Chapter 7

Cysteine Desulfurase-Mediated Sulfur Donation Pathways in Plants and Phototrophic Bacteria

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Summary

Cysteine is the sulfur donor for a number of important cofactor biosynthetic pathways including the synthesis of iron–sulfur clusters, thiamine, biotin and molybdenum cofactor. NifS-like cysteine desulfurase enzymes are key components in these pathways, catalyzing the initial release of S from cysteine. NifS-like enzymes do not work alone but are the first component of a sulfur transfer pathway from cysteine to cofactor. *In vivo*, NifS-like cysteine desulfurases work in concert with assembly factor proteins to which they transfer the released S and which serve to regulate the cysteine desulfurase activity and orchestrate the delivery of S to downstream targets. In plants, the chloroplast localized iron–sulfur assembly machinery resembles at least in part a machinery that in bacteria is responsible for the synthesis of iron–sulfur clusters under oxidative stress and iron limitation. A similar system operates

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in photosynthetic bacteria. While we are just beginning to unravel the mechanisms of S-dependent cofactor assembly systems it is already evident that these pathways play pivotal roles in cellular metabolism, and particularly are important to the function of plant plastids.

I. Introduction

The amino acid cysteine plays an important role as a component of proteins and glutathione. In addition, cysteine is required for the synthesis of essential sulfur-containing cofactors. The sulfur in Iron–Sulfur [Fe–S] clusters, thiamine, molybdenum cofactor (Moco), lipoic acid and biotin is derived from cysteine, as is the S in some modified tRNA species. A key enzymatic activity in these reactions is provided by NifS-like cysteine desulfurase enzymes, which decompose cysteine to alanine and sulfide (see Fig. 1). The first of these pyridoxal 5′-phosphate (PLP) dependent enzymes identified was the NifS enzyme of *Azotobacter vinelandii* which is required for the Fe–S cofactor assembly in dinitrogenase. It is now evident that NifS-like enzymes are ubiquitous and play key roles in the synthesis of a variety of S containing compounds and cofactors. Because free sulfur is potentially toxic, cells need to couple the biosynthesis of S-containing cofactors to the activation of S from cysteine (see Hell and Wirtz, chapter 4). Therefore, NifS-like enzymes pair up with downstream-acting protein partners that may serve to activate the cysteine desulfurase and that form sulfur transfer pathways mediating the transfer of S from cysteine into the various target compounds. We are just beginning to see how these cysteine desulfurase dependent systems are regulated. This chapter aims to review our current knowledge of cysteine desulfurase mediated sulfur activation pathways and its role in cofactor assembly, with emphasis on photosynthetic organisms.

II. Iron–Sulfur Cluster Assembly

A. Overview of Iron–Sulfur Cluster Function

Iron occurs in a large variety of cofactors, but we can distinguish three main groups: iron sulfur, heme plus siroheme, and finally non-heme iron. Iron–sulfur ([Fe–S]) clusters are an ancient class of prosthetic groups, consisting of iron and sulfur atoms. The architecture of various types of [Fe–S] clusters is described by Beinert and coworkers (1997, 2000). As a protein cofactor, [Fe–S] clusters are usually bound to polypeptides by covalent bonding between iron atoms of the [Fe–S] cluster and sulfur of cysteine residues in the polypeptide. An exception is the Rieske-type [2Fe-2S] cluster, in which one iron atom of the [Fe–S] cluster is coordinated to two histidines. Since the iron in [Fe–S] clusters can easily gain or lose an electron, switching between Fe^{2+} and Fe^{3+}, [Fe–S] clusters are ideal cofactors for proteins that function in electron transport chains (photosynthesis and respiration) and catalyze redox reactions. Iron–sulfur clusters also act as catalytic centers, as sensors of iron and oxygen, and as regulators of gene expression (for a review see Beinert and Kiley 1999).

B. Iron–Sulfur Cluster Assembly Systems in Microbes

Iron–sulfur cluster assembly can be divided into three steps: (i) mobilization of S and Fe, (ii) cluster assembly, and (iii) insertion in apo-proteins. Many aspects of [Fe–S] cluster assembly are conserved from bacteria to eukaryotes. Much of the nomenclature for [Fe–S] assembly components is derived from bacterial systems where components were first discovered. To put the plant machinery in perspective we start here with a brief overview of microbial [Fe–S] assembly systems. For more details about Fe–S assembly in microbes see reviews by Frazzon et al. (2002), Johnson et al. (2005) and Lill and Muhlenhoff (2005).

In bacteria, four machineries have been found to assemble [Fe–S] clusters, each of which is encoded by a gene cluster. The first Fe–S assembly machinery studied in detail was the *nif* system of *Azotobacter vinelandii*, which is responsible for the formation of Fe–S clusters for dinitrogenase, required under nitrogen fixation conditions (Zheng et al., 1993). The *A. vinelandii nif* gene cluster includes a cysteine desulfurase (CysD) encoding gene, *nifS*, as well as the other

Abbreviations: CysD – cysteine desulfurase; [Fe–S] – iron–sulfur cluster; Moco – molybdenum cofactor; NiR – nitrite reductase; PLP – pyridoxal 5′-phosphate; SiR – sulfite reductase

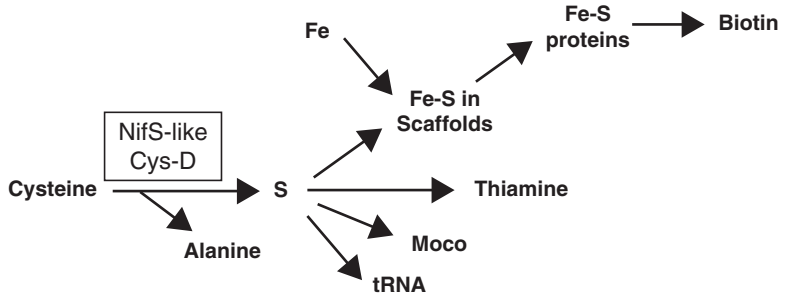

Fig. 1. Overview of cysteine desulfurase dependent biosynthetic pathways.

genes *nifU, iscA*^{nif}, *nifV* and *cysE*, all thought to be involved in Fe–S cluster formation. NifS-like proteins are pyridoxal 5′-phosphate (PLP)-dependent, enzymes that produce elemental sulfur from cysteine or selenium from selenocysteine, leaving alanine (Mihara et al., 1997; Mihara and Esaki 2002). Some enzymes also show activity with L-cystine as substrate and produce pyruvate, ammonia and elemental sulfur, via the formation of an enzyme complexed cysteine persulfide intermediate (Clausen et al., 2000). *In vitro*, most NifS-like enzymes show equal or higher activity on selenocysteine compared to cysteine.

On the basis of sequence similarity (Mihara et al., 1997) the NifS-like proteins are divided into group I (NifS/IscS-like) and group II (SufS-like) proteins. Purified group I enzymes efficiently use L-cysteine as a substrate and produce alanine and elemental sulfur as products via the formation of an enzyme bound cysteinyl persulfide intermediate (Zheng et al., 1994). Enzymes acting by this mechanism thus have high endogenous cysteine desulfurase activity. Purified group II enzymes have much lower cysteine desulfurase activity compared to selenocysteine lyase activity. The cysteine desulfurase activity of many of these enzymes is now known to be activated *in vivo* by other proteins.

A second NifS-like protein, IscS, occurs in *A. vinelandii*, and has a housekeeping function in the formation of cellular Fe–S proteins other than dinitrogenase (Zheng et al., 1998). The *iscS* gene is present in a gene cluster that contains paralogs of some of the *nif* genes (*iscU*, similar to the N-terminus of *nifU*, and *iscA*); thus the *nif* and *isc* clusters share a similar organization (Zheng et al., 1998). The IscU- and perhaps IscA-like proteins are thought to serve a scaffold function for the [Fe–S] cluster during its synthesis and before its transfer to the target protein, and conserved cysteines play a pivotal role in this process (Agar et al., 2000; Krebs et al., 2001). IscA may be an alternative scaffold for IscU because it can carry a transient [2Fe–2S] cluster, which subsequently can be transferred to ferredoxin or biotin synthase (Ollagnier-de-Choudens et al., 2004). However, alternative functions for IscA have been proposed. Some studies supported a role of IscA as an iron-binding protein, which subsequently donates iron for the [Fe–S] cluster assembly on IscU (Ding et al., 2004a, 2004b, 2005). Other data support a role of IscA type proteins in iron sensing (see below). The Isc gene cluster further includes an Hsp70 and Hsp40 and a ferredoxin type protein. Based on work done in yeast mitochondria (Muhlenhoff et al., 2003), the Hsp type proteins may be involved in the transfer of clusters from IscU scaffold proteins with which they interact (for a review on eukaryotic [Fe–S] assembly with a focus on yeast, see Lill and Muhlenhoff, 2005). Homologues of the *nif/isc* genes have been discovered in several other bacteria including *E. coli* (Zheng et al., 1998) and are also present in the mitochondria of eukaryotes (Lill and Kispal, 2000), as described below.

A third set of genes involved in Fe–S cluster formation that was first described for *E. coli* and *Erwinia chrysanthemi* is present in the *suf* operon (Takahashi and Tokumoto, 2002). The *suf* operon of *E.coli* is upregulated in response to oxidative stress and iron-limitation (Zheng et al., 2001, Outten et al., 2004) whereas expression of the *isc*-operon requires iron sufficient conditions. A major function of the *suf*-genes may be in protecting the cell from oxidative stress and iron starvation (Nachin et al., 2003; Outten et al., 2004). Besides a NifS-like protein (SufS/CsdB) the Suf operon encodes SufA, SufB, SufC, SufD and SufE. SufA

is related in sequence to IscA^{nif} and IscA and may have a scaffold function (Ollagnier-de Choudens et al., 2003), while SufE was shown to activate SufS (Loiseau et al., 2003; Outten et al., 2003). SufC constitutes a non-intrinsic cytosolic member of the ABC domain transporter superfamily. SufC forms a complex with SufB and SufD but the precise biochemical role of this complex is not yet clear (Nachin et al., 2003; Outten et al., 2003).

Finally, a fourth bacterial [Fe–S] machinery may be present in *Escherichia coli* (Loiseau et al., 2005). This simple gene cluster called *csd* is composed of *csdA* and *csdE*. The *csdA* gene encodes a cysteine desulfurase (Mihara et al., 2000; Loiseau et al., 2005) and *csdE* encodes a SufE-like protein that activates the cysteine desulfurase. The Csd system is proposed to supply [Fe–S] clusters for quinolinate synthase, NadA (Loiseau et al., 2005).

C. Functions of [Fe–S] Proteins in Chloroplasts

Chloroplasts use [Fe–S] proteins in photosynthetic electron transport, nitrogen and sulfur assimilation, and various other plastidic processes. In chloroplasts five cluster types have been found thus far. These [Fe–S] cluster types include [2Fe–2S] (found in for instance ferredoxin), Rieske-type [2Fe-2S] (found in the cytochrome-b/f complex and TIC55, a protein involved in precursor import), [3Fe–4S] (found in FD-GOGAT or glutamate synthase), [4Fe–4S] (found in PSI and in ferredoxin dependent thioredoxin reductase), and finally siroheme-[4Fe–4S], a unique cofactor in which the [4Fe–4S] is covalently bound to a siroheme (found in nitrite reductase and sulfite reductase). A more extensive overview of the various functions of iron–sulfur proteins in plants is given by Ye et al., (2006b). The function and synthesis of [Fe–S] proteins in plants (mitochondria, plastids and cytosol) were reviewed recently by Balk and Lobreaux (2005).

D. [Fe–S] Cluster Biogenesis in Chloroplasts and Photosynthetic Bacteria

1. Overview

Iron–sulfur cluster insertion into apoferredoxin was observed in isolated spinach chloroplasts and chloroplast fractions (Takahashi et al., 1986). Moreover, *in vitro* synthesized ferredoxin acquires a [2Fe–2S] cofactor after it is imported into isolated pea chloroplasts (Li et al., 1990; Pilon et al., 1995), suggesting that [Fe–S] clusters can be synthesized within this compartment independent of cytosol or other sub-cellular organelles. Cysteine was identified as the source of sulfur for the *in vitro* insertion of a [Fe–S] cluster in ferredoxin (Takahashi et al., 1986, 1990). As a specialized system, the chloroplast [Fe–S] biosynthetic machinery has more recently been investigated using *Arabidopsis thaliana* as a model. With the availability of complete genome sequences of cyanobacterial species, the existence of NifS-like proteins, present in suf-type operons and possibly isc operons have been discovered largely by comparing the genomes of *Synechocystes*, with those of *E.coli, A.vinelandii* and *A. thaliana*.

2. Cysteine Desulfurase

Because cysteine is required as a source of sulfur, a cysteine desulfurase is an essential component of any [Fe–S] biosynthetic machinery. In *Arabidopsis, CpNifS* (At1g08490) encodes a plastidic cysteine desulfurase (Leon et al., 2002; Pilon-Smits et al., 2002). CpNifS converts cysteine to alanine and provides sulfur for [Fe–S] assembly. Like all other NifS-like proteins, CpNifS has also selenocysteine lyase activity. Its selenocysteine lyase activity is much higher than its cysteine desulfurase activity. CpNifS is a class II NifS-like protein and most similar to SufS among all NifS-like proteins in *Escherichia coli*. CpNifS is essential for the [Fe–S] cluster formation activity of chloroplast stroma (Ye et al., 2005). In stroma, CpNifS is detected both in a 600 kDa complex and in dimeric form as indicated by gelfiltration (Ye et al., 2005). Repression of *CpNifS* expression by RNAi is lethal and causes a defect in the maturation of plastidic but not mitochondrial FeS proteins (Van Hoewyk et al., 2007).

CpSufE, a SufE-like protein encoded by At4g26500, is a cysteine desulfurase activator for CpNifS (Ye et al., 2006a). CpSufE forms a heterotetrameric complex with CpNifS, stimulating cysteine desulfurase activity 40–60-fold and increasing the substrate affinity of CpNifS toward cysteine. *In vitro* reconstitution experiments show that the CpNifS [2Fe-2S] cluster assembly activity in ferredoxin is enhanced 20-fold

by CpSufE (Ye et al., 2006a). These activities need an essential cysteine residue in CpSufE, which is likely the acceptor site for intermediate sulfur. This cysteine is not required for binding to CpNifS. Therefore, excess cysteine-mutated SufE displays a dominant negative effect over the wild-type protein. CpSufE function is essential for seedling viability (Xu and Moller, 2006; Ye et al., 2006a). The subcellular location of SufE is a matter of interest. Whereas Ye et al. (2006a) detected the protein in chloroplasts by analyzing GFP-fusions as well as by immunoblotting, data presented by Xu and Moller (2006) suggest that in *Arabidopsis* CpSufE may have a dual localization in both plastids and mitochondria. The dual localization reported by Xu and Moller (2006) is supported by GFP-fusions, complementation of a T-DNA KO only by intact precursor SufE or by both a mitochondrial targeted and plastid targeted SufE and by interaction of SufE with both CpNifS and the mitochondrial NifS, Nfs2. If correct, this would be the first reported case of a type-I, IscS-like, cysteine desulfurase acting with a SufE-like protein.

The cyanobacterium *Synechocystis* sp. PCC 6803 has four NifS-like proteins. The genes slr0387 and sll0704 encode enzymes (Sscsd 1 & 2) similar to NifS of *A.vinelandii* and IscS of *E. coli*. (Kato et al., 2000). They catalyze the desulfuration of L-cysteine, producing alanine and elemental sulfur, and likely play an important role in [Fe–S] synthesis in this cyanobacterium. Typical for NifS-like proteins, the two enzymes also show selenocysteine lyase activity. Sscsd1 has a higher substrate specificity for L-selenocysteine and a much higher specific activity towards the selenium substrate. Sscsd1 and 2 also produced [2Fe-2S] *in vitro* that facilitated the formation of holoferredoxin (Kato et al., 2000). These cyanobacterial IscS related proteins most likely work in concert with an Isc-type assembly machinery.

In addition to the two iscS-like genes described above, *Synechocystis* sp. PCC 6803 also has genes encoding type-II NifS-like proteins. The *slr2143* gene encodes a cystine C-S lyase (Clausen et al., 2000) and *slr0077*, encodes a protein which acts as both a cysteine desulfurase and cystine lyase (Kessler, 2004). Slr0077 is essential (Seidler et al., 2001) but the IscS-like type-I cysteine desulfurases are not. Also called SufS, the Slr0077 enzyme is most related in predicted protein sequence to the *Arabidopsis* chloroplast NifS-like enzyme CpNifS (Pilon-Smits et al., 2002). Slr0077 shows cysteine desulfurase activity under reducing conditions and cystine lyase activity in a partially oxidizing environment (Kessler, 2004). This twin reaction mechanism of Slr0077 might be a significant feature of [Fe–S] synthesis in the cyanobacterium that responds to the redox status of the cell.

3. IscA and Nfu Scaffolds Proteins

CpIscA (At1g10500) may serve as a scaffold protein for [Fe–S] synthesis in plastids (Abdel-Ghany et al., 2005). After acquiring sulfur from cysteine via CpNifS and ferrous iron from media, CpIscA is able to assemble a [2Fe–2S] cluster resulting in dimeric holo-CpIscA *in vitro*. This holo-CpIscA can be isolated by gelfiltration holding its transient cluster. Upon incubation with apoferredoxin CpIscA can transfer its cluster, resulting in holoferredoxin, which is then active when tested for electron transfer (Abdel-Ghany et al., 2005). The presence of the CpIscA scaffold improves the [2Fe–2S] reconstitution in ferredoxin. Interestingly, CpIscA is mostly present in a Ð600 Kda chloroplast stromal complex, similar to CpNifS (Abdel-Ghany et al., 2005). Recent work by Yabe and Nakai (2006) reported that the chloroplast IscA in *A.thaliana* is a non-essential or redundant scaffold. The authors showed that a chloroplast Nfu-like protein called Nfu2 acts as an essential scaffold whose deficiency affected the production or accumulation of CpIscA. Thus CpIscA seems to operate downstream of NfU (see below).

In the cyanobacterium *Synechocystis* sp. PCC 6803, *sufA* and *sufE* exist in a separate operon that is not contiguous with the other *suf* genes as is the case in *E. coli* (Wang et al., 2004). A recent and interesting report proposes a model for the function of the *suf* and *isc* regulons and the regulatory role of *sufA* and *iscA* in cyanobacteria (Balasubramanian et al., 2006). An *iscA* null mutant had upregulated expression of the *suf* and *isc* genes and *isiA* (a marker for iron limitation), meaning the iscA mutant mistakenly senses iron-limitation under normal conditions and responds to it. Therefore iscA may be part of iron sensing and homeostasis in cyanobacteria (Balasubramanian et al., 2006). The mRNA levels of *suf* genes were increased in a sufA null mutant during

oxidative stress (Balasubramanian et al., 2006). Therefore the Suf system has been proposed to be involved in [Fe–S] synthesis/repair under oxidative stress. The cyanobacterial and *Arabidopsis* sufA homologs contain two additional highly conserved cysteine residues (absent in non-photosynthetic bacteria) which could possibly act as a redox sensitive regulatory switch by reversible disulfide bond formation, and set in motion the suf, isc and possibly other factors of cyanobacterial [Fe–S] biosynthesis machinery. This recent work by Balasubramanian et al. (2006) proposes new roles for *iscA* and *sufA* in iron homeostasis and oxidative stress response, in addition to the role of alternative scaffolds earlier assigned to them (Ollagnier de Choudens et al., 2004; Abdel-Ghany et al., 2005). In view of these recent reports and the cyanobacterial origin of chloroplasts, it would be interesting to look into the roles of IscA and/or SufA in iron sensing/homeostasis and oxidative stress responses.

In addition to CpIscA, Nfu1–3 are scaffold proteins active in chloroplasts (Leon et al., 2003; Touraine et al., 2004; Yabe et al., 2004). Nfu1 (At4g01940) and Nfu2 (At5g49940) are able to restore the growth of a scaffold-mutated yeast, strain Δ*isu1*Δ*nfu1*, suggesting a role as a scaffold. Recombinant Nfu2 contains a labile [2Fe–2S] cluster, which can be transferred to apo-ferredoxin resulting in holo-ferredoxin formation (Leon et al., 2003; Yabe et al., 2004). The analysis of T-DNA insertion lines revealed that Nfu2 is required for assembling [4Fe–4S] clusters of photosystem I and the [2Fe-2S] cluster of ferredoxin in chloroplasts (Touraine et al., 2004; Yabe et al., 2004). Interestingly, a subset but not all chloroplast [Fe–S] proteins are affected in the *Nfu2* mutant, suggesting alternative scaffolds are functional in chloroplasts. Two more Nfu-like proteins, Nfu4 and Nfu5, are located in mitochondria (Leon et al., 2003). Notably, proteins with similarity to the N-terminus of NifU (IscU-like proteins) are all localized in mitochondria in *Arabidopsis* and are absent in plastids (Leon et al., 2005).

In cyanobacteria there is a gene denoted nfu encoding a protein similar to the C-terminal domain of NifU of *A.vinelandii* (Nishio and Nakai 2000). It was shown to be an essential scaffold protein (Seidler et al., 2001, Balasubramanian et al., 2006). On the other hand, null mutants of *iscA, sufA* and the *iscA-sufA* double mutant expressed wild-type levels of Nfu and growth rates comparable to wild-type under normal conditions, iron-limited conditions, or oxidative stress (Balasubramanian et al., 2006).

4. Other Factors

Components of the SufBCD complex have been found in plants and are active in the plastids. In Arabidopsis, *SufB* (At4g04770) encodes a protein with ATPase activity (Xu et al., 2005). This gene can complement *sufB* deficiency in *Escherichia coli. Arabidopsis SufC* (At3g10670) is an ABC type ATPase (Xu and Moller, 2004), which can partially rescue growth defects in an *Escherichia coli sufC* mutant. Finally, the *Arabidopsis SufD* (At1g32500) encodes a protein with homology to the bacterial SufD, mutation of which results in impaired embryogenesis and abnormal growth of *Arabidopsis* (Hjorth et al., 2005). Like their bacterial homologues, the chloroplast SufBCD proteins form a complex (Xu and Moller, 2004; Xu et al., 2005), displaying ATPase activity. Although it is likely that SufBCD play some role in [Fe–S] cluster biogenesis, the exact role in the process remains to be characterized not just in plants but in any organism.

Two additional components were identified in screens for mutants that are pleiotropically affected in photosynthesis. One such protein is HCF101 for High Chlorophyll Fluorescence (Stockel and Oelmuller, 2004). *HCF101* (At3g24430) encodes a protein with sequence similarity to P-loop ATPases (Lezhneva et al., 2004). It is required for the biogenesis of [4Fe–4S] clusters for photosystem I (PSI) and ferredoxin-thioredoxin reductase (FTR) in chloroplasts. The exact biochemical function of HCF101 remains to be elucidated. Another factor which was originally identified in a photosystem I mutant screen is *APO1* (Accumulation of Photosystem One1). *APO1* (At1g64810) is a member of a novel gene family so far found only in vascular plants, (Amann et al., 2004). It is involved in the assembly of [4Fe–4S] cluster-containing complexes of chloroplasts, e.g. PSI. However, a direct connection between APO1 and [Fe–S] assembly needs to be shown because the protein could also affect PS1 maturation without acting directly on [Fe–S] assembly.

The existence of a complete *suf* operon has been reported in the cyanobacteria *Synechocystis, Synechococcus* and *Anabaena* (Wang et al., 2004). DNA sequence comparison shows that the cyanobacterial *sufBCDS* cluster is homologous to the suf operon of *E. coli* (Wang et al., 2004). The *sufBCDS* genes are cotranscribed in *Synechococcus* sp. PCC 7002. A gene named *sufR* is located upstream of the *sufBCDS* cluster in cyanobacteria and has been proposed to be a transcriptional repressor of the operon (Wang et al., 2004). SufR has a DNA binding domain at its N-terminus and a metal binding region with conserved cysteines at the C-terminus, which could be acting as a sensor of oxidative stress or iron limitation. *sufR* null mutants of *Synechococcus* sp. PCC 7002 had elevated transcript levels of *sufBCDS* compared to wild type and also showed higher growth rates under iron limitation (Wang et al., 2004).

Little is known about [Fe–S] biosynthesis mechanisms in other photosynthetic prokaryotes, though the existence of [Fe–S] clusters and hence their biosynthetic machineries can be predicted. The occurrence of *nifS, nifU* and ferredoxin as part of the *nif* gene cluster for maturation of [Fe–S] and Mo cofactor containing nitrogenase was reported in the photosynthetic bacterium *Rhodopseudomonas palustris* (Oda et al., 2005). Microarray data indicate upregulation of [Fe–S] assembly/repair genes in a facultative phototrophic bacterium *Rhodobacter sphaeroides* upon exposure to hydrogen peroxide (Zeller et al., 2005).

5. Summary of Plastidic and Cyanobacterial [Fe–S] Assembly Systems

In summary, chloroplasts and photosynthetic bacteria conserve a complete SUF-type machinery, including homologs to all components encoded by the *E. coli sufABCDSE* gene cluster for [Fe–S] biogenesis. Because green chloroplasts produce saturated oxygen levels in the light, it is not unexpected that a SUF-like system operates in chloroplasts. However, the complete chloroplast [Fe–S] machinery is more complex than bacterial [FeS] assembly systems. The chloroplast system also includes three NifU-like proteins and HCF101, a protein that has no homologue in bacterial [Fe–S] machineries. Furthermore APO1, a protein that is unique in vascular plants is required for the accumulation of a subset of [Fe–S] proteins in plastids. The severe phenotypes caused by mutations of individual machinery components point to the general importance and complexity of the chloroplast [Fe–S] machinery. For instance, loss of function mutants for *CpNifS* are inviable (Van Hoewyk et al., 2007). An insertion in *SufE* causes embryonic lethality (Xu and Moller, 2006; Ye et al, 2006a). *Nfu2* T-DNA insertion mutants are dwarfed and yellowish (Touraine et al., 2004; Yabe et al., 2004). Abnormal plastid structure and impaired embryogenesis is observed for *SufC* or *SufD* mutants (Xu and Moller, 2004; Hjorth et al., 2005). Finally, seedling lethality and high chlorophyll fluorescence is observed for *HCF101* mutants (Lezhneva et al., 2004).

6. The Integration of Iron and Sulfur Metabolism in Plastids

Because the CpNifS-dependent machinery supplies [Fe–S] clusters for a range of chloroplast activities, plastid [Fe–S] biogenesis is essential for photosynthetic carbon fixation, nitrogen assimilation, sulfur assimilation, and other biosynthetic processes. Because of the importance of iron and sulfur assimilation for agricultural crops, the effects of the plastid [Fe–S] machinery on the homeostasis of iron and sulfur in plants is of particular interest.

In *Arabidopsis* leaf tissue, about 70% of the iron is located in chloroplasts and at least half of that iron is associated with thylakoids (Shikanai et al., 2003). Fe^{2+} transport across the chloroplast inner envelope membrane is measurable, and determined to be light-dependent. Furthermore, Fe^{2+} transport across inner envelope membrane is stimulated by an electrochemical proton gradient, and reduced by negating the potential gradient, suggesting that iron transport into chloroplasts is via a Fe^{2+}/H^{+} symport mechanism (Shingles et al., 2002). In thylakoid membranes, iron is predominantly used for electron transfer. Approximately half of the thylakoid iron is in the photosystem I in form of [4Fe–4S] clusters. The remaining half is present as non-heme iron, iron in cytochromes in the form of heme and Rieske-type [2Fe–2S] clusters (Raven et al., 1999). In the stroma, iron is used in [Fe–S] clusters, hemes, and non-heme iron in for instance Fe–SOD. Because excess

free iron would be toxic, surplus iron is stored in stromal ferritin (Petit et al., 2001). Because iron is one of the most limiting micronutrients to plants yet potentially toxic in excess, we expect a sophisticated homeostasis mechanism to control cellular iron, particularly in plastids. Would there be cross-talk between the various biosynthetic pathways that involve Fe cofactors in plastids? We consider this more than likely and possible interactions are indicated in Fig. 2. The pathways for inserting iron in non-heme Fe proteins such as FeSOD are largely unknown. However, much is known about the synthesis of heme and siroheme cofactors, which are produced through the tetrapyrrole pathway starting from glutamate. This pathway also serves to synthesize chlorophyll A and B (for review see Cornah et al., 2003). Feedback regulation in the tetrapyrrole pathway is well established. Heme accumulation may be an important factor in this process. Furthermore, the accumulation of the intermediate Mg-protoporphyrin IX is in

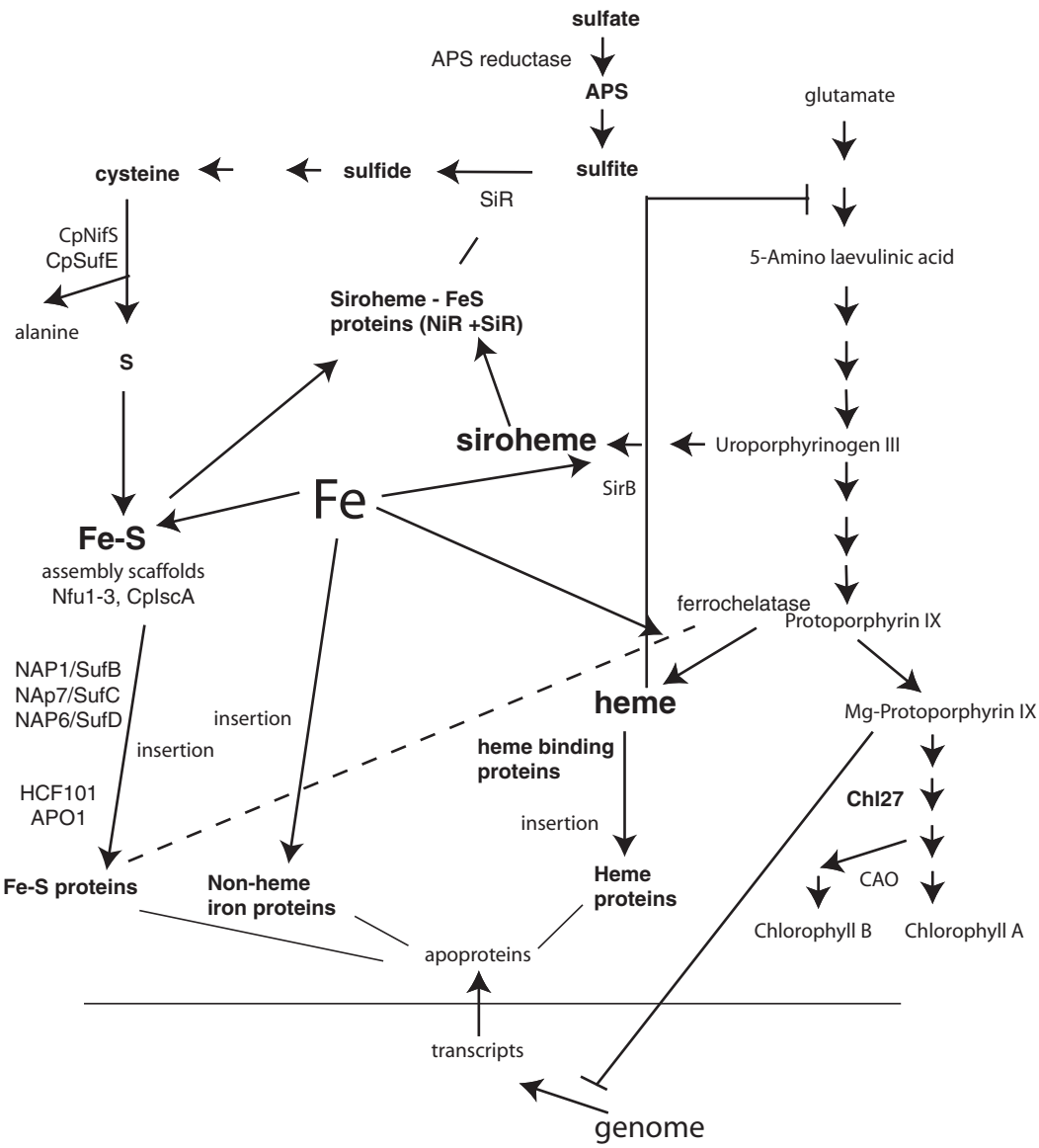

Fig. 2. Interactions of Fe and S metabolism in plastids. See text for details.

all likelihood an important signal affecting the expression of nuclear genes for chloroplast precursor proteins (Cornah et al., 2003). Finally, there may be cross-talk between [Fe–S] assembly and heme synthesis because ferrochelatase activity is downregulated in [Fe–S] assembly mutants, at least in mitochondria of yeast (Lange et al., 2004); a similar regulation may occur in plastids. Cross-talk is also suggested by the phenotype of the *laf6* (= *SufB*) mutant of *Arabidopsis*, which accumulates protoporphyrin IX an intermediate in chlorophyll and heme synthesis (Moller et al., 2001).

The plastid is also a key compartment for sulfur assimilation from sulfate to cysteine (for reviews see: Hawkesford, 2007; Hell and Wirtz, 2007). Notably, out of four enzymes involved in S assimilation in plastids, two are [Fe–S] proteins: APS reductase and sulfite reductase (SiR) (Fig. 2). Thus, sulfur assimilation is likely dependent on the plastid [Fe–S] machinery. The step catalyzed by sulfite reductase is even more dependent on the [Fe–S] assembly machinery. This enzyme employs a unique siroheme-[4Fe–4S] as its prosthetic group, requiring not only a direct incorporation of a [4Fe–4S] cluster but also a sirohydrochlorin ferrochelatase (SirB), which is also a [2Fe–2S] protein, for synthesizing the siroheme. Moreover, the six electron reduction catalyzed by SiR needs ferredoxin, a [2Fe–2S] protein, for providing electrons. Thus, the SiR-catalyzed step in sulfur assimilation is completely dependent on the plastid [Fe–S] machinery, strongly suggesting a central role of the [Fe–S] machinery in plastid sulfur homeostasis.

E. Function and Assembly of Fe–S Clusters in Plant Mitochondria and Cytosol

Iron sulfur clusters play crucial roles in mitochondrial function. For instance, aconitase, a key enzyme in the Krebs cycle in the matrix, is an Fe–S protein. Furthermore respiratory electron transport chain complexes I, II and III all have Fe–S cofactors (for review see Balk and Lobreaux, 2005). In Arabidopsis, a second cysteine desulfurase, Nfs2, is present in mitochondria (Kushnir et al., 2001), where another [Fe–S] biogenesis machinery is present. In addition, a third cysteine desulfurase activity is found in the cytosol (Heidenreich et al., 2005). This activity is due to the NifS-like domain of the ABA3 protein, which functions in Moco synthesis and is most likely not relevant to [Fe–S] synthesis. Work in yeast, which can grow anaerobically using fermentation, suggested that Fe–S cluster formation is the only essential function of mitochondria (Lill and Kispal, 2000) and cytosolic Fe–S clusters depend on the mitochondrial Isc machinery involving homologues of the genes encoded by the *nif/isc* clusters of bacteria. The yeast mitochondrial NifS-like protein, IscS, is essential for this reason (Kispal et al., 1999). A similar mitochondrial machinery, dependent on Nfs2, may be present in plants (Kushnir et al., 2001).

In *Arabidopsis* the mitochondrial ABC transporter Sta1 could be involved in the transport of [Fe–S], or an unknown precursor of these clusters, from mitochondria to the cytosol (Kushnir et al., 2001). The *sta1* mutants were dwarfed, chlorotic, had distorted nuclei, and accumulated higher amounts of free (non heme, non protein) iron in mitochondria and showed increased expression of mitochondrial cysteine desulfurase: effects collectively known as the '*starik*' phenotype (Kushnir et al., 2001). Sta1 is a functional ortholog of Atm1p, the mitochondrial ABC transporter in yeast (Kushnir et al., 2001). Complementation of mutant *atm1* yeast cells with *Arabidopsis Sta1* restored maturation of cytosolic [Fe–S] protein Leu1p (Kushnir et al., 2001). However, in *Arabidopsis sta1* mutants, the activities of cytosolic and mitochondrial isoforms of aconitase were similar to wild type, probably due to the presence of redundant mitochondrial ABC transporters such as Sta2 and Sta3 that perhaps compensated the loss of Sta1. The *starik* phenotype is thought to be a pleiotropic effect resulting from an imbalance in the intracellular iron homeostasis (Kushnir et al., 2001). This report indicates that there is a mechanism of iron movement between mitochondria and cytosol and that [Fe–S] generated in mitochondria could be exported to cytosol for the maturation of cytosolic Fe–S proteins as seen in *atm1* yeast cells (Kushnir et al., 2001). In that case, there could be scaffold proteins in the cytosol that help transfer mitochondrial [Fe–S] to cytosolic apoproteins. The mechanisms for the maturation of cytosolic [Fe–S] proteins remain to be elucidated.

The existence of Fe–S proteins in the cytosol (e.g. an aconitase) raises the question if there is

a separate machinery for the synthesis of [Fe–S] clusters and their incorporation into cytosolic apo-proteins. We do not yet know of a cytosolic [Fe–S] assembly system in plants. Recently, components of such a machinery have been reported in mammalian (Pondarre et al., 2006; Tong and Rouault 2006) and yeast cells (Balk et al., 2004, 2005; Hausmann et al., 2005). A matter of debate is the presence of an IscS activity in the cytosol or nucleus. The observation in yeast that IscS is required for the thio-modification of cytoplasmic tRNA *in vivo*, strongly supports the presence of IscS outside the mitochondria (Nakai et al., 2004).

III. Iron–Sulfur Cluster Dependent Cofactor Assembly Pathways

A. Radical SAM Enzymes

Radical SAM enzymes are [4Fe–4S] containing proteins that use S-adenosyl methionine (SAM) as a cofactor (Layer et al., 2004). These enzymes form a superfamily of proteins that now includes more than 600 members, present in a diverse group of organisms from bacteria to plants and humans (Sofia et al., 2001). The functions supported by radical SAM proteins so far identified include a diverse set of reactions such as sulfur transfer reactions, heme and chlorophyll biosynthesis, ring forming reactions like thiazole formation, antibiotic and herbicide biosynthesis and DNA repair (Sofia et al., 2001). The activities of most SAM radical enzymes are extremely oxygen sensitive. All these enzymes contain a [4Fe–4S] cluster which is thought to be the main catalytic site (Walsby et al., 2005). Some enzymes like biotin synthase and lipoyl synthase have an additional [2Fe–2S] cluster. Other examples of the radical SAM enzymes identified are HemN (oxygen independent coproporphyrinogen synthase, involved in tetrapyrrole biosynthesis), Biotin synthase, MoaA (involved in molybdenum cofactor biosynthesis), littorine mutase (an alkaloid generating enzyme in *Datura*) and HydE/G, two enzymes required for the maturation of an Fe-hydrogenase in the chloroplast of *Chlamydomonas reinhardtii* (Layer et al., 2005). This large class of iron–sulfur proteins is probably very important for plant metabolism, but thus far only few radical SAM enzymes have been characterized in plants and other photosynthetic organisms.

A characteristic feature of the radical SAM superfamily members is the presence of a conserved three-cysteine motif binding the [4Fe–4S] cluster. Three of four iron atoms bind to the three cysteines. The fourth iron, bound to a non-cysteine ligand, provides a unique site for coordination with the cofactor S-adenosyl methionine. Electron transfer from the iron–sulfur cluster to SAM results in SAM cleavage and generates a highly reactive 5′-deoxyadenosyl radical that abstracts a H atom from the substrate and initiates the reaction, a feature common to all SAM radical enzyme catalysed reactions (Walsby et al., 2005).

It is obvious that this important class of enzymes must depend on the iron–sulfur synthesis machinery for their [Fe–S] clusters. However, to date the processes involved in formation and transfer of [Fe–S] to SAM radical proteins have not been studied in detail. In one report the SAM radical enzyme lipoyl synthase from *E.coli* when coexpressed with the *A.vinelandii* Isc operon is active without *in vitro* reconstitution (Cicchillo et al., 2004). It would be interesting to investigate if a specialized mechanism exists for the [Fe–S] supply to SAM radical proteins.

B. Biotin Synthesis

Biotin, a member of the vitamin B family is a water soluble cofactor for certain enzymes involved in fatty acid and carbohydrate metabolism. Biotin could also be involved in modulating gene expression (Che et al., 2003). Plants and bacteria possess a similar biosynthetic pathway (Schneider and Lindqvist, 2001) for the synthesis of this essential nutrient. The mechanism of biotin synthase reaction has been well studied in *E. coli*. Biotin synthase, also known as BioB contains a [4Fe–4S] and a [2Fe–2S]. It is a radical SAM enzyme requiring S-adenosyl methionine as a cofactor. The [2Fe–2S] is proposed to be the immediate sulfur donor to dethiobiotin (Jameson et al., 2004), resulting in the loss of sulfur from the [2Fe–2S] and consequent inactivation of the enzyme. *In vitro*, NifS from *A.vinelandii* and C-DES from *Synechocystis* could mobilize sulfur from cysteine for reconstitution of the [2Fe–2S] into the apoprotein of *E. coli* biotin synthase and restore its activity (Bui et al., 2000).

In plants, biotin synthesis occurs in the cytosol and mitochondria. The last step involves the conversion of dethiobiotin to biotin and involves the insertion of a sulfur atom into dethiobiotin. The enzyme catalyzing this reaction, biotin synthase, occurs in the mitochondrial matrix (Baldet et al., 1997). The identity of the sulfur donor in plants remains unknown but based on analogy with bacterial systems this may involve S from a 2Fe–2S cluster, ultimately derived from cysteine and activated via the mitochondrial NifS-like enzyme. Indeed it has been shown that in *Arabidopsis* mitochondria, biotin synthase requires other mitochondrial components for its activity (Picciocchi et al., 2003). It is highly probable that mitochondrial cysteine desulfurase, using cysteine as the substrate could replenish sulfur for [2Fe–2S] of biotin synthase.

C. NAD Synthesis

NAD and NADP are important coenzymes in biological redox reactions. In all NAD biosynthetic pathways known thus far, quinolinate is a precursor of nicotinic acid, which is a component of NAD. Quinolinate is synthesized from aspartate or tryptophan. In the aspartate pathway, aspartate is oxidized by L-aspartate oxidase (product of the NadB gene) to iminoaspartate, which is converted to quinolinate by the enzyme quinolinate synthase (NadA). NadA has been characterized in *E. coli*. It is a [4Fe-4S] containing enzyme. The iron–sulfur cluster is sensitive to oxygen and is essential for the quinolinate synthase activity (Ollagnier-de Choudens et al., 2005). An *E. coli* strain lacking IscS was unable to synthesize NAD, and required nicotinic acid for growth (Lauhon and Kambampati 2000). A recent report (Katoh et al., 2006) shows that in *Arabidopsis* NadA, along with two other enzymes of the aspartate to quinolinate pathway (NadB and quinolinate phosphoribosyl transferase) is essential. T-DNA disruption of the corresponding genes was embryonic lethal. The three proteins were found to be located in the plastid (Katoh et al., 2006). Most likely, the *Arabidopsis* plastid NadA is also an iron–sulfur protein like its *E. coli* counterpart, and could be sensitive to oxidative stress and plastid iron status. Therefore this enzyme could be a link between NAD synthesis, redox status and photosynthesis in chloroplasts. The *Arabidopsis* NadA protein has an extra amino terminal SufE-like domain that may provide SufE activity to recruit and enhance the cysteine desulfurase CpNifS specifically for the activation of quinolinate synthase.

IV. Synthesis of Thiamine

Thiamine or vitamin B1 is an essential cofactor in all cells. Thiamine consists of pyrimidine and thiazole moieties. The mechanism of biosynthesis of this essential vitamin, especially its thiazole ring has been a matter of interest over the past 15 years (Julliard and Douce, 1991; Belanger et al., 1995; Park et al., 2003; Dorrestein et al., 2004). In higher plants, the chloroplast is a site of thiamine synthesis. When stromal proteins from spinach chloroplasts were incubated with substrates such as glyceraldehyde-3-phosphate, pyruvate, tyrosine, cysteine and MgATP, thiazole was synthesized *in vitro* (Julliard and Douce, 1991). Stromal proteins formed thiamine when provided with thiazole and pyrimidine moieties, showing that chloroplast stroma has all the enzymes and substrates required for thiazole synthesis and condensation of thiazole and pyrimidine, to form thiamine (Julliard and Douce, 1991).

Cysteine is an essential substrate for thiazole formation. It is quite likely that the chloroplast NifS is involved in the mobilization of sulfur from cysteine, for thiazole synthesis. It has been shown in bacterial systems that in addition to the components of a multi-enzyme complex involved in the biosynthesis of the thiazole ring (Leonardi et al., 2003), a NifS-like protein is essential, along with cysteine and other substrates for *in vitro* thiazole synthesis (Park et al., 2003, Leonardi and Roach, 2004). An *E.coli* IscS-deletion strain required thiazole in the medium for growth (Lauhon and Kambampati, 2000). Recently, a thiazole biosynthetic enzyme Thi1 has been identified in *A. thaliana*, that seems to be targeted to both chloroplasts and mitochondria (Ribeiro et al., 2005). It would be interesting to know if thiamine is synthesized in both chloroplasts and mitochondria since both organelles contain cysteine desulfurases.

V. Synthesis of Molybdenum Cofactor

Plants have only four Mo requiring proteins: nitrate reductase, xanthin dehydrogenase, and

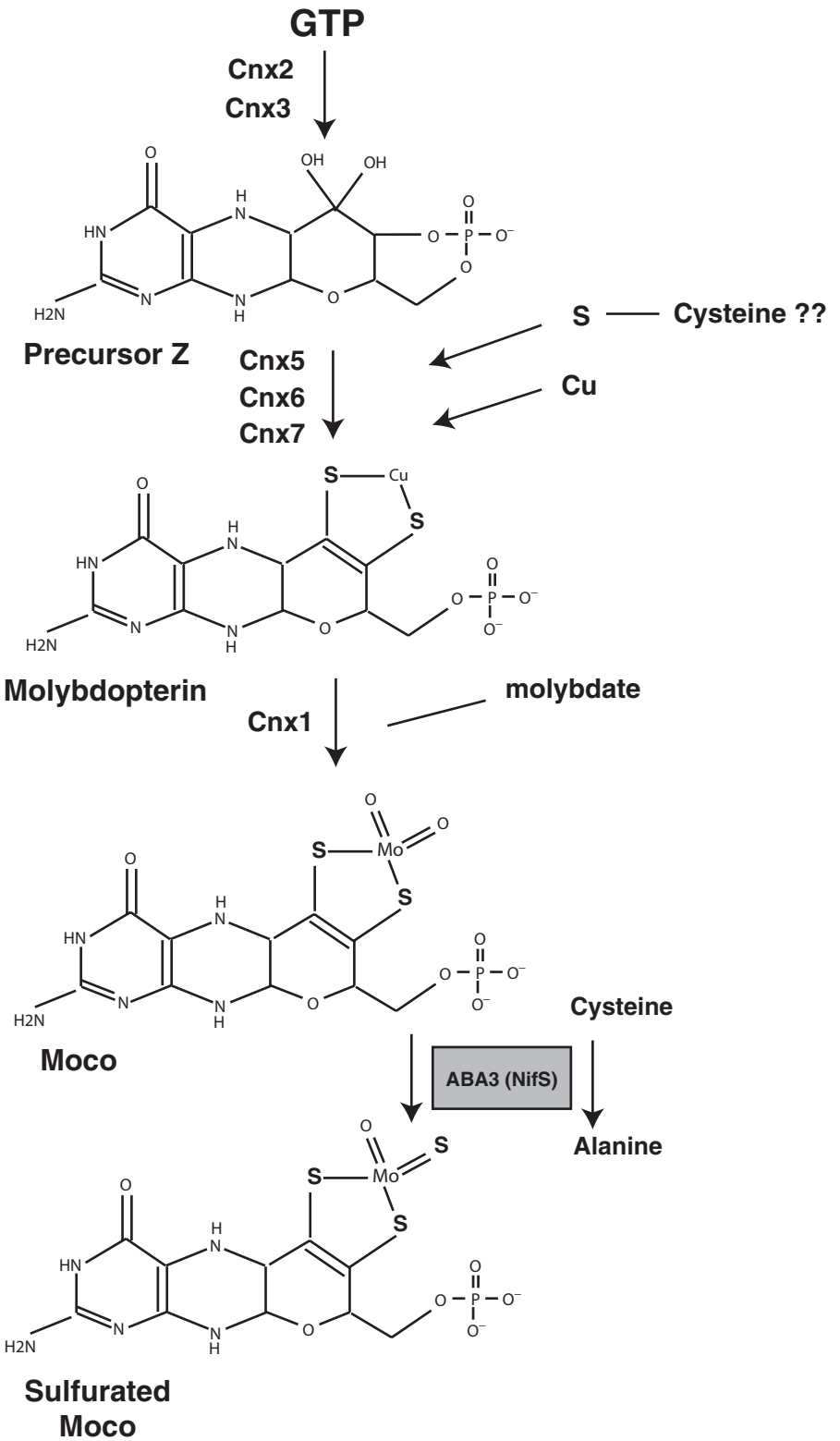

Fig. 3. Molybdenum cofactor biosynthesis. See text for details.

aldehyde oxidase are active in the cytosol while sulfite oxidase is most likely active in peroxisomes (Mendel and Hansch, 2002). With the exception of bacterial nitrogenase, all biologically active Mo occurs in a special pterin-derived cofactor termed molybdenum cofactor or Moco, which is assembled in the cytosol (Mendel, 2005). In plants, MoCo deficiency leads to reduced nitrate reductase activity and N depletion, as well as reduced phytohormone synthesis (Mendel and Hansch, 2002). The studies performed in plants have significantly contributed to what we know about Moco synthesis and this is now one of the best-understood cofactor assembly systems (Mendel, 2005). Seven enzymes are required for Moco synthesis in plants (see Fig. 3). The bacterial homologue of the first of these enzymes, Cnx2, is called MoaA and is a member of the radical SAM enzyme family in bacteria. Therefore, since radical SAM enzymes are [4Fe–4S] enzymes, we can expect that the activity of the Fe–S assembly machinery is required for Moco synthesis. The conversion of precursor-Z to molybdopterin involves the insertion of two S atoms, that will later hold Mo in Moco. Presently the source of these two S atoms is unknown, but it can be speculated that they derive from cysteine. The insertion of molybdenum in the molybdopterin skeleton requires the CNX1 protein. The structure of CNX1 with the molybdopterin bound revealed that the enzyme makes an adenylated intermediate, molydopterin-AMP, which was found to be bound to the enzyme. Furthermore, Cu was found to be bound to the sulfhydryls of the molybdopterin moiety and it was suggested that the presence of Cu served to protect the thiols and facilitate Mo insertion (Kuper et al., 2004). The activities of xanthin dehydrogenase and aldehyde oxidase require a special modification of Moco: the replacement of O by S to form sulfurated Moco (see Fig. 3). The S for this modification is derived from cysteine. In *Arabidopsis*, a specialized cytosolic enzyme, ABA3 catalizes this reaction (Xiong et al., 2001; Heidenreich et al., 2005). ABA3 contains a PLP containing NifS-like domain required specifically for this activation. ABA3 is the 3rd NifS-like enzyme in *Arabidopsis*, next to CpNifS and mitochondrial Nfs2. The activity of ABA3, which is induced by drought stress and cold treatment, may regulate the activities of xantine dehydrogenase and aldehyde oxidase and therefore cellular phytohormone levels through this sulfurylation step.

VI. Conclusions and Outlook

Cysteine is the sulfur donor in a wide spectrum of biosynthetic reactions mediated by NifS-like and PLP-dependent cysteine desulfurase enzymes. This is a critical role of cysteine next to its role as an amino acid subunit of peptides and proteins. A central role of cysteine desulfurases is in the synthesis of [FeS] clusters and other S containing cofactors. Because [FeS] containing enzymes play such pivotal roles in assimilation reactions (including those for sulfur) and in the biosynthesis of other cofactors acting both as catalysts but also as non-catalytic donors, using transient [FeS] clusters, the machinery for [FeS] synthesis is essential. Iron–sulfur clusters are ancient cofactors that probably evolved very early in the evolution of life or even before that time in a reducing atmosphere where these clusters may have formed spontaneously. The accumulation of oxygen, due to oxygenic photosynthesis, presented cells with a challenge with respect to their [FeS] assembly because the clusters are very sensitive to oxygen. Several types of NifS-like protein dependent [FeS] machinery, perhaps with different sensitivities to oxygen have evolved. We still have not identified all the components needed for [FeS] and other S containing cofactors but we are getting closer. Once all components are known we can try to understand the regulation of cysteine desulfurase dependent pathways and how cofactor assembly and cysteine availability for protein synthesis are balanced.

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