverted repeat sequences upstream of *sufBCDS* with a higher affinity than that of apo-SufR. Reduction of the [4Fe-4S] cluster in holo-SufR decreased the DNA binding affinity to the same level as that of apo-SufR, suggesting that modulation of the redox state of the cluster may be sufficient to regulate SufR activity. SufR was also shown to autoregulate its own expression by binding to a lower-affinity site upstream of the *sufR* gene.

Interestingly, it was also shown that an *iscA* deletion mutant causes constitutive expression of the *sufBCDS* genes in cyanobacteria, presumably due to a loss of the Fe-S cluster in SufR (7). Surprisingly, deletion of both *sufA* and *iscA* restores *sufBCDS* transcription to normal levels. In contrast, a *sufA* single deletion has no effect on basal *suf* transcription but does lead to elevated transcription of *iscS1* and *iscS2* (7). These results suggest that IscA and SufA may play mutually antagonistic roles in regulating the *suf* and *isc* genes. This integrated regulation may be explained by the presence of an IscR homologue in cyanobacteria. The complicated transcriptional changes seen in *iscA* and *sufA* gene deletion strains could be explained if IscA maintains Fe-S clusters in SufR while SufA maintains Fe-S clusters in IscR. Thus, the loss of IscA would lead to a loss of holo-SufR and upregulation of *sufBCD-sufS*. Loss of SufA would lead to a loss of holo-IscR, causing upregulation of genes that IscR represses, possibly such as *iscS1* and *iscS2*. The restoration of normal *suf* transcription in an *iscA sufA* double mutant is more difficult to explain, but in this model, it might suggest that IscR directly coregulates the *suf* genes, as seen in *E. coli*. This intricate model awaits testing in vivo and would require extensive characterization of the target promoters for both SufR and IscR transcriptional regulation and experimental confirmation that IscR can actually coordinate an Fe-S cluster.

FUNCTIONAL CROSS TALK BETWEEN PATHWAYS

Organisms ranging from bacteria to higher eukaryotes have evolved with multiple Fe-S cluster assembly pathways, namely, the Isc, Suf, and Nif pathways. For example, *E. coli* contains both Isc and Suf pathways, while *A. vinelandii* contains Isc and Nif, but they are utilized under different growth conditions via differential regulation (92). In other bacteria, only a single system is present. For example, in *Mycobacterium tuberculosis*, the Suf system appears to be the only Fe-S cluster assembly pathway (43).

While the Suf and Isc pathways can both accomplish Fe-S cluster assembly in *E. coli*, the two systems are divergent. The Suf pathway contains four gene products, SufB, SufC, SufD, and SufE, that have no direct sequence homologues in the Isc system. Similarly, the Isc pathway utilizes four gene products, IscU, HscA, HscB, and Fdx, that are not present in the Suf system. Both pathways contain a cysteine desulfurase enzyme (IscS and SufS), and both pathways contain a protein that may be an iron donor or Fe-S scaffold (IscA and SufA). If the *sufABCDSE* operon is deleted from the *E. coli* genome, the resulting mutant strain is sensitive to oxidative stress and iron starvation, despite the presence of the *iscRSUA-hscBA-fdx* system (92, 115). The growth defects of the *suf* deletion strain under oxidative stress or iron starvation result from reduced activities of dehydratase enzymes that contain [4Fe-4S] clusters (92, 115). The reduced activities of these [4Fe-4S] enzymes appear to result from a block in de novo synthesis rather than a lack of Fe-S cluster repair (30). In addition, simultaneous deletion of both the *suf* and *isc* operons is lethal in *E. coli*, indicating that there is some functional redundancy between the two pathways (92, 115, 119). These results show that Suf and Isc can both assemble Fe-S clusters in *E. coli* but that the Suf pathway is better adapted to do so under oxidative stress and iron starvation conditions.

Gene deletion and plasmid complementation experiments show that *sufB*, *sufC*, *sufD*, *sufS*, and *sufE* are each absolutely essential for Fe-S cluster assembly under stress in vivo. Deletion of any of them results in the same growth defects as deletion of the entire *suf* operon, and each single-gene deletion is lethal if combined with *iscS* or *iscRSUA* deletions (92, 115, 119). In contrast, *sufA* seems to be somewhat dispensable for Suf function, since a *sufA* mutant displays less severe intermediate growth defects under oxidative stress and iron starvation. A *sufA* deletion is also not synthetically lethal with *iscS* or *iscRSUA* gene deletions (92, 115, 119).

For *A. vinelandii*, careful genetic experiments have shown that overexpression of *nifU* by use of a heterologous promoter rescues an *iscU* deletion strain (47). Similarly, the lethal phenotype of an *iscU* deletion strain was rescued under nitrogen fixation conditions when *nif* was normally expressed, but only if oxygen levels were low so as to reduce the overall Fe-S cluster demand. Furthermore, elevated expression of *iscU* in a *nifU* deletion strain partially restored diazotrophic growth. Together, these results indicate that IscU and NifU can functionally substitute for each other under certain conditions, most notably if expression levels of the remaining scaffold are elevated. Interestingly, the C-terminal domain of NifU was not required for NifU to functionally substitute for IscU, indicating that the N-terminal "IscU-like" domain of NifU likely functions as a scaffold domain within NifU. Neither the molecular chaperones HscA and HscB nor IscFdx was required for NifU replacement of IscU, suggesting that NifU can function without these accessory proteins. In the case of IscFdx, its possible role in reductive coupling assembly of [4Fe-4S] clusters might be carried out by the central, stable [2Fe-2S] cluster in NifU.

In contrast to the U-type scaffolds, the cysteine desulfurases were not as functionally redundant (47). Elevated levels of IscS could partially replace NifS in vivo, but NifS was not able to rescue the lethality of an *iscS* deletion strain. Although other IscS-dependent sulfur trafficking pathways might be affected, the failure of NifS to rescue an *iscS* deletion strain was clearly linked to disrupted Fe-S cluster assembly.

Analysis of Fe-S cluster assembly in cyanobacteria has been most well studied with *Synechocystis* species. The relevant im-

portance of the Suf and Isc pathways appears to be reversed in *Synechocystis* compared to that in *E. coli*. The *sufB*, *sufC*, *sufD*, *sufS*, and *sufE* genes are all essential in *Synechocystis* sp. strain PCC 6803, while the *isc* genes are not required for viability (7, 11, 86, 118, 127). The increased importance of the Suf pathway in *Synechocystis* likely stems from innate differences between photosynthetic and nonphotosynthetic organisms. Cyanobacteria have a high iron quota, since both PSI and PSII require iron in the form of heme or Fe-S clusters. Thus, cyanobacteria, especially in iron-limited marine environments, are chronically iron starved. In addition, PSI and PSII also generate significant levels of ROS. For example, superoxide forms when oxygen reacts with the acceptor side of PSI, while H_2O_2 is generated from incomplete water oxidation on the donor side of PSII (reviewed in reference 76). Production of ROS from PSI and PSII is in addition to ROS production from aerobic respiration, resulting in a high level of constitutive oxidative stress in cyanobacteria relative to that in other nonphotosynthetic microbes. Since the Suf pathway functions better than the Isc pathway under iron starvation and oxidative stress conditions, it appears that cyanobacteria have evolved with Suf as their primary Fe-S cluster biogenesis system and relegated Isc to a more ancillary role.

OUTLOOK

In Vivo Iron Donation

Mechanistically, the most important unanswered question in Fe-S cluster biogenesis is the following: how is iron donated for cluster assembly in vivo? Several proteins can provide iron for in vitro Fe-S cluster assembly on IscU, including the frataxin homologue CyaY and IscA (27, 60, 134). However, to date, the identity of the in vivo iron donor remains murky. Unlike in eukaryotic organisms, deletion of the frataxin homologue encoded by *cyaY* does not cause severe phenotypes unless it is combined with *yggX* and *apbC* mutations (124). As mentioned above, deletion of *iscA* results in a mild phenotype compared to deletion of other *isc* genes. At best, these genetic phenotypes suggest a great deal of redundancy in the iron donation step for cluster assembly. At worst, they call into question any in vivo role for these proteins in direct iron donation.

The iron donation step is further complicated by the presence of the Suf pathway. Suf assembles Fe-S clusters under conditions where bioavailable iron is likely limiting. The ability of Suf to function under such conditions, when the Isc system is perturbed, raises the possibility that Suf may have its own pathway for iron acquisition. Possible candidates for iron donation to Suf under stress include iron storage proteins, such as ferritin or its stress-specific homologue, Dps. Alternatively, iron recycled from Fe-S and heme proteins might be channeled to the Suf pathway by SufD or an unidentified iron chaperone. Clearly, answering these questions in vivo represents a major challenge in the field of Fe-S cluster biogenesis.

Target Specificity

In vitro, most Fe-S scaffold proteins, such as IscA and IscU, can transfer clusters to a variety of Fe-S apoproteins, including some that are not native to the organism being studied. This raises the question of how specificity for target apoproteins is maintained in vivo. In the reducing environment of the cytoplasm, thiols likely predominate, providing an abundance of potential targets for Fe-S cluster ligation. The Isc and Suf proteins may interface with an as yet unidentified system that directs cluster insertion to specific enzymes. The recent discovery that glutaredoxins may catalyze Fe-S cluster transfer raises the possibility of an entirely new layer of cluster transfer proteins that might help to convey target specificity for cluster insertion (1, 5, 80, 84, 100, 101). Transcriptional or posttranscriptional regulation of Fe-S proteins by Fur, IscR, FNR, and RyhB must also play a role in this process by limiting the amount of available apoproteins under a given growth condition.

Role of Accessory Proteins

A number of accessory proteins have been implicated in Fe-S cluster biosynthesis or repair in vivo. In *E. coli* alone, there are multiple genes that are either regulated in response to Fe-S cluster status (*yadR*, *yhgI*, and *yfhJ-pepB-sseB*) or lead to Fe-S cluster defects if they are mutated or deleted (*yggX*, *cyaY*, *apbC*, and *ytfE*) (48). Establishing clear roles for accessory proteins in Fe-S cluster assembly and/or repair is an important issue to be addressed. Only by integrating the complexity of in vivo Fe-S cluster metabolism will a truly accurate picture of Fe-S cluster assembly emerge.

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