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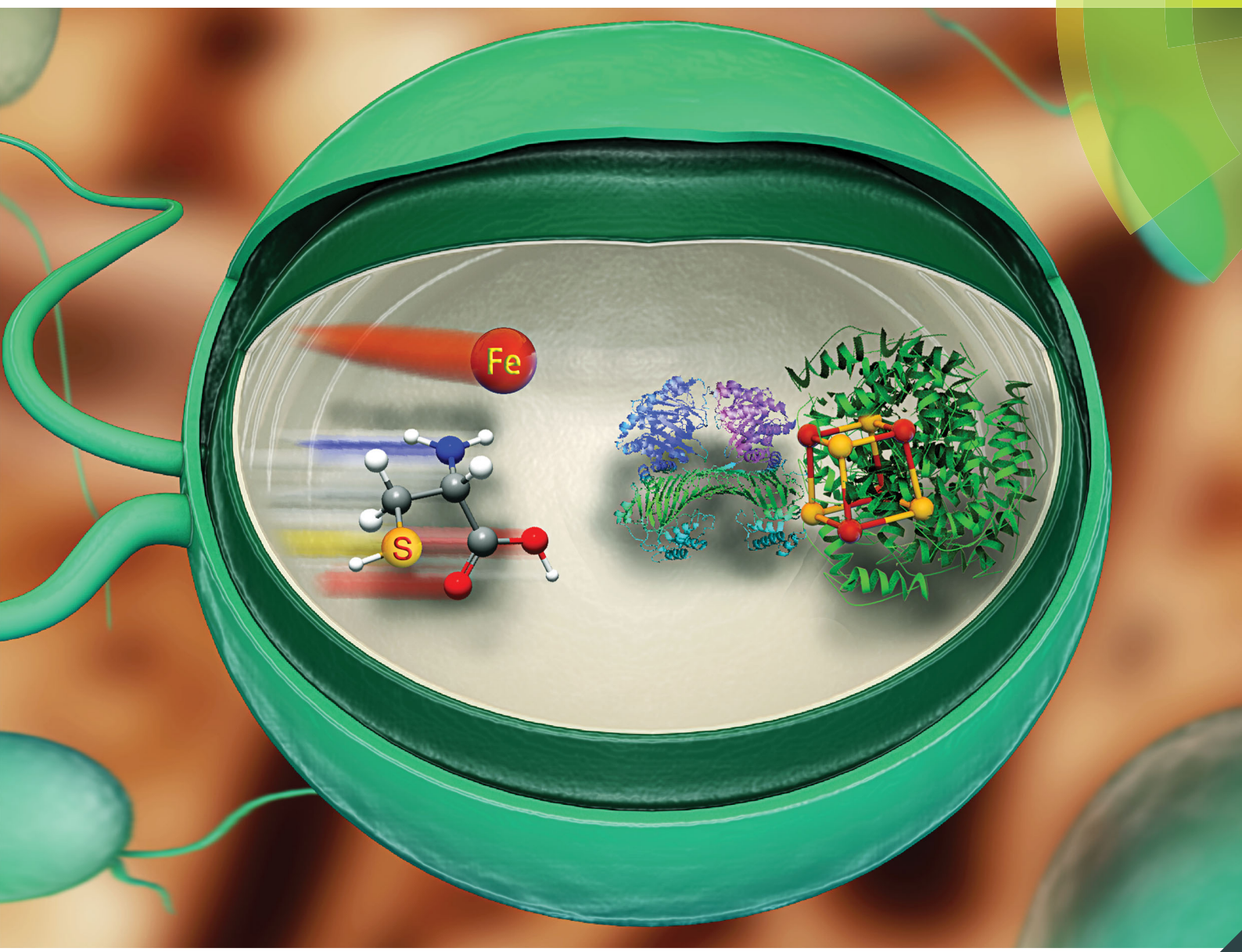

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Iron–sulphur cluster biogenesis *via* the SUF pathway

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Iron–sulphur (Fe–S) clusters are versatile cofactors, which are essential for key metabolic processes in cells, such as respiration and photosynthesis, and which may have also played a crucial role in establishing life on Earth. They can be found in almost all living organisms, from unicellular prokaryotes and archaea to multicellular animals and plants, and exist in diverse forms. This review focuses on the most ancient Fe–S cluster assembly system, the sulphur utilization factor (SUF) mechanism, which is crucial in bacteria for cell survival under stress conditions such as oxidation and iron starvation, and which is also present in the chloroplasts of green microalgae and plants, where it is responsible for plastidial Fe–S protein maturation. We explain the SUF Fe–S cluster assembly process, the proteins involved, their regulation and provide evolutionary insights. We specifically focus on examples from Fe–S cluster synthesis in the model organisms *Escherichia coli* and *Arabidopsis thaliana* and discuss in an *in vivo* context the assembly of the [FeFe]-hydrogenase H-cluster from *Chlamydomonas reinhardtii*.

Introduction

Iron (Fe) and sulphur (S) were two of the most bioavailable elements on the ancient Earth before the presence of oxygen (O_2) in the atmosphere, with Fe and S existing in the soluble forms of Fe^{2+} and S^{2-}, respectively.[1] The ancestors of some widely-distributed Fe–S proteins, such as ferredoxins, nitrogenases, reductases and aconitase, were thus likely present in early biological systems.[2–4]

In light of the geochemical ubiquity of Fe and S, Wächtershäuser postulated an "iron–sulphur-world" theory, in which pyrite (FeS_2) formation from iron sulphide and hydrogen sulphide is proposed as the first energy source for thermophilic life.[5–7] Recently however, it was reported that ultraviolet light can drive both [2Fe2S] and [4Fe4S] cluster synthesis through the photo-oxidation of ferrous ions and the photolysis of organic thiols,[8] suggesting that high temperatures may not have been required for Fe–S enzyme biogenesis, opening a novel view into the origin of life.

Due to their versatile redox abilities, Fe–S clusters primarily function as cofactors in many enzymes. The major functions of protein-bound Fe–S clusters are electron transfer, enzyme catalysis and environmental sensing (or metabolic regulation).[9–12] The most common forms of Fe–S clusters are rhombic ([2Fe2S]), such as that found in plant-type ferredoxin[13] and cubane ([4Fe4S]), *e.g.* in aconitase.[14] More complex Fe–S clusters containing other metal ions also exist, such as nitrogenase's $[Mo_7Fe_9S]$ cluster[15] and *Desulfovibrio* hydrogenase's [NiFe] cluster.[16]

In vivo, Fe–S cluster assembly is highly regulated due to the intracellular toxicity of free iron (Fe^{2+}) and sulphides (S^{2-}),[17,18] while *in vitro*, assembly is relatively straightforward and can be achieved by simply adding inorganic Fe^{2+}, S^{2-} and a reducing equivalent.[19] In bacteria, three Fe–S cluster assembly systems have been identified and characterised: the NIF (nitrogen fixation), ISC (iron–sulphur cluster) and SUF (sulphur utilization factor) systems. The NIF system was first observed in *Azotobacter vinelandii* and appears to be dedicated to nitrogenase maturation,[20–23] while the ISC and SUF machineries assemble the Fe–S clusters of housekeeping proteins under normal or stress conditions, such as oxidation or Fe starvation, respectively.[24–26] Additionally, *Escherichia coli* has a CSD (cysteine sulfinate desulfinase) system, which contains similar components to the ISC and SUF systems but which lacks a scaffold protein.[27]

Eukaryotes also contain the ISC and SUF systems – the ISC system is located in the mitochondria and assembles mitochondrial Fe–S clusters, while the SUF system is in the chloroplast and assembles chloroplastic Fe–S clusters. An additional assembly machinery, known as the CIA (cytosolic iron–sulphur cluster assembly) system, is responsible for cytosolic and nuclear Fe–S protein maturation.[28] This pathway is essential for the synthesis and repair of DNA, protein translation and RNA modification. The activity of the CIA pathway is highly dependent on the activity of the ISC system, which transports a S-containing substrate into the cytoplasm *via* the ATP-binding

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cassette (ABC) transporters. Functional studies on *Arabidopsis thaliana* ATM3 and *Saccharomyces cerevisiae* Atm1 indicate that this substrate may be glutathione polysulphide, which contains persulphide.[29–34]

In this review, we provide an overview of the SUF pathway, detailing the general Fe–S cluster assembly process, the proteins involved, and its regulation, discussing the most recent findings. We focus on the model organisms *E. coli* and *A. thaliana* but also highlight the maturation of the *Chlamydomonas reinhardtii* [FeFe]-hydrogenase, HYDA1.

Fe–S cluster assembly process

While the ISC pathway is widely distributed across almost all domains of life, from archaea and Gram-negative bacteria to yeasts, plants and animals,[35–38] the SUF system is less widespread. For example, the SUF system is found in most Gram-positive bacteria, which generally only have this pathway for Fe–S cluster generation,[39,40] as well as in archaea, with nearly all of the available genomes containing SufBC and a few containing SufD,[25] the chloroplasts of plants and green algae and cyanobacteria, but not in yeast or animals.[25,41–44]

The ISC and SUF machineries share a similar assembly process, which proceeds as follows (Fig. 1): (1) sulphane suphur (S^0) acquisition from L-cysteine *via* a cysteine desulphurase (IscS or SufS) and Fe from a still unknown Fe donor; (2) preassembly of the Fe–S cluster on a scaffold protein (the most highly conserved are IscU and SufU); (3) Fe–S cluster release and transfer to the apo-target or onto a carrier protein where, in the case of IscU, the preassembled [2Fe2S] can be reductively coupled to form [4Fe4S], while for SufU, [4Fe4S] is possibly oxidised to [2Fe2S]; (4) the carrier-bound Fe–S cluster is transferred to the apo-target.

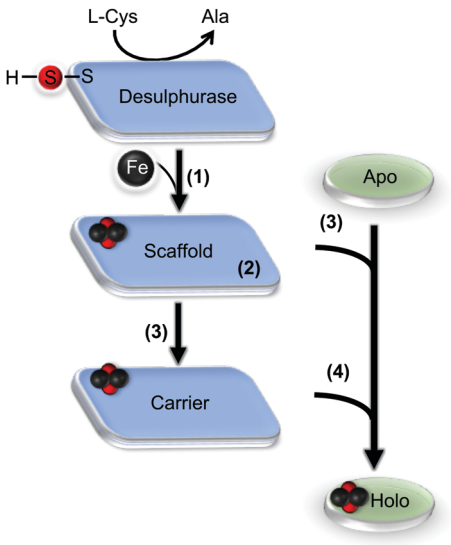

Fig. 1 General process of iron–sulphur (Fe–S) cluster assembly. (1) Sulphide is derived from a cysteine desulphurase that catalyses the removal of sulphur from L-cysteine to form a sulphane sulphur (S^0), which is bound to the active site of the enzyme as a persulphide (R–S–S^0–H) and which is transferred to the scaffold protein along with Fe. (2) The Fe–S cluster is preassembled on the scaffold protein and then (3) transferred onto the carrier protein or directly to the apo-target protein ("Apo") to form the holo-protein ("Holo"). (4) If a carrier protein is being used, the carrier-bound Fe–S cluster is transferred to the apo-protein ("Apo"). The red and black spheres represent S and Fe, respectively, while the cluster of red and black spheres represents the Fe–S cluster. This model fits most assembly pathways.

The well characterised *E. coli* and *A. thaliana* Fe–S cluster machineries have enabled the identification of orthologous components in eukaryotes whose sequenced genomes are available through BLAST similarity searches.[45] For example, by comparing the *C. reinhardtii* genome with the known Fe–S cluster assembly sequences from bacteria, yeast and *A. thaliana*, Godman *et al.* predicted a set of genes likely involved in compartmental Fe–S cluster assembly in *C. reinhardtii*.[45]

SUF proteins

The specific process of Fe–S cluster assembly *via* the SUF pathway is shown schematically in Fig. 2: (1a) the cysteine desulphurase SufS uses its pyridoxal 5′-phosphate (PLP) cofactor to mobilise S from L-cysteine, forming a persulphide intermediate and then (1b) SufE, which has been found to enhance SufS activity, receives the persulphide from SufS *via* a conserved cysteine residue; (2) the Fe–S cluster is pre-assembled on the scaffold complex $SufBC_2D$ after obtaining the S from SufE and the Fe from the Fe source; (3) the pre-assembled Fe–S cluster can then be transferred directly onto the apo-target protein or (4) to the target *via* carrier proteins. The process differs slightly for different proteins and is thus explained in more detail in the following section.

SufS is one of five characterised cysteine desulphurases (the others are NifS, IscS, CsdA and ABA3), and belongs to the NifS-like proteins, which are named after NifS, the first desulphurase identified to be required for nitrogenase activity in *A. vinelandii*.[46–48] In addition to their cysteine desulphurase activity, these NifS-like desulphurases can also catalyse the decomposition of selenocysteine to selenium (Se) and L-alanine, providing Se for the formation of selenoproteins containing SeCys.[48,49]

The desulphurisation activity of *E. coli* SufS is low alone, but it is significantly enhanced when in a complex with SufE or SufE-$SufBC_2D$.[50,51] An *in vitro* study showed that SufE binds tightly to SufS independent of the ligation of persulphide to SufS.[52] Compared with IscS, the persulphide formed on SufS is more buried.[53] Furthermore, the *E. coli* SufE crystal structure shows that the side-chain of persulphide-bound SufE Cys51 is located in a hydrophobic cavity. This likely plays a role in protecting the fragile persulphide from oxidative damage.[54] The binding of SufE and SufS stimulates conformational changes, likely leading to the exposure of the conserved residue Cys51 on SufE, which is essential for the enhancement of SufS desulphurisation activity, allowing SufE access to the SufS active sites, especially to the PLP and Cys364, enabling the transfer of S.[54,55]

When cysteine is used as a substrate, S is transferred from SufS onto SufE in the form of persulphide, with SufE forming a heterotetrameric complex with the dimeric SufS in *E. coli*.[56] This is a PLP-mediated process, in which the PLP forms an

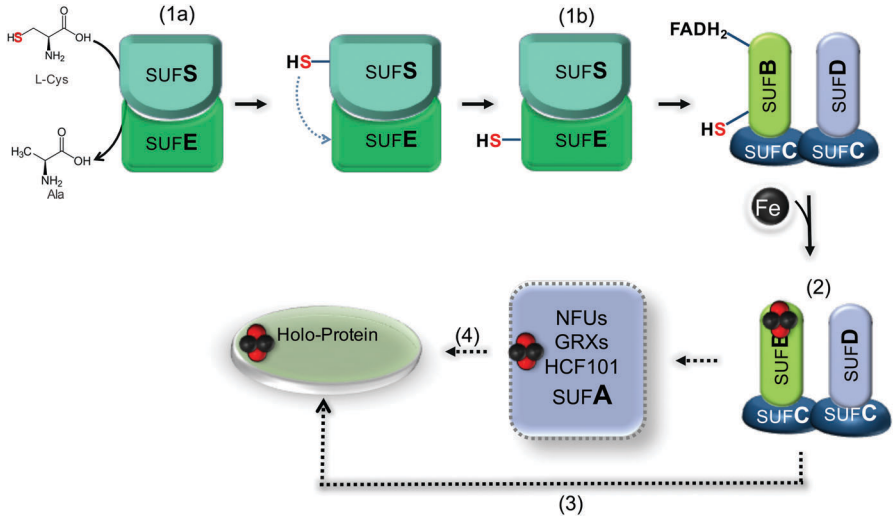

Fig. 2 Overview of the sulphur utilization factor (SUF) iron–sulphur (Fe–S) cluster assembly pathway. The complexing of the cysteine desulphurase SufS with SufE promotes S extraction and delivery in the form of sulphane sulphur (S^0) which is then transferred to SufB and reduced to S^{2-}. Powered by the activity of the SufC ATPase, Fe–S cluster (probably [4Fe4S]) synthesis occurs on the scaffold complex $SufBC_2D$. Carrier protein (Nfus, glutaredoxins (Grxs), high-chlorophyll-fluorescence protein 101 (HCF101) or SufA) transfers preassembled Fe–S cluster to the apo-protein and converts it into a holo-protein (Holo-protein). The protein maturation process depends on the target. During this process, the conformation of the preassembled Fe–S cluster can alter *via* unknown mechanisms. The cluster of red and black spheres represents the Fe–S cluster. The dotted line indicates the possible pathways of Fe–S cluster delivery.

internal aldimine with SufS Lys226, which then reacts with the L-cysteine substrate to form an external aldimine. This is followed by nucleophilic attack of SufS Cys364 on the L-cysteine thiol and S exaction from the latter to generate a persulphide on SufS.[53]

The function of SufS appears to be restricted to Fe–S cluster biosynthesis in *E. coli*. A recent study showed that overexpressing the *suf* operon in *E. coli* strains deficient in IscU, CyaY and IscS, respectively, could restore Fe–S protein synthesis. However, SufS was unable to replace IscS in its role in molybdenum cofactor biosynthesis or tRNA thiolation.[57] These results lead to the proposed function of the SUF system, which might exist to maintain basic levels of Fe–S assembly for survival under specific conditions.

In *A. thaliana*, SUFS is also a PLP dependent desulphurase and its crystal structure shows an internal aldimine is formed between the conserved Lys241 and the PLP at the resting state, like the *E. coli* SufS.[58] SUFS (cpNIFS/NFS2, PDB ID:4Q75) was identified to be necessary for maturation of all plastidic Fe–S proteins through the phenotype of *AtSUFS* RNAi silenced plant lines.[59] However, it is also possible that AtSUFS plays a role in plant selenium metabolism. Purified AtSUFS was found to display an almost 300-fold higher activity towards SeCys than Cys[60] and its overexpression in *A. thaliana* resulted in an increased tolerance to and accumulation of Se, which is toxic to plants at high levels, suggesting that AtSUFS might play a role in preventing Se toxicity in plants.[59,61] *In C. reinhardtii*, which unlike higher plants contains selenoproteins, SUFS might contribute to Se supply.[62] However, direct evidence is still required.

In higher plants, there are three isoforms of SUFE: SUFE1, SUFE2 and SUFE3, but there is only one SUFE protein in prokaryotes and algae. The *Arabidopsis SUFE1* sequence was identified by a BLAST search of the *E. coli sufE* gene against the *A. thaliana* genome.[63] In contrast to the other SUF components, SUFE1 localises to both the mitochondria and chloroplast *via* a dual N-terminal targeting sequence and stimulates the desulphurase in both organelles.[64] SUFE2 is found mainly in pollen and consists of a SUFE domain but does not seem to function in Fe–S cluster assembly. SUFE3 possesses a SUFE domain able to enhance desulphurase activity and a NadA (quinolinate synthase) domain homologous to *E. coli* NadA.[65]

A similar effect has been described for *A. thaliana* desulphurase SUFS, with all three *A. thaliana* SUFE isoforms enhancing its activity drastically.[60,63,65] In *A. thaliana*, SUFE1 Cys65 is critical for the stimulation and is most likely the S accepting site.[63]

Compared with ISC Fe–S cluster assembly on the IscU scaffold protein, cluster assembly on the SUF scaffold protein SufB is less understood. The core component, SufB, complexes with SufC and SufD to facilitate the preassembly of the Fe–S cluster. Sulphur is transferred from SufE onto the scaffold, exactly on SufB, with the participation of an unidentified Fe to form the Fe–S cluster. In *E. coli* and *A. thaliana*, biochemical and spectroscopic analyses have shown that the scaffold complex occurs mainly in the form of $SUFBC_2D$/$SufBC_2D$, which was able to further enhance SufS/SUFS desulphurase activity.[51,66–68]

It is demonstrated from the *E. coli* $SufBC_2D$ crystal structure that in this complex, two SufC subunits form a head to tail dimer upon ATP binding, which drives a structural change in SufB and SufD to expose the active site residues (Cys405 in SufB and His360 in SufD) and enable Fe–S cluster assembly.[69] SufB is the core component of Fe–S cluster assembly, SufC is an atypical ABC ATPase functioning as the power generator during

the process and SufD is the paralogue of SufB, but does not appear to function as a scaffold protein. Both SufC and SufD are required for Fe acquisition for Fe–S cluster assembly, with the absence of either resulting in diminished Fe content in the complex SufBCD.[70] *In vitro* assays showed that when alone or fused in the $SufBC_2D$ complex, SufB can bind a [4Fe4S] which can be converted to [2Fe2S] upon exposure to O_2.[71] However, Blanc *et al.* reconstituted the apo-SufB expressed from *E. coli* by adding ferric iron and sulphide anaerobically *in vitro* and obtained [2Fe2S] SufB, which is more stable than [2Fe2S] IscU, the ISC scaffold protein and possibly the resting state of SufB. This [2Fe2S] cluster on SufB could be converted to a [4Fe4S] cluster under reducing conditions when needed.[72] In contrast to prokaryotic SufB, the *A. thaliana* homologue, SUFB, which could restore the growth of a SufB-deficient *E. coli* mutant under oxidative conditions, contains the ABC signature motif and displays Fe-stimulated ATPase activity, catalysing ATP hydrolysis.[73,74] The [4Fe4S] cluster of IscU, the scaffold protein in the ISC pathway, is assembled by the coupling of two adjacent [2Fe2S] clusters.[75] However, there is no similar report of this process in SufB [4Fe4S] cluster assembly.

SufC is an ABC-ATPase, however instead of translocating substrates across membranes,[76,77] SufC initiates Fe–S cluster assembly. Upon ATP binding, SufC forms a transient dimer that elicits a conformational change of the entire $SufBC_2D$ complex and this is proposed to result in the exposure of SufB Cys405 and likely SufD His360 to the surface, enabling the formation of the nascent Fe–S cluster.[78,79] The crystal structure of *E. coli* SufC has been determined at 2.5 Å resolution, showing that despite a low sequence identity ($\leq$25%) to other ABC-ATPase members, the secondary structure and overall topologies are similar.[80] The *A. thaliana* homologue SUFC could rescue the growth of a SufC-deficient *E. coli* mutant under oxidative stress and interact with AtSUFB.[73] However, an *A. thaliana* SUFC crystal structure is still not available.

A SufD structure has also been determined, with the protein found to have a novel topology and $\sim$20% sequence similarity to SufB. The exact function of SufD is unclear, but it is proposed to be involved in Fe acquisition.[81] It is speculated that the $FADH_2$ bound to the $SufBC_2D$ complex plays a role in Fe acquisition with SufC and SufD, due to its ability to reduce Fe^{3+} to Fe^{2+}. Saini *et al.* proposed that $FADH_2$ might also help to reduce the persulphide to produce a bridging S^{2-} or drive the reductive coupling of [2Fe2S].[66,70]

Carrier proteins

The preassembled Fe–S cluster is delivered to the apo-target protein directly from the scaffold or indirectly *via* carrier proteins.[82,83] This process is not inhibited by an Fe chelator, indicating that the Fe–S cluster is intact during the delivery and that there is no transient disassembly, thereby avoiding the toxicity of free Fe and S. The most common carrier proteins are the A-type carriers (ATCs), which are generally grouped into three subfamilies: ATC-I, II and III.[84,85] *E. coli* has three ATCs: ErpA (ATC-I), SufA and IscA (both of which belong to ATC-II). Members of the ATC-III subfamily are involved in nitrogenase maturation.[85] There are also some other carrier proteins, such as Nfus, high-chlorophyll-fluorescence protein 101 (HCF101) and Grxs, which can also function as scaffold proteins *in vitro*. However, their exact roles in Fe–S cluster synthesis are still unclear. It has been reported that SufA, IscA (and its homologues), Nfus, Grxs and HCF101 are able to assemble Fe–S clusters *de novo* when supplied with proper substrates.[86–90] The co-existence of these carrier and scaffold proteins indicates their redundancy. Considering the low concentration of accessible Fe and S in cells, and the diversity of the target apo-proteins, these similarly functioning proteins may have evolved to ensure the maturation of these crucial Fe–S proteins under stress conditions.

SufA

It appears that ATC proteins may play different roles in different organisms. In *Synechococcus* sp. *PCC 7002*, the single and double mutants *sufA*, *iscA* and *sufA iscA* showed no obvious phenotype when grown photoautotrophically, whereas a *nfu* null mutant could not be created, suggesting that Nfu, but not SufA or IscA is a scaffold protein. It instead appears that SufA and IscA are involved in redox sensing and Fe homeostasis, respectively.[91] Under redox stress conditions, *suf* transcript levels were higher in the *sufA* and the *sufA iscA* mutants than in the wild type. Meanwhile, *iscA* mutant strains were found to be less chlorotic than the wild type following Fe deprivation and under Fe-replete growth, *suf* and *isc* transcript levels were significantly higher in the *iscA* mutant.[91]

In *E. coli*, SufA and IscA share a high similarity, both in structure and function.[92,93] Vinella *et al.* found that the double *iscA sufA* mutant was conditional lethal under aerobic conditions, while the single mutants only showed mild growth effects, suggesting that *E. coli* IscA and SufA are functionally redundant carriers under these conditions.[85] However, it seems that the ATC proteins cannot be completely substituted for one another. In another report, three ATC *E. coli* mutants were isolated, *iscA*, *sufA* and *erpA*, as well as an *iscU* mutant, and the enzymatic activities of the anaerobic respiratory Fe–S enzymes formate dehydrogenase N (Fdh-N) and nitrate reductase (Nar) were analysed.[94] In the *sufA* mutant, both enzymes displayed increased activities, in the *iscA* mutant, both showed reduced activities, while no activity could be detected for either enzyme in the *erpA* and *iscU* mutants. These results indicated the irreplaceable roles of different ATC proteins and that an ATC protein was unable to fulfil the functions of the scaffold protein IscU.

In another study looking at the maturation of the [NiFe]-hydrogenase, the *E. coli iscA* and *erpA* mutants did not show any hydrogen (H_2)-uptake [NiFe]-hydrogenase activity, while the *sufA* mutant was not deficient in [NiFe]-hydrogenase, suggesting that biogenesis of the small subunit's Fe–S cluster is dependent on the ISC pathway rather than the SUF pathway.[95]

Nfus

Another series of U-type proteins are the Nfus, which are presumed to be involved in the oxidative stress response, and

which contain the typical Nfu domain that shares high sequence identity to the C-terminal domain of NifU.[88] The Nfu family shares the highly conserved CXXC motif in their Nfu domain, which coordinates Fe–S clusters. Nfu domain-containing proteins can be found in a broad range of organisms, *e.g.* in yeast (Nfu1), cyanobacteria (NifU/NfuA), *E. coli* (NfuA), humans (Nfu) and plants (NFU1-5).[96–98] These proteins have been reported to be capable of transiently binding Fe–S clusters and transferring them to apo-targets *in vitro*.[99,100]

Four subfamilies have been identified for Nfu-containing proteins, with *E. coli* NfuA being the most represented example of class I. NfuA from *E. coli* contains two domains, an N-terminal 'degenerate' ATC (A-type carrier) domain (ATC*), which lacks the three coordinating cysteines, and a C-terminal Nfu domain that binds a [4Fe4S] cluster.[101] It is reported that *E. coli* NfuA is able to reconstitute the destructed lipoyl synthase (LIPA) by donating its [4Fe4S] cluster to provide sulphur for lipoyl cofactor synthesis.[102] It is reported that the NfuA from cyanobacteria *Synechocystis PCC6803*, which shares a high sequence similarity to the *A. vinelandii* carboxyl-terminal domain of NifU, is able to transfer its labile [2Fe2S] cluster to an apo-ferredoxin *in vitro*.[103] However, NfuA from another cyanobacteria *Synechococcus* sp. *PCC 7002* has been confirmed to contain [4Fe4S] clusters which could be transferred to the photosystem I (PSI) component PSA *in vitro*.[100]

A. thaliana has five Nfu-containing proteins, AtNFU1-5, with plastid-localised NFU1-3 representing the plant-specific class of NFUs, which have an N-terminal redox-active Nfu domain with the conserved CXXC motif and a C-terminal redox-inactive Nfu domain. These chloroplastic Nfu proteins are unique to plants. The mitochondrial NFU4-5 belong to Nfu class II. Except for NFU3, the other four Nfu proteins have been shown to restore the growth of a double yeast mutant Δ*isu* Δ*nfu1*, indicating their potential functions as scaffolds for Fe–S cluster assembly. It has been proven that [4Fe4S] and [2Fe2S] can be reconstituted *in vitro* on NFU2 and transferred to the corresponding apo-targets. *NFU2* knockout mutants displayed dwarf phenotypes and decreased levels of ferredoxin and photosystem I (PSI) and photosystem II (PSII).[104–106]

In contrast, the *NFU3* mutant showed drastically decreased levels of chloroplastic [2Fe2S] ferredoxin, [3Fe4S] ferredoxin-dependent glutamine oxoglutarate aminotransferases and PSII activity and significantly decreased levels of PSAA, PSAB and PSAC, resulting in a nearly absent PSI activity.[107] Recombinant NFU3 displayed features characteristic of [4Fe4S] and [3Fe4S] clusters. Further studies revealed its essential role in overall plant fitness.[107,108]

Grxs and HCF101

Other proteins involved in Fe–S cluster assembly or transfer are the Grxs and HCF101. Grxs are subdivided into four classes, with class I and II exhibiting the signature motifs CPXC and CGFS, respectively, and are present in all photosynthetic organisms. GRXS14 and GRXS16 are plastidial glutaredoxins and belong to class II. GRXS14 has been confirmed to be capable of efficiently accepting a pre-assembled [2Fe2S] cluster and transferring it to A-type proteins, *e.g. A. vinelandii* ^{Nif}IscA and AtSUFA1.[109] Additionally, it has been suggested that the Grx family plays a role in regulating Fe homeostasis and Fe–S cluster trafficking in cells. However, there is no direct evidence that reveals their involvement in SUF Fe–S cluster synthesis.[109–111]

HCF101 has been proposed to act as a scaffold protein due to its ability to assemble the [4Fe4S] cluster and transfer it to apo-proteins *in vitro*.[90,112] High-chlorophyll-fluorescence *A. thaliana* mutants were isolated and characterised. The *hcf101* mutant seedlings were deficient in PSI and the mutation was seedling lethal.[113] The chloroplastic HCF101 protein belongs to the FSC-NTPase ([4Fe4S] cluster containing P-loop NTPase) family and does not encode PSI components, indicating that the mutated gene is involved in the assembly of PSI [4Fe4S] clusters.[113,114] The PSAA subunit of PSI is suggested as the HCF101 functional target, which would explain the failure of the mutants to accumulate PSI.[115] However, as of yet there is no direct evidence to prove this.

Regulation of iron–sulphur cluster assembly

In cyanobacteria, the SufR protein coordinates two $[4Fe4S]^{2+,1+}$ clusters and acts as the main transcriptional repressor of the *sufBCDS* operon under Fe replete conditions, while *sufBCDS* transcription is de-repressed under Fe deprivation. The *sufR* gene is transcribed in the opposite direction to the *suf* operon, with a regulatory region in between. The binding of Fe–S cluster-bound SufR to the *sufBCDS* promoter represses transcription of the *suf* operon, whereas the apo-form or redox state change of SufR induces transcription, revealing its role in maintaining the physiological levels of Fe–S protein in cells in response to diverse environmental conditions, like IscR, a component of the ISC pathway.[44,116,117]

In Gram-negative bacteria, the ISC and SUF pathways co-exist in the same space, but respond to different environmental conditions. The ISC pathway takes more responsibility for the basic requirement of cell growth but is replaced by the SUF system under stress conditions. This switch is coordinated by three regulators: the ferric uptake regulator (Fur), IscR (iron sulphur cluster regulator)[118,119] and OxyR (a transcriptional activator related to redox regulation).[120] IscR plays a central role in this process. Under oxidative conditions, the IscR-bound [2Fe2S] is destroyed and its apo-form activates the SUF pathway. OxyR also senses the oxidative state of the cell and activates the SUF pathway.[84,121] Fur indirectly affects the mechanisms' interconversion by activating *ryhB* (a non-coding small RNA which regulates the expression of many genes under iron starvation conditions) transcription, which could lead to *iscSUA* mRNA degradation when Fe is limited.[122] Most Gram-positive bacteria only encode the SUF pathway, and therefore do not contain an IscR regulator, however, *Thermincola potens*, which contains both ISC and SUF, encodes an IscR involved in the regulation of Fe–S cluster biogenesis.[41]

In contrast, in plants, the SUF pathway is the sole Fe–S cluster assembly pathway present in plastids,[43] meaning that it

is separated from the ISC pathway and thus responsible for maintaining numerous essential and regulatory Fe–S proteins. Iron depletion appears to regulate the abundance of some SUF proteins in plants. In a recent study, SUFA and SUFB displayed a decreased abundance following Fe depletion, which was mirrored on the transcript level for *SUFB*. This is perhaps another protection mechanism against oxidative damage whereby in the absence of sufficient Fe, the risk of forming incomplete clusters is avoided to prevent oxidative damage.[123] To protect the Fe–S cluster from damage *e.g.* from oxidative agents, the major products in plant chloroplasts, cells have evolved a series of protective mechanisms. These include the proteins adopting conformations which result in the sensitive Fe–S clusters being buried inside the proteins, instead of remaining on the solvent-exposed surface,[124,125] as well as cells undergoing a rapid acquisition of Fe and S to repair or supplement damaged or degraded Fe–S clusters to sustain a sufficient turnover of active Fe–S proteins.

In *E. coli*, the *suf* genes are encoded in the *sufABCDSE* operon while in *A. thaliana*, these genes are distributed across different chromosomes (Table 1). The *A. thaliana* SUF system is responsible for the maturation of chloroplastic Fe–S proteins. SUFS, SUFE, SUFB, SUFC and SUFD are all essential for growth, with deficiencies in any of these proteins resulting in chlorotic phenotypes, growth defects and even lethality.[59,64,67,68,126–129] The proteins involved in Fe–S cluster assembly are highly conserved. The ISC pathway consists of at least 19 proteins, while the SUF pathway contains about 14 (Table 1). Some of the ISC proteins are homologous with those from the SUF system. For example, the ISC desulphurase NFS1 is homologous with SUFS (or NFS2). Activity and stability of yeast Nfs1 require the Isd11 protein,[130] which plays a similar role to SufE/SUFE in the SUF system in *E. coli* or *A. thaliana*. Both mechanisms rely on Nfus, A-type carriers and Grxs, all of which are cluster carrier proteins. Compared with the ISC scaffold protein IscU, cluster assembly on the *E. coli* SUF scaffold protein SufB is less understood.

Hydrogenase Fe–S cluster assembly

In addition to containing conserved Fe–S proteins, some species contain unique Fe–S proteins and assembly factors. One example is the [FeFe]-hydrogenase from the unicellular green microalga *C. reinhardtii*, HYDA1.[131]

HYDA1 is capable of reversibly catalysing the reduction of protons to H_2.[132–137] It contains a standard [4Fe4S] cluster and a unique 2Fe sub-cluster with a bridging dithiolate ligand, three CO ligands and two CN ligands. A model of the hydrogenase holo-HYDA1 structure (Fig. 3a) and its 2Fe sub-cluster assembly process (Fig. 3b) are detailed in Fig. 3.[138–141] HYDA1 is linked to the photosynthetic electron transfer chain by the ferredoxin PETF. It has a high H_2 production efficiency and is the simplest and smallest hydrogenase in nature, as it does not contain any additional Fe–S clusters.[142–144] It is also the best candidate for analysing diverse hydrogenase-related metabolisms in microalgae, as the unicellular green algae *C. reinhardtii* is a model organism with three fully sequenced genomes and a large library of molecular tools and techniques.[145–148]

The active site of *C. reinhardtii* HYDA1, also known as the H-cluster, consists of a cubane [4Fe4S] bridged to a unique 2Fe

Table 1 Components of the sulphur utilization factor (SUF) pathway from *Escherichia coli*, *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* with corresponding functions

	E. coli		*A. thaliana*		*C. reinhardtii*[a]	
Proposed function	Protein	Gene ID	Protein	Locus tag	Protein	Gene ID
Desulphurase, provides sulphur	SufS	946185	SUFS/NFS2/CPNIFS	AT1G08490	SUFS	5722498
Activator of desulphurase	SufE	946173	SUFE1 (CPSUFE1) SUFE2 (CPSUFE2) SUFE3 (CPSUFE3)	AT4G26500 AT1G67810 AT5G50210	SUFE	5716815
Fe–S cluster assembly scaffold	SufB	945753	SUFB (ATNAP1)	AT4G04770	SUFB	5726263
NTPase, Fe–S cluster assembly component, provides energy	SufC	946128	SUFC (ATNAP7)	AT3G10670	SUFC	5717947
Proposed iron source, Fe–S assembly component	SufD	944878	SUFD (ATNAP6)	AT1G32500	SUFD	5728519
Fe–S cluster assembly scaffold or carrier	SufA	949014	SUFA	AT1G10500	SUFA	5716996
NFU domain, scaffold	NfuA	947925	NFU1 NFU2 NFU3	AT4G01940 AT5G49940 AT4G25910	NFU1 NFU2 NFU3	5720786 5716443 5717130
Hypothetic scaffold			HCF101 GRXS14 (CXIP1) GRXS16 (CXIP2)	AT3G24430 AT3G54900 AT2G38270	HCF101	
Regulator	IscR	945279				

[a] Genes were identified from open reading frames which displayed similarity to known genes.[45] Specific gene and protein characterisation is needed to confirm their functions.

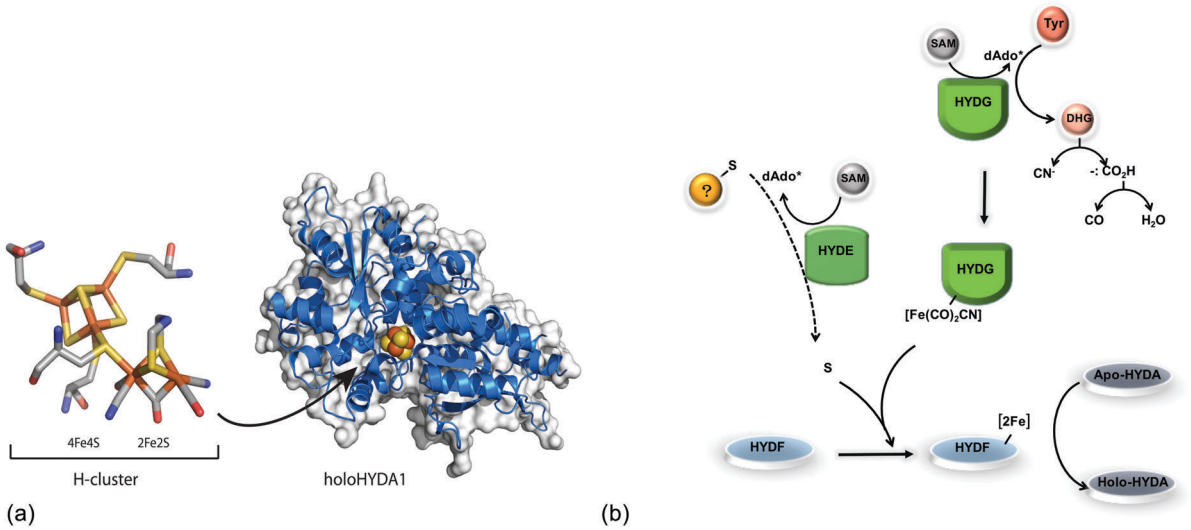

Fig. 3 (a) Model of the *Chlamydomonas reinhardtii* holo-[FeFe]-hydrogenase structure ("holoHYDA1") and its active site H-cluster. HYDA1 (PDB ID 3LX4) is composed of an active site that contains an inorganic iron–sulfur (Fe–S) cluster consisting of a [4Fe4S] cluster and a 2Fe sub-cluster. The [4Fe4S] cluster is bound to the protein by four cysteines and linked to the 2Fe sub cluster *via* one of these four cysteines. Each Fe of [2Fe2S] binds a CO and CN ligand and an additional CO links up the two Fe atoms. Colour scheme: Fe, orange; S, yellow; C, grey; O, red; N, navy. The HYDA1 structure was generated in pymol. (b) The proposed process of HYDA1 [2Fe] assembly. 2Fe sub cluster biosynthesis requires three maturases: HYDF, a GTPase that serves as a scaffold or carrier and offers energy for the process and HYDE and HYDG, both radical *S*-adenosylmethionine (SAM) enzymes. HYDG cleaves SAM and generates a 5′-deoxyadenosyl radical (dAdo*) which then cleaves tyrosine to synthesise an $[Fe(CO)_2CN]$, which is ultimately transferred to HYDF to form the 2Fe sub-cluster. HYDE is assumed to be involved in the formation of dithiomethylamine bridge, likely offering sulphur atoms. HYDF receives components from HYDE and HYDG and assembles a di-iron unit, which is then transferred to the hydrogenase for maturation.

sub-cluster *via* the cysteine thiolate. Studies in which the [FeFe]-hydrogenase was heterologously produced in *E. coli*, which is unable to produce the 2Fe sub-cluster, have shown that the [4Fe4S] cluster is incorporated first by the standard bacterial Fe–S cluster machinery (it is yet to be elucidated whether this is the SUF or ISC system),[149] followed by the 2Fe sub-cluster, which is assembled by the specific *C. reinhardtii* maturases HYDE, HYDF and HYDG heterologously co-expressed in *E. coli*.[150–153]

In *C. reinhardtii*, it appears that both the [4Fe4S] and the 2Fe sub-cluster are assembled in the chloroplast (where the enzyme is localised), as *hydA1-1 hydA2-2* mutant cells transformed with chloroplast-expressed/targeted *HYDA1* produced active HYDA1,[154,155] indicating a fully-assembled Fe–S cluster, while those in which HYDA1 was targeted to the cytoplasm produced inactive enzyme, indicating the absence of a fully-assembled Fe–S cluster. The cytoplasmic HYDA1 could be activated by [4Fe4S] reconstitution and addition of a 2Fe mimic ($[2Fe]^{MIM}$).[155] These results indicate that the SUF mechanism is most likely responsible for [4Fe4S] cluster assembly, followed by 2Fe sub-cluster binding facilitated by the maturases HYDE, HYDF and HYDG.[156–158] This result is also consistent with the report in which the cytosolic CIA pathway in *Saccharomyces cerevisiae* was unable to assemble a [4Fe4S] cluster for the heterologously expressed apo-NifH from *A. vinelandii*.[159]

In contrast to the poor knowledge on the [4Fe4S] cluster, to date, far greater insight has been gained into the assembly of the 2Fe sub-cluster *in vivo* (Fig. 3b). The absence of HYDEF or HYDG leads to inactive HYDA1 due to the lack of a 2Fe sub-cluster, indicating the essential role of these maturases in HYDA1 maturation.[156] It also shows that the conserved SUF mechanism cannot fulfil this function.

The maturases HYDE and HYDG are both radical *S*-adenosyl-l-methionine enzymes and contain the characteristic CX3CX2C motif. It is proposed that HYDE offers the sulphur for dithiomethylamine (DTMA) ligand production,[160,161] while HYDG catalyses the tyrosine cleavage and generates an $[Fe(CO)_2CN]$, providing not only CO and CN but also Fe for 2Fe sub-cluster assembly.[162–165] HYDF acts as a scaffold or carrier of the 2Fe sub-cluster to the apo-HYDA1.[166–169] Due to its central role and speciality, HYDF is of high interest. It has been reported that dimerised $HYDF^{\Delta EG}$ expressed without HYDE or HYDG coordinates an active 2Fe sub-cluster and then interacts with HYDE and HYDG. The exact process of how these maturases interact and how the 2Fe sub-cluster is assembled is still unclear.[170,171]

Proteomic studies have identified SUFS, SUFB, SUFC, SUFD, SUFA, and NFU3 peptides in the *C. reinhardtii* chloroplast,[172,173] however, experimental data is required to confirm their activity. Research on the synthesis of the HYDA1 [4Fe4S] cluster, or of the maturases HYDE, HYDF and HYDG in their native host *C. reinhardtii* will help us to further understand the hydrogenase maturation mechanism. Like in higher plants, the SUF components in *C. reinhardtii* are not included on the same chromosome.[174] Furthermore, despite genomic and proteomic data being available, the *C. reinhardtii* SUF genes and proteins are yet to be properly characterised.[172,175,176] It is also possible that additional components are involved in FeS cluster assembly, *e.g.* regulatory proteins and cofactors, therefore, future work will involve analysing these pathways in more detail.

Conclusion

As ubiquitous cofactors in nature, Fe–S clusters have been a hot topic for decades and extensive studies on the structure, function and assembly process in organisms such as *E. coli* and *A. thaliana* have led to significant insights into the process of Fe–S cluster assembly. It is widely accepted that in *E. coli* Fe–S cluster biogenesis mainly relies on the ISC pathway, while the SUF pathway is induced to compensate ISC deficiency for survival under stress conditions.[177,178] In plants, Fe–S clusters synthesised *via* the SUF pathway are incorporated into plastidial proteins, while the ISC pathway is responsible for Fe–S protein maturation in mitochondria and plays an essential role in cytosolic and nuclear Fe–S protein maturation.[29,30]

The identities and functions of most of the components of these systems have now been elucidated. New components are still being discovered and unsolved questions remain. The compartmentalisation of Fe–S assembly in eukaryotic cells allows us to study the mechanisms in parts. In the case of the SUF pathway, the Fe donor is still to be identified, with the proposed Fe donor ferritin likely functioning as the Fe scavenger.[179,180] Also, it is difficult to precisely differentiate the scaffold proteins from carrier proteins, with some of them acting in both ways *in vitro*. The question of how the SUF pathway is able to function in the chloroplast in the presence of oxygenic products generated from photosynthesis is another important point worth pursuing.

Progress in *in vitro* protein interaction allows us to start identifying these components in other organisms, *e.g.* through mutagenesis and further *in vivo* studies. For example, *C. reinhardtii* would make an excellent model system for the study of eukaryotic Fe–S cluster assembly, as many versatile tools have been developed for this organism in recent years.[181–183] As a promising biofuel source, hydrogenases are being extensively studied, however, the main focus is on 2Fe sub-cluster assembly. The predicted *C. reinhardtii* SUF homologues might further enhance our understanding of Fe–S metabolism and the hydrogenase [4Fe4S] cluster assembly mechanism.

Although there are still a number of difficulties to be faced, it is likely that these mysteries will be solved in the not so-distant future.

Conflicts of interest

The authors declare that this review was written without any potential financial conflict of interest.

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