Synthesis, Delivery and Regulation of Eukaryotic Heme and Fe-S Cluster Cofactors

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Abstract

In humans, the bulk of iron in the body (over 75%) is directed towards heme- or Fe-S cluster cofactor synthesis, and the complex, highly regulated pathways in place to accomplish biosynthesis have evolved to safely assemble and load these cofactors into apoprotein partners. In eukaryotes, heme biosynthesis is both initiated and finalized within the mitochondria, while cellular Fe-S cluster assembly is controlled by correlated pathways both within the mitochondria and within the cytosol. Iron plays a vital role in a wide array of metabolic processes and defects in iron cofactor assembly leads to human diseases. This review describes progress towards our molecular-level understanding of cellular heme and Fe-S cluster biosynthesis, focusing on the regulation and mechanistic details that are essential for understanding human disorders related to the breakdown in these essential pathways.

Keywords

Iron; Heme; Fe-S Clusters; Fe Homeostasis; Fe-Cofactor Biosynthesis

1. The Role of Iron in Biology

Iron's abundance and unique chemical characteristics are often exploited by nature to drive the complex chemistry required by cells to maintain life. Iron is the fourth most abundant element on the earth's crust[1], so its high prevalence during early evolution is certainly a factor for its current ubiquitous presence in nature. The human body contains 3 to 4 grams of the metal and absorbs 1 to 2 mg of it each day[2]. The reactivity and ability of the metal to cycle between the Fe(II), (III) and (IV) oxidation states makes it extremely useful for driving intricate reactions in biology that include substrate activation, electron transfer, and

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oxidation-reduction reactions. Because of this chemical versatility, iron plays a role in nearly every biological pathway. While its utility within biology is apparent, the Achilles heel of this essential metal is its tendency to precipitate in aqueous solutions[3]. Through coordination to biomolecules, however, the solubility of the metal can be controlled and its reactivity attenuated. At the elemental level, first coordination sphere ligands stabilize the solubility of the metal while at the same time tune its chemical properties for selective participation in only desired reactions [4]. Ligand variability in this first coordination sphere helps control how the metal behaves, however at the cellular level a complex network of proteins, controlled at the genetic level, helps ensure metal availability to specific protein partners in the cell[5].

Pathways related to eukaryotic iron homeostasis are shown in Figure 1[6]. Iron is brought into cells either via the transferrin Fe uptake pathway or through direct membrane transporter (ex. the DMT1 pathway)[7]. Once in the cell, imported iron can bind to iron regulatory proteins where it serves to control concentration, it can be stored within the ferritin complex, it can be exported, or it can be incorporated as cofactors into apoprotein partners. In humans, the bulk of iron in the body (over 75%) is directed towards heme-[2] or Fe-S cluster cofactor[8] biosynthesis. Assembly of the Fe-cofactors follows highly regulated pathways that have evolved to safely build and load these inorganic Fe-containing cofactors into the apoprotein partners while controlling metal reactivity. In eukaryotes, heme biosynthesis is initiated and completed within the mitochondria, while Fe-S cluster assembly is controlled by separate but correlated pathways within both the mitochondria and the cytosol. Iron plays a central role in a wide array of metabolic processes, so it is not surprising that defects in cofactor assembly leads to human diseases. This review details progress towards elucidating the molecular details of cellular heme and Fe-S cluster biosynthesis. A focus on both regulation and mechanism will be critical for understanding human disorders related to the breakdown in these essential pathways.

2. Heme Cofactors

2.1 Introduction

Among the many metalloporphyrins found in nature, heme is one of the most abundant. It is utilized in many vital biological processes including photosynthesis, oxygen transport, biological oxidation and reduction, and many more. Most of the total iron content in human body is incorporated into heme-containing proteins such as hemoglobin, myoglobin, catalases, peroxidases, nitric oxide synthases, and cytochromes[9]. The unique structure of heme, which consists of the iron cation coordinated to four nitrogen atoms from a tetrapyrrole ring, allows for fine tuning of the metal's reactivity to carry out the function of the biomolecule to which it is attached. Here we discuss the different heme types, their biosynthesis and regulation, their function in biology, and finally the diseases linked to a loss of heme function.

2.2 Heme Structure and Types of Heme in Nature

Modifications to the basic heme molecule allow for the diverse array of heme functions found in nature. The parent heme molecule, also known as heme b, protoheme IX or

protoporphyrin IX, serves as the platform in each case. The tetrapyrrole unit of heme consists of four pyrrole units linked by four methine bridges. A nitrogen atom from each pyrrole coordinates the iron atom in the center of the planar tetrapyrrole ring. Distortions from planarity can be critical to heme function and reactivity (examples include hemoglobin[10] and myeloperoxidase, MPO[11]). Fifth and sixth ligands to the Fe can be provided by amino acid side chains from the apoprotein or by small inorganic molecules coordinating above and below the plane of the ring. Eight positions in the tetrapyrrole carry side chain modifications, particularly methyl groups on carbons 2, 7, 12, and 18, vinyl groups on carbons 3 and 8, and propionyl groups on carbons 13 and 17 (Figure 2). Substitutions to the same carbons with various other side chains create several biologically important heme types found in nature (Figure 3). Heme A, B, and C are found in a wide spectrum of organisms and take part in vital biochemical processes such as respiration, photosynthesis and oxygen transport. The additional cofactor types (Heme D, D1, I, M, and O) are species-specific and carry out highly specialized functions. Table 1 summarizes the structural features, occurrences, and known functions of each heme type.

2.3 Heme Biosynthesis Pathway

Since heme serves as an essential cofactor to several proteins involved in central metabolic processes, all organisms have established a conserved biosynthetic pathway to synthesize the cofactor. Atomic detail is available for many of the enzymes involved in bioassembly of heme, and these enzyme structures have provided key insights into reaction mechanisms. The general assembly process consists of four stages: the synthesis of a single pyrrole, the assembly of four pyrroles to make the tetrapyrrole ring, modification of the side chains, and the insertion of iron into the ring (Figure 4)[12]. Specific details for this conserved pathway are outlined below.

2.3.1 Important Steps in Heme Biosynthesis—The first step in eukaryotic heme biosynthesis is the mitochondrial formation of 5-aminolevulinic acid (ALA), which is the precursor that serves as the only source of carbon and nitrogen atoms required to build the basic heme unit. Depending on species, one of two major pathways directs formation of this precursor leading to production of tetrapyrroles. In the Shemin pathway, the enzyme ALA synthase (ALAS) that resides on the matrix side of the inner mitochondrial membrane catalyzes the condensation of glycine and succinyl-CoA[13–16]. ALAS is found in animals, fungi, non-photosynthetic eukaryotes, and α-proteobacteria (organisms that resemble bacterial ancestors of mitochondria). Mammals have two ALAS isoforms; the first isoform (ALAS1) provides housekeeping functions and a second erythroid specific isoform (ALAS2) provides for the more robust heme requirements in erythrocytes[17]. Both forms of ALAS are homodimers and require pyridoxal 5′-phosphate (PLP) bound as a Schiff base covalent adduct to a catalytic lysine. During the joining of glycine and succinyl CoA on the PLP scaffold, both CO_2 and Coenzyme A are released as byproducts[18]. Upon formation, ALA exits the mitochondria to serve as the substrate for the subsequent four-enzymatic conversions that occur in the cytosol. Little is known about the specifics of export, but it has been suggested that SLC25A38, a member of the SLC25 family of inner mitochondrial membrane transporters, facilitates import of glycine into the mitochondria in exchange for ALA transfer across mitochondrial inner membrane[19].

In the second major pathway for ALA synthesis, also known as the C_5 pathway, glutamylt-RNA reductase (GluTR) converts glutamyl-tRNA to glutamate-1-semialdehyde (GSA) in the first of the two-step ALA production pathway[20]. The second step involves the conversion of GSA to ALA by the enzyme glutamate-1-semialdehyde-2, 1-amino mutase (GSAM)[21–23]. This second major pathway is found in plants, archaea and most bacteria.

Once exported to the cytosol, ALA serves as the building block to synthesize uroporphyrinogen III following three consecutive steps. The first step involves condensation of two ALA molecules to form porphobilinogen (PBG) by porphobilinogen synthase (PBGS), also known as ALA dehydratase (ALAD)[24,25]. PBGS is a homooctamer in which each dimer contains one catalytic site[26]. Each active site binds an ALA molecule at two distinct sites and each subunit binds one zinc atom[27]. Of the eight Zn atoms on PBGS, four zinc atoms stabilize the enzyme structure while the other four engage in catalytic activity[28,29]. Four molecules of porphobilinogen undergo polymerization to form 1-hydroxymethylbilane in a reaction catalyzed by porphobilinogen deaminase (PBGD). PBGD uses a covalently attached dipyrromethane cofactor (made of two linked PBG molecules) to prime the polymerization of four PBG molecules. Six PBG molecules form a linear hexapyrrole covalently bound to PBGD, which is then cleaved to yield 1-hydroxymethylbilane and the protein bound cofactor[30]. This unstable tetrapyrrole serves as the substrate for uroporphyrinogen synthase (UROS), the enzyme that synthesizes uroporphyrinogen III. UROS functions as a monomer and completes ring inversion and the subsequent closure of the tetrapyrrole, yielding uroporphyrinogen III[31,32]. Spontaneous ring closure is also possible, but the resulting product uroporphyrinogen I cannot be converted to heme. Uroporphyrinogen III also serves as the branching point for the synthesis of chlorophylls and corrins. In heme biosynthesis, this cyclic intermediate is converted to coproporphyrinogen III, a product that lacks four acetic side chains. Subsequent decarboxylation steps are carried out by uroporphyrinogen decarboxylase (UROD) within the cytosol to yield four methyl groups in place of the acetic side chains[33]. UROD is a homodimer with each subunit carrying an active site cleft that faces the other in the dimer interface[34].

Three enzymes associated with the mitochondrial inner membrane complete the terminal steps in the eukaryotic heme biosynthesis. Their arrangement suggests they act as a multiprotein complex that facilitates substrate channeling[35]. Coproporphyrinogen III entry into the mitochondria is likely mediated by the peripheral-type benzodiazepine receptor (PBR), located on the outer mitochondrial membrane[36]. In the first of the three terminal steps, coproporphyrinogen III undergoes oxidative decarboxylation of its propionyl side chains on two pyrrole rings to form protoporphyrinogen IX. This reaction is completed in higher eukaryotes and in a few bacterial species by the oxygen-dependent coproporphyrinogen III oxidase (CPO), an enzyme located in mitochondrial intermembrane space[37]. CPO requires molecular oxygen as the terminal electron acceptor[38].

In the next step, protoporphyrinogen IX is oxidized to protoporphyrin IX, following the removal of six hydrogen atoms from the porphyrinogen ring, to provide an alternating double bond structure to the macrocycle; this reaction is catalyzed by protoporphyrinogen IX oxidase (PPO)[39]. In eukaryotes, the oxygen-dependent PPO is a homodimer that utilizes

molecular oxygen as the terminal electron acceptor and functions as an integral membrane protein located in the inner mitochondrial membrane[40]. The active site of the protein faces the intermembrane space and uses a FAD cofactor for electron transfer. The complete reaction uses three O_2 molecules that are reduced to three H_2O_2 molecules.

Ferrochelatase (FC) is responsible for the insertion of ferrous iron into protoporphyrin IX to form Fe-protoporphyrin IX or heme, in the next step of heme biosynthesis. FC is an inner mitochondrial membrane protein with its catalytic site located on the mitochondrial matrix side. Channeling of protoporphyrin IX from PPO to FC may occur via direct interaction between the two proteins that allows efficient substrate transfer[40]. FC functions as a homodimer and each monomer contains a 2Fe-2S cluster whose function is unknown[41,42]. The reaction mechanism involves distortion of the planar porphyrin molecule into a saddle conformation to facilitate the insertion of ferrous iron[43]. Once synthesized, heme can undergo side chain modifications (ex: heme A and heme O synthesis) or covalent attachments (ex: heme C in cytochrome c biogenesis) to produce additional heme types depending on cellular needs.

2.3.2 Regulation of Heme Biosynthesis—In humans, heme is synthesized at two locations, in erythroid progenitors within bone marrow to provide for developing red cells, and in the liver to provide for numerous heme-containing enzymes. Liver responds to various metabolic states in the body, hence heme synthesis in liver is regulated accordingly. Erythroid progenitors on the other hand maintain heme production at a steady pace to meet the demand of red blood cells. Mechanisms for regulation of heme synthesis in these two origins therefore differ.

The first step, catalyzed by two different isoforms of ALAS in liver (ALAS1) and bone marrow (ALAS2), is the rate-limiting step of heme synthesis. Both isoforms are post-transcriptionally regulated by two different mechanisms. Heme has a negative feedback effect on ALAS1 transcripts and two alternate splice forms of ALAS1 facilitate this regulatory mechanism. One splice form is subjected to heme-mediated decay[44–46] but the other is resistant to this effect and requires translation in order to be regulated by heme-mediated decay[47,48]. Heme itself blocks translocation of a precursor form of ALAS1 from cytoplasm to mitochondria contributing to its downregulation[49]. Transcription of ALAS1 is upregulated by the peroxisome proliferator activated coactivator 1α (PGC-1α) in correlation to cellular glucose levels[50]. In contrast, erythroid-specific transcriptional factors such as GATA1 regulate ALAS2 transcription by binding to the promoter region[51]. In translational regulation of ALAS2, iron responsive elements (IRE) in ALAS2 transcripts can bind iron regulatory proteins (IRP). This prevents ALAS2 mRNA translation, allowing heme synthesis in differentiating red cells to be regulated in relation to cellular iron availability and mitochondrial function[52,53].

In addition, three enzymes within the heme biosynthesis pathway (PBGS, PBGD, and UROS) are transcriptionally regulated, yet they utilize a dual promoter system that allows erythroid specific or non-erythroid specific regulation of a single gene. Alternative splicing of 10 exons creates two different PBGS transcripts, either with a housekeeping promoter or with an erythroid–specific promoter that binds erythroid-specific transcription factors

including GATA1[54]. Both PBGS transcripts encode identical proteins because they share the same translational start site despite their difference in lengths. On the contrary, alternative splicing of transcripts of the PBGD gene produces proteins of different lengths[55,56]. Erythroid-specific promoters of PBGD contain several specific cis-acting sequences (including GATA1, NF-E2 and CACCC motifs) that are not seen in the housekeeping promoter[57]. Similarly, alternative splicing of UROS gene creates two transcripts. Erythroid-specific promoters of UROS contain eight GATA binding sites while the non-erythroid promoter contains NF1, AP1, Oct1, Sp1 and NRF2 binding sites. Both transcripts produce identical proteins[58]. The remaining enzymes within the pathway have single promoters; regardless, they exhibit erythroid and non-erythroid expression differences. The UROD gene carries a promoter of non-erythroid origin but UROD levels in erythroid tissue are significantly elevated compared to ubiquitous tissue and the specific mechanism resulting in this upregulation has yet to be determined[59]. The human CPO gene contains a single active promoter with six Sp1 sites, four GATA binding sites and a novel regulatory element named CPRE that aids in the elevated expression in erythroid tissue[60]. The PPO gene contains a GATA1 binding site in its single promoter, suggesting potential erythroid-specific regulation[36]. Cis-elements like NF-E2, GATA1 and the Sp1 binding sites are present in the human FC gene promoter and they have been found to induce FC expression during erythroid differentiation while the GC box maintains housekeeping expression of the gene[61]. Expression of ferrochelatase is also regulated by iron availability of the cells, related to the Fe-S cluster of FC [62].

It is evident that the heme biogenesis enzymes in the erythroid pathway are transcriptionally induced by erythroid-specific transcription factors in coordination with iron uptake. Liver on the other hand maintains sufficient heme levels by combining synthesis and degradation in response to changes in cellular heme pools. Both systems are important however for maintaining cellular iron homeostasis and they are therefore areas of interest for understanding heme related diseases.

2.4 Incorporation of Heme into Apoproteins Recipients

Although diverse in their function, heme-containing proteins share a structurally conserved element, the heme cofactor. Primary factors contributing to the functional diversity of heme proteins are the protein ligands coordinating Fe in the proximal/distal positions and the covalent linkage of the heme to the biomolecule. Insertion and stabilization of the heme unit within a heme containing protein has been studied extensively within cytochrome c. Heme C forms two thioether bonds between C3 and C8 vinyl groups of heme and the cysteine residues of CXXCH motifs in the apocytochrome c. Three systems driving this association have been studied extensively (reviewed in[63,64]). System I/CCM (Cytochrome C Maturation) is most prevalent in α and γ proteobacteria, all plant mitochondria, some protozoal mitochondria and red algae, and typically involves nine assembly proteins including CcmA through CcmH[65]. Although system II/CCS (Cytochrome C Synthesis) was originally studied in green algae *Chlamydomonas reinhardtii*, it can also be found in chloroplasts, most Gram-positive bacteria, cyanobacteria and some β, δ, and ε proteobacteria. Several of the components in system II vary in different organisms but the major components include CcsA, CcsB and CcsX. System III/CCHL (Cytochrome C Heme

Lyase) is mostly restricted to fungal, vertebrate and invertebrate mitochondria and some protozoal mitochondria. This system employs a cytochrome c heme lyase enzyme, which is now known as holocytochrome c synthase (HCCS)[66] to convert apocytochrome c to holocytochrome c. These three systems carry out a similar function: to keep both Fe in heme and sulfur in cysteine residues of the apoproteins in the reduced state to facilitate correct covalent attachment. Mechanisms of incorporation of additional heme types 12 into different heme binding proteins can be quite diverse and species specific: hence their discussion was eliminated from this review but is discussed in additional sources[67–70].

2.5 Heme Function

Heme proteins are ubiquitous in nature and perform a wide variety of functions. One abundant class of heme proteins is the photosynthetic and respiratory cytochromes. Other classes include globins, catalases, peroxidases, cytochrome P450s, oxygenases and others. Here we discuss the versatile chemistry of the heme unit in several categories of heme proteins.

Peroxidases use H_2O_2 to oxidize substrates without oxygen transfer. Their catalytic cycle includes three steps: 1) H_2O_2 oxidizes Fe^{3+} and porphyrin to generate a Fe(IV)oxo porphyrin π cation radical with water as a product; 2) oxidation of substrate reduces the Fe(IV)oxo porphyrin π cation radical; and 3) a second substrate reduces Fe^{4+} to Fe^{3+} [71]. Examples from mammalian peroxidases include myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO)[72], and these enzymes can oxidize a wide variety of substrates due to their high reduction potentials. The covalent vinyl sulfonium heme linkage in heme M of MPO enables heme distortion and the resulting reduction in electron density in the heme best explains the unusually high reduction potential of MPO[73]. These peroxidases are capable of generating oxidants such as hypohalous acids, hypothiocyanous acid, reactive nitrogen species, singlet oxygen, phenoxyl and hydroxyl radicals, all which are key components in antimicrobial properties exerted by the immune system[72].

Cytochromes P450 represent another subgroup of heme proteins in high abundance in nature; humans alone carry more than fifty P450 enzymes. Despite the immense diversity of P450s in nature, as per published structures, the overall fold in these biomolecules is basically the same. The typical electron transfer catalytic cycle of a P450 requires electrons derived from redox protein partners such as flavin and Fe-S containing proteins. Substrate binding shifts low-spin hexacoordinate heme to high-spin heme while displacing the axial water ligand, which allows electron transfer from the redox partner and oxygen binding to heme to form the oxy complex. A second electron transfer step is responsible for generating the heme Fe(III)dihydroperoxy species which finally undergoes heterolytic cleavage to give heme Fe(IV)oxo species[71]. Fe(IV)oxo porphyrin π cation radicals have been proposed as reactive species in catalytic oxygenation reactions. Although both peroxidases and cytochromes P450 form Fe(IV)oxo intermediates during their catalytic cycles, profound differences in protein environments create different products. Cytochromes P450 are mostly known for xenobiotics detoxification in liver, where drugs and other xenobiotics are hydroxylated and made more soluble, facilitating their conversion to easily eliminated

products. In addition, P450s also participate in the biosynthesis of steroids, highlighting their importance in metabolism.

Additional noteworthy heme enzymes include nitric oxide synthase (NOS) and heme oxygenase (HO). NOS catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO), a signaling molecule important for regulation of the cardiovascular and nervous systems as well as participating in immune responses. NOS catalysis is similar to P450 mechanism in the first step in which L-arginine is converted to N^{ω}-L-hydroxyarginine except that an unusual cofactor, tetrahydrobiopterin or BH4 is required. Oxidation of N^{ω}-L-hydroxyarginine to L-citrulline in the second step is proposed to be carried out via peroxy or superperoxy species as opposed to the Fe(IV)oxo intermediates discussed before. Heme oxygenase is responsible for the degradation of free heme, resulting in its efficient elimination and the recycling of iron. This conversion is carried out in three distinct steps; heme is first converted to α–*meso*-hydroxyheme, which in turn gets converted to verdoheme and subsequently to billiverdin. Studies show it is highly unlikely for an Fe(IV)oxo species to serve as an oxidant in the catalytic action of heme oxygenase since it uses a histidine as an axial ligand[71].

The complete list of heme proteins is extensive and exceeds the limits of this review. Instead, we will focus on human disorders that result from loss of heme protein function, many of which are severe, and merit detailed discussion.

2.6 Diseases of Heme Synthesis

Several human diseases are associated with disruption of the heme biosynthetic pathway. Many of these diseases are associated with inherited mutations in heme biosynthesis genes, however some are caused by environmental factors affecting their enzyme products. Along with nine major porphyrias, we will focus on diseases associated with disruption of the heme biosynthesis pathway.

2.6.1 Porphyrias—The clinical presentation of porphyria includes skin lesions and acute neurovisceral attacks which are related to the accumulation of specific intermediates in the heme biosynthetic pathway. Nine such diseases have been identified and they are categorized as hepatic or erythropoietic, pertaining to the organ in which the intermediates accumulate. However, a more clinical classification of porphyria divides these into three groups: acute, cutaneous and rare recessive.

Even though no disease causing mutations for ALAS1 has ever been found in humans, many mutations affecting the function of the ALAS2 isoform have been identified. Although not commonly seen as loss of function mutations in the heme biosynthesis pathway, gain of function deletions in ALAS2 are found to be causative of a cutaneous porphyria called *X-linked dominant erythropoietic protoporphyria (XLDPP)*. This disease is characterized by increased ALAS2 activity and excessive protoporphyrin production. ALA production is increased such that the final step catalyzed by ferrochelatase is rate-limiting, resulting in the accumulation of protoporphyrin. Liver damage and photosensitivity are the clinical manifestations of this disease[74].

Deficiency of ALAD causes a rare recessive porphyria named *ALAD porphyria,* for which only less than ten cases have been reported. In this autosomal disorder, loss of ALAD activity in liver and erythroid precursors leads to excretion of ALA and coproporphyrinogen III into the urine. Patients suffer from intermittent acute neurovisceral attacks and/or chronic neuropathy, and onset of this disorder ranges from childhood to adulthood[75].

An additional disease related to the first steps in heme biosynthesis is *acute intermittent porphyria (AIP).* As in all acute porphyrias, acute life-threatening complications occur mostly in the adulthood, however in rare cases severe attacks can occur in the childhood. Urinary excretion of ALA and porphobilinogen is increased due to decreased PBGD activity. Activity loss is caused by mutations in the PBGD gene and over 200 mutations have been identified. This disorder is seen in homozygous dominants for the trait and a majority of heterozygotes remain disease-free[76].

Congenital erythropoietic porphyria (CEP) is another rare recessive type of porphyria caused by the deficiency of UROS. This autosomal recessive disease that is a result of loss of UROS function leads to the spontaneous formation of uroporphyrinogen I, the isomer of uroporphyrinogen III that cannot be converted to heme, so it is accumulated and excreted[77]. Clinical features of this disease include chronic hemolysis and cutaneous photosensitivity caused by diffusion of uroporphyrinogen I to plasma, and these conditions begin to manifest in early infancy. There have been over twenty UROS mutations that cause this disease identified to date[78].

Porphyria cutanea tarda (PCT) is the most common porphyria and it falls under the category of cutaneous porphyrias. This disease only shows symptoms in the skin, including lesions in areas exposed to sun, skin fragility leading to secondary infections, and hypertrichosis. Ocular pain and photophobia have also been reported rarely[79]. The cause of these symptoms is accumulation of excessive porphyrin in the skin. Tetrapyrroles are highly photoreactive and hence they absorb energy from the visible region of electromagnetic spectrum. Excited ring structures reach ground state by transferring energy, which drives peroxidation and oxidation of biological macromolecules such as membrane lipids, nucleic acids and proteins. Familial PCT, one of the two forms of PCT, is an autosomal dominant trait similar to AIP in that only a minority of heterozygotes is affected. In familial PCT, uroporphyrinogen III is accumulated and excreted in urine due to UROD mutations. Patients with sporadic PCT, although not associated with UROD mutations, show decreased UROD activity and hepatic Fe overload, similar to that seen in familial PCT[80].

Hepatoerythropoietic porphyria (HEP) is a rare porphyria associated with UROD deficiency. Symptoms are similar to that seen in PCT (skin lesions, red urine, hypertrichosis and scarring) however they can be much more severe. This rare recessive porphyria often onsets in infancy or childhood and shows high porphyrin concentrations in erythrocytes and less than 10% UROD activity[81].

Hereditary coproporphyria (HCP), another acute porphyria, is caused by mutations that destabilize the enzyme CPO. Clinical characteristics are very similar to other acute porphyrias (i.e., AIP) and show a high coproporphyrinogen III content in urine and feces, as well as increased photosensitivity. Also similar to other acute porphyrias, HCP is mostly

seen in dominant homozygotes for the trait, with only some heterozygotes developing the disease[82].

An additional porphyria caused by mutations in a heme biosynthesis enzyme includes *variegate porphyria (VP)*, characterized by dysfunctional PPO. Symptoms are again very similar to any acute porphyria, showing acute neurovisceral attacks and cutaneous photosensitivity. Onset of the symptoms is in adulthood and over 100 PPO gene mutations (including nonsense, missense, deletion, insertion and splice mutations) have been identified[83]. As in AIP, urinary excretion of ALA and porphobilinogen and fecal content of protoporphyrinogen IX and coproporphyrinogen III is increased during acute attacks. In addition, PPO activity is dramatically reduced (50%) in tissues of VP patients[84].

The last of the nine porphyrias associated with heme synthesis is another cutaneous porphyria named *erythropoietic protoporphyria (EPP)*. Mutations in the ferrochelatase gene cause a deficiency in mitochondria leading to accumulation of free protoporphyrin IX, primarily in erythrocytes. Excess protoporphyrin IX is behind the predominant clinical feature of photosensitivity, which begins in childhood[85,86].

2.6.2 Additional Diseases—Loss of function mutations of ALAS2 is the cause of the disorder *X-linked sideroblastic anemia (XLSA)*. A group of point mutations alter the protein's ability to bind the PLP cofactor, however other mutations affect protein domains outside the cofactor-binding region. The decrease in heme synthesis efficiency prevents normal erythroblast development in the bone marrow. The resulting overload of iron results in characteristic iron granules surrounding the nucleus (a sideroblast). This disease is characterized by microcytic hyperchromic anemia, the presence of mature but pale and smaller than normal erythrocytes and Fe overloaded mitochondria in erythroblasts in the bone marrow[87].

ALAD requires Zn for function, and Zn deficiency makes ALAD susceptible to Pb inhibition. Pb replaces Zn in ALAD and the inhibited protein leads to high levels of ALA in blood, which is responsible for neurological manifestations due to its toxicity at high concentrations[88]. A similar disease, named *hepatorenal tyrosinemia,* shows neurotoxicity due to high ALA levels, with abnormal liver and kidney function. This disease is caused by mutations in fumarylacetoacetate hydrolase gene, which hydrolyzes fumarylacetoacetate to fumarate and acetoacetate during the tyrosine catabolism pathway. In the event of enzyme deficiency, fumarylacetoacetate is metabolized to succinylacetone, which is structurally similar to ALA, hence it acts as a potent inhibitor for ALAD[89]. Excess ALA and succinylacetone can be seen in patients' urine and blood samples.

3 Fe-S Clusters

3.1 Introduction

Iron-sulfur (Fe-S) clusters are the second major form of complex iron cofactors found in biology. Due to the high abundance of iron and sulfur on the earth's surface, and the easy association of these atoms under anaerobic conditions, Fe-S clusters likely developed early in evolution before the earth's transition to an aerobic atmosphere. Consequently, these

cofactors are ubiquitous in all organisms and play a role in almost every biological pathway. Here we provide an overview of the structure, formation, and function of Fe-S cluster cofactors.

3.2 Fe-S Cluster Structure

In many ways, Fe-S clusters are simpler than heme. While heme is a mixture of organic (protoporphyrin) and inorganic (iron) components, Fe-S clusters are strictly inorganic. Iron atoms in biological Fe-S clusters interact directly with protein residues and sulfur atoms bridge neighboring iron atoms. Fe-S clusters exist in a variety of configurations depending on their respective number of iron and sulfur atoms. The three most common forms (2Fe-2S, 3Fe-4S and 4Fe-4S) are illustrated in Figure 5. More complex Fe-S clusters have also been observed, including the 7Fe-8S and 8Fe-8S clusters identified in ferredoxins from *Desulfovibrio africanus*[90].

The Fe atoms within the Fe-S cluster can exist in either ferric or ferrous forms and cycling between these redox states allows the transfer of electrons for redox reactions. The tendency of the oxidized FeS cluster to gain an electron is termed the "reduction potential". By convention, this potential is expressed in comparison to a reference standard hydrogen electrode, which is assigned a potential of 0V. Depending on Fe-S cluster type, interactions with neighboring amino acids, and solvent accessibility, a single Fe-S cluster can up to two electrons with a reduction potential spanning ~1000 mV[91]. This remarkable range of accessible reduction potentials can largely explain the biological utility of the FeS cluster.

Fe-S clusters do not exist freely but are intimately connected to their apoprotein partner. Free iron will form an insoluble complex when bound to sulfide, so the protein plays a critical role in solubilizing the Fe-S unit. Fe-S proteins usually bind their corresponding Fe-S cofactor via ionic interactions between cysteine thiols and iron in the Fe-S cofactor. In some cases, Fe-S clusters are alternatively ligated via histidine residues. Subsets of 2Fe2S clusters, such as those found in Rieske proteins (see section 3.5), are coordinated by two cysteine and two histidines (Cys_2His_2)[92] and a common coordination theme of proteins involved in Fe-S cluster biogenesis is Cys_3His_1 coordination[93].

3.3 General Fe-S Cluster Biogenesis Pathways

Production of Fe-S clusters must be highly regulated to prevent unwanted reactions of both free iron and sulfur. Similar to heme-proteins, Fe-S proteins are synthesized in their *apo*-state and obtain their Fe-S cluster cofactor from a dedicated Fe-S cluster formation pathway. At present, there are three known general pathways for Fe-S cluster formation: the iron sulfur cluster (ISC), cytosolic iron sulfur assembly (CIA), and sulfur assimilation (SUF) pathways. These three pathways provide Fe-S clusters for the majority of Fe-S proteins in almost all organisms. While dedicated Fe-S cluster forming pathways can exist for individual Fe-S proteins, such as the nitrogen fixation (Nif) pathway that provides an Fe-S cluster for the nitrogenase enzyme in nitrogen-fixing bacteria[94], this review will focus only on the general Fe-S cluster production pathways.

3.3.1 Iron Sulfur Cluster (ISC) Pathway—The most robust and best-characterized pathway for Fe-S cluster biosynthesis is the iron sulfur cluster (ISC) pathway. A simplified description of this pathway is provided in Figure 6. The ISC pathway, present in bacteria and in the mitochondria of eukaryotes, provides general housekeeping Fe-S clusters to a large number of Fe-S proteins. In eukaryotes, this pathway provides Fe-S clusters for several key mitochondrial Fe-S proteins. ISC was initially identified in the *Azotobacter vinelandii* and *Escherichia coli* bacterial species, where ISC genes are arranged in the *isc* operon. Additional early work in eukaryotes (in *Saccharomyces cerevisiae* and human proteins) revealed a highly homologous system localized to the mitochondria. In addition to providing for mitochondrial Fe-S proteins, the ISC system provides a component to the cytosolic and nuclear Fe-S cluster formation pathways, thus ISC is essential for the maturation of all cellular Fe-S proteins in eukaryotes[95,96].

ISC serves as a template for understanding general Fe-S cluster production. In human ISC, *de novo* 2Fe-2S synthesis occurs on the dedicated scaffold protein ISCU[97]. Sulfur for this reaction is provided by ISC's dedicated cysteine desulfurase enzyme ISCS via a persulfide intermediate that gets transferred to ISCU[98] upon formation of an ISCU-ISCS complex. In eukaryotes, ISCS has a dedicated protein co-factor ISD11 that is essential for ISCS function[99]. Electrons for ISCS persulfide release are provided by the 2Fe-2S cluster containing ferredoxin FDX, which in turn gets reduced by the ferredoxin reductase FDXR that uses NADPH as its final electron source[100,101]. FDX also interacts with ISCU, providing 2 reducing equivalents for assimilation of two 2Fe-2S clusters on an ISCU dimer to form a single 4Fe-4S cluster[97]. An additional Fe-binding protein Frataxin interacts with the ISCS-ISCU complex and regulates ISCS activity[102]. Additionally, there are alternate scaffold proteins (ISCA[103] and NFU1[104]) that interact with ISC proteins and these are believed to be required for the maturation of a specific subset of Fe-S proteins.

Despite intense study, the physiologic source of iron for ISC remains a subject of debate. Several potential iron donors have been investigated and iron delivery to ISCU has been demonstrated from several potential sources *in vitro* within a variety of systems. The protein frataxin[105,106] interacts with the ISCU-ISCS complex and could be the source of iron for the pathway[106]. In the bacterial system, two additional members of the *isc* operon have been investigated as potential iron donors, IscX and IscA. The small acidic protein IscX binds iron and regulates cysteine desulfurase activity in a manner very similar to Frataxin[107]. The alternative scaffold IscA is also an interesting candidate because of its tight ($K_D{\sim}10^{19}$) binding affinity for mononuclear iron[108] and its capability of delivering iron to IscU[109,110]. An additional interesting hypothesis is that iron may come from a glutathione-glutaredoxin complex[111]. The lack of conclusive evidence for a specific iron source suggests that *in vivo*, there could be multiple iron sources or that the mode of iron delivery may be atypical.

A detailed mechanism for Fe-S cluster delivery from ISCU to downstream targets is currently under investigation. The Fe-S cluster bound to ISCU is transferred to the glutaredoxin GLRX5[112,113]. Efficient transfer from ISCU to GLRX5 requires involvement of the ATPase SSQ1, which binds to both ISCU and GLRX5[114]. Binding of SSQ1 to ISCU, along with the interaction of a DnaJ-like co-chaperone JAC1[115], destabilizes the Fe-S cluster on ISCU facilitating its transfer from ISCU to GLRX5[116]. GLRX5 is considered the

end of the ISC pathway because it is the last common Fe-S cluster carrier for all mitochondrial Fe-S proteins. GLRX5 continues the cluster transfer process, however, and it is able to interact with a variety of downstream Fe-S proteins[117–119]. The specific recipient depends on the ultimate destination of the Fe-S cluster. For example, 4Fe-4S cluster conversion and delivery is facilitated by GLRX5's interaction with two other Fe-S proteins, ISCA and IBA57[120].

Despite being mostly localized to mitochondria[121], ISC is required for maturation of all cellular Fe-S proteins[96]. The mechanism by which cytosolic Fe-S proteins depend on mitochondrial ISC is actively being investigated. Because Fe-S clusters are not able to cross the inner mitochondrial membrane[122], this mechanism likely involves the transport of an Fe-S cluster precursor out of the mitochondria and into the cytosol. Recent work has identified an unknown compound produced by mitochondrial ISCS (named 'X-S') that may provide reduced sulfur for cytosolic Fe-S cluster formation[123].

3.3.2 Cytosolic Iron-Sulfur Assembly (CIA) Pathway—Recent studies have revealed the involvement of another essential and highly conserved Fe-S biosynthetic pathway that is present within the cytosol and nucleus of eukaryotes. This pathway is called cytosolic iron-sulfur assembly (CIA)[124]. This pathway has been identified in many eukaryotic systems and is essential in almost all cases[125]. CIA is unique among Fe-S maturation pathways in that it does not obtain reduced sulfur via a dedicated cysteine desulfurase. A simplified description of the CIA pathway is provided in Figure 6. Instead of an ISCS analog, CIA relies on mitochondrial export of a sulfur-containing compound, 'X-S', via the mitochondrial export protein ABCB7[126] and the intermembrane space protein ALR[127]. The identity of X-S is currently unknown, but may be glutathione-complexed to an Fe-S cluster[125]. In human CIA, the primary scaffold for *de novo* Fe-S assembly is a tetrameric complex formed between CFD1 and NBP35, which binds a bridging 4Fe-4S cluster between the CFD1 and NBP35 subunits[128]. Reducing equivalents for this reaction are provided by an Fe-S containing protein CIAPIN1 (similar to FDX in ISC), which in turn gets reduced by the diflavin reductase NDOR1 (similar to FDXR in ISC), utilizing reducing equivalents from NADPH[129,130].

The 4Fe-4S clusters from the NBP35-CFD1 complex get transferred to another Fe-S protein IOP1, which binds two 4Fe-4S clusters per monomer[131,132]. IOP1, in turn, delivers its Fe-S clusters to a multi-component complex called the CIA targeting complex, which consists of at least three proteins CIA1, CIA2B, and MMS19[133]. The CIA targeting complex is able to interact with a variety of recipient Fe-S proteins, likely an indication of its function in downstream Fe-S cluster delivery.

3.3.3 Sulfur Assimilation (SUF) Pathway—Of the three Fe-S general cluster formation pathways, the sulfur assimilation (SUF) pathway is probably the most ancient. SUF predominantly exists in prokaryotes, however it is found in specific locations in eukarya, including the chloroplasts in some plants[134] and the apicoplasts in some plasmodium species[135], and recently proteins homologous to bacterial SUF were discovered in the cytosol of a blastocystis species[136]. At present, SUF has been best characterized in the

Gram-negative bacteria *Escherichia coli* and *Erwynia chrysanthemi* where its genes are organized into the *suf* operon (Figure 7).

The SUF pathway is similar to ISC in many ways. Like in ISC, SUF provides general Fe-S cluster formation to accommodate a variety of Fe-S proteins. In fact, SUF and ISC seem to be redundant in Gram-negative bacteria, as the removal of the entire *isc* or *suf* operon results in no deleterious effects. Simultaneous *suf/isc* operon deletion, however, is lethal[137]. While ISC and SUF follow the same general mechanism for Fe-S cluster formation (Figure 6), SUF seems to be favored under conditions of oxidative stress and iron limitation[138] and the SUF proteins are correspondingly more stable under adverse conditions *in vitro*[139]. In *E. coli*, the SUF pathway centers around two heteromeric complexes called SufBC and SufSE.

The primary scaffold SufB requires a binding partner SufC for activity, forming the SufBC complex in a $SufB_2C_2$ arrangement. The SufBC complex can form a 4Fe-4S cluster on SufB that can be transferred to recipient proteins[140]. The exact role of SufC in this process is unknown, but it has ATPase activity that is essential for Fe-S cluster formation on SufB[141]. SufB, on its own is relatively unstable and prone to spontaneous oligomerization. There is also a paralogue of SufB, named SufD that is able to replace a SufB in the SufBC complex, resulting in the SufBCD complex[142]. However, SufBC is likely the most active form[143]. The SUF cysteine desulfurase SufS functions in a similar manner to ISCS, accepting sulfur from cysteine via a persulfide intermediate. SufS has an essential binding partner SufE, which is required for activity, forming the SufSE complex[144]. While it may seem SufE is similar to ISD11 from eukaryotic ISC, SufE functions differently from ISD11 in that it accepts the persulfide from SufS and allows the SufS enzyme to complete its turnover[145].

Details of SUF's downstream cluster delivery are not as well established as in the ISC pathway. The 4Fe-4S cluster formed by SufBC can be transferred to the A-type carrier protein SufA *in vitro*[140,143], but SufBC also may be able to transfer directly to recipient apo-proteins. *In vivo*, SufA is functionally redundant with the ISCA bacterial homologue[146] and possibly acts as an intermediate carrier of the 4Fe-4S cluster from SufB, passing it off to downstream apoproteins[147,148]. Another protein involved in this process (ErpA) has redundant function with SufA but is necessary for the development of active Fe-S proteins[149].

Several important details of the SUF pathway remain to be identified. As in ISC, the *in vivo* source of iron is unknown. Also of interest is SufD's incorporation in the SufBCD complex, which allows SufB to accept iron *in vivo*[141] and facilitates binding of a $FADH_2$ cofactor[142]. This cofactor may be able to reduce ferric iron, facilitating potential Fe^{3+} sources such as ferritins or ferric citrate[150]. While SufA can deliver mononuclear iron to SufBC *in vitro*[109], it is currently believed to function downstream of *de novo* Fe-S formation (Figure 6). The source of electrons for SufS turnover and for Fe-S formation remains in question[151].

3.4 Fe-S Cluster Biogenesis Regulation

The best-developed model for Fe-S biogenesis pathway regulation comes from work done in Gram-negative bacterial systems, where both the ISC and SUF pathways are present. This work reveals a fascinating interplay between ISC and SUF, where the necessary ISC and

SUF genes are organized into their respective *isc* and *suf* operons (Figure 7). While this review has focused on eukaryotic systems, regulatory mechanisms in bacteria may provide insight into how this regulation occurs in eukaryotes. At the center of *E. coli* Fe-S cluster biogenesis regulation is a DNA-binding protein IscR, the first member of the *isc* operon, which directly regulates both the ISC and SUF systems.

Under non-stressed conditions, ISC is favored over SUF for general housekeeping of Fe-S cluster biosynthesis[152]. The transcriptional regulator IscR can bind a 2Fe-2S cluster (forming *holo*-IscR), obtaining its Fe-S cluster from the same ISC machinery utilized by other Fe-S proteins[153]. In the *holo* configuration, IscR binds to the *isc* promoter and prevents binding of RNA polymerase[154]. Thus, *holo*-IscR acts as a feedback regulator, inhibiting transcription of the entire *isc* operon when ISC activity is sufficient[155]. IscR is a relatively poor substrate for ISC-mediated Fe-S cluster loading[156] and *holo*-IscR can only form after the ISC proteins have exhausted their interactions with other *apo*-Fe-S proteins. In addition to being a weak ISC substrate, IscR does not bind its Fe-S cluster tightly and effectively acts as a sensor of cellular iron and oxygen conditions[157]. Under high-oxygen or low-iron conditions, *holo*-IscR quickly reverts to *apo*-IscR. Therefore under typical aerobic conditions, high oxygen levels cause *holo*-IscR to revert to *apo*-IscR. *Apo*-IscR dissociates from the *isc* promoter and *isc* is uninhibited.

In the *apo* configuration, IscR does not bind to the *isc* promoter but instead favors binding to the *suf* promoter, activating transcription of SUF genes[158]. Appropriate interaction of *apo*-IscR with the *suf* promoter involves two additional transcription factors: the ferric uptake regulator (Fur) and the peroxide responsive regulator (OxyR). *Suf* expression is constitutively repressed by Fur, which binds Fe^{2+} under non-stressed conditions when iron levels are sufficient and oxidative stress is low. With its Fe^{2+} cofactor, Fur binds to the *suf* promoter at the same site as *apo*-IscR, inhibiting *suf* expression[159]. When the cell faces iron deficiency, Fur loses its iron cofactor, dissociates from the suf promoter, thus triggering transcription of *suf*. The cell's preference for SUF over ISC in the presence of oxidative stress also reveals the involvement of another transcription factor (OxyR), as oxidized OxyR recruits RNA polymerase to the *suf* promoter.

There are additional regulatory mechanisms for cluster bioassembly beyond the level of gene expression. The small non-coding RNA RyhB, for example, is encoded just upstream of the SUF promoter and can bind to the iscRSUA mRNA to prevents its translation160. RyhB expression, however, is constitutively repressed by Fur-Fe^{2+}, so RyhB effectively inhibits ISC when conditions favor SUF[161].

3.5 Fe-S Cluster Function

Fe-S clusters are versatile biological cofactors found in the most fundamental biochemical pathways, including aconitase and succinate dehydrogenase of the citric acid cycle and respiratory complexes I-III of the electron transport chain[121]. Nuclear Fe-S proteins also have a unique role in DNA damage recognition and repair. Several forms of DNA polymerase, helicase, glycosylase, and primase all contain Fe-S clusters[125]. Considering their remarkable range of functions, a thorough summary of various Fe-S proteins is well beyond the limited scope of this review. New Fe-S proteins continue to be discovered but in

many cases, the role of the Fe-S cluster within the Fe-S protein remains unknown, even if the cluster's presence is essential for proper protein function.

Fe-S clusters can be found in the active site of many essential enzymes and usually are involved directly in catalysis. Being stable in a variety of redox states, Fe-S clusters are best known for their role as electron carriers. Fe-S clusters can carry usually one, but sometimes two electrons and are, subsequently, stable in various reduced states. 2Fe-2S clusters, for example, can exist in oxidized (Fe^{3+}/Fe^{3+}) or reduced (Fe^{3+}/Fe^{2+}) forms while 4Fe-4S clusters are stable in oxidized ($Fe^{3+}/Fe^{3+}/Fe^{3+}/Fe^{2+}$), intermediate ($Fe^{3+}/Fe^{3+}/Fe^{2+}/Fe^{2+}$), and reduced ($Fe^{3+}/Fe^{2+}/Fe^{2+}/Fe^{2+}$) forms[162]. The reduction potential of an Fe-S cluster is often modulated by interactions with nearest neighbor protein residues and by access to solvent, allowing for a large range of biological functions. Ferredoxins are considered the archetypical Fe-S cluster electron carriers and were the earliest Fe-S proteins to be functionally characterized[163]. Ferredoxins are involved in many essential biochemical pathways, transferring electrons for cellular respiration, photosynthesis, and nitrogen fixation[164]. A ferredoxin is even involved in the ISC iron sulfur cluster biogenesis pathway, as discussed previously (see section 3.3.1)[165].

But Fe-S cluster-mediated electron transfer is not limited to ferredoxins. In fact, one of the most the fundamental electron transfer processes, the electron transport chain (ETC), utilizes numerous Fe-S clusters. Respiratory complexes I, II, and III of the ETC all contain Fe-S clusters. Respiratory complex I uses a network of 8 Fe-S clusters for step-wise electron transfer[166]. Similarly, complex II contains an Fe-S protein component called SDHB with a 2Fe-2S, 3Fe-4S, and 4Fe-4S cluster[167]. Lastly, complex III utilizes a unique Fe-S cluster called a "Rieske center"[168]. The Rieske center is a 2Fe-2S cluster where one of the iron atoms is coordinated by histidines instead of cysteines, resulting in a Cys_2His_2 coordination[169].

Fe-S clusters can also be involved in non-redox reactions. The 4Fe-4S cluster in aconitase, for example, catalyzes a hydration-dehydration reaction, ligating directly to the citrate substrate[170]. In some cases, Fe-S clusters appear to only serve a structural function and not participate in chemistry directly, as is the case in endonuclease III[171].

3.6 Fe-S Clusters in Human Disease

Unlike in Gram-negative bacteria, where ISC/SUF redundancy allows for removal of an entire pathway, in humans the absence or mutation of a single component is often incompatible with life. In select cases there are human diseases that have been linked to defective Fe-S cluster biogenesis pathways. Below we describe several diseases directly linked to dysfunctional Fe-S cluster formation.

3.6.1 Friedreich's Ataxia—With an incidence of 1 in 50,000[172,173], and a carrier prevalence of 1 in 100[174], *Friedreich's ataxia* (FRDA) is by far the most prevalent disease linked to defective Fe-S cluster formation. FRDA is an autosomal recessive genetic disease caused by a GAA-trinucleotide repeat expansion in an intron of the frataxin gene, a protein involved in the ISC pathway[175]. This trinucleotide repeat expansion leads to under-expression of the frataxin gene and subsequently, low levels of frataxin[176]. These

insufficient frataxin levels are responsible for the pathophysiology of FRDA, but the precise role of frataxin is still unknown[177]. Frataxin may deliver iron to the ISC pathway[178], be an allosteric activator of the ISCU-ISCS complex[102], or may have a combination of roles. FRDA tissues demonstrate increased mitochondrial iron deposits[179] which leads to increased oxidative stress and cell death in metabolically active tissues such as cardiomyocytes and neurons of the dorsal root ganglia. FRDA presents early in adolescence with progressive ataxia, or difficulty coordinating movement, sensory loss, weakness, and dysarthria. FRDA patients are usually wheelchair bound in their teens with a significantly reduced quality of life and life expectancy[180]. Median age of survival is 35 years with cardiac dysfunction usually being the cause of death[181].

3.6.2 ISCU Myopathy—*ISCU myopathy* (IM) is an additional condition related to a defect in Fe-S cluster biogenesis. It is the 2nd most common disorder linked to defective Fe-S cluster synthesis but is much less common than FRDA with only 25 known cases. To date, all patients identified with IM have come from families of Swedish ancestry[121]. Similar to FRDA, the IM phenotype is inherited in an autosomal recessive pattern. IM is caused by a splicing defect during ISCU post-transcriptional processing that leads to defective ISCU protein[182,183]. Loss of ISCU leads to lower ISC activity and a resulting deficiency of essential Fe-S proteins, including succinate dehydrogenase and aconitase of the citric acid cycle[184]. Symptoms are exacerbated in cells that are metabolically active, such as the myocytes of skeletal muscle during exercise, and patients with IM experience exercise intolerance[185]. Prolonged activity can lead to tachycardia, tachypnea, and muscle pain[186]. Unlike FRDA, IM is not progressive and most cases have a normal life expectancy.

3.6.3 GLRX5 Sideroblastic Anemia—*GLRX5 Sideroblastic Anemia* (GSA), a disease caused by mutated GLRX5, has only been identified in a single patient to date. While GSA is exceedingly rare, this particular case study has revealed a unique mechanism linking Fe-S cluster production to general iron homeostasis[52,187]. GLRX5, involved in the last step of the ISC pathway, directs Fe-S cluster delivery from ISCU to downstream targets. One target is the iron-responsive protein IRP1, an Fe-S protein activated when its Fe-S cluster is absent[187]. Defective GLRX5, therefore, leads to constitutively active IRP1. IRP1 regulates several proteins involved in iron homeostasis[188], including those involved in heme production. In particular, *apo*-IRP1 inhibits expression of the final enzyme in heme synthesis, aminolevulinate synthase (ALAS2). Defective GLRX5, therefore, leads to insufficient heme and impaired erythropoiesis, resulting in anemia. Iron that would be directed towards heme production accumulates in the cytosol of erythroblasts, creating the characteristic ringedsideroblasts[151].

3.6.4 Additional Diseases—Succinate Dehydrogenase (SDH) subunit B, the Fe-S cluster containing protein of the succinate dehydrogenase complex, is a known tumor suppressor. Succinate, the substrate for SDH, stabilizes hypoxia-inducible factor (HIF), which regulates key processes in cell division and blood vessel growth under hypoxic conditions. Mutations in SDHB or in fact any of the other main subunits of SDH (SDHA, SDHC, and SDHD) cause susceptibility to tumor formations known as paragangliomas or phaeochromocytomas in a disorder called *Hereditary Paraganglioma-Pheochromocytoma*[189], stemming from the

accumulation of succinate and stabilization of HIF. SDHAF2, an assembly protein that flavinates SDH, is also implicated in paragangliomas. Recently, two additional Fe-S assembly proteins, SDHAF1 and SDHAF3, were found to stabilize Fe-S cluster assembly in SDH[190]. The later two proteins have LYR-motifs (Leu-Tyr-Arg) common to proteins involved in Fe-S cluster assembly. SDHAF1 deficiency is known to cause leukoencephalopathy[191].

Lastly, mutations in either NFU1 or BOLA3, two different genes involved in Fe-S cluster biogenesis, leads to *multiple mitochondrial dysfunction syndrome*, a condition characterized by defects in Complexes I, II, and III and pyruvate/α-ketoglutarate dehydrogenases. NFU1 is thought to be an alternative to ISCU as a scaffold for Fe-S assembly. Both BOLA3 and NFU1 appear to be involved in lipoate synthesis, possibly related to a role in assembling Fe-S clusters in lipoic acid synthase (LIAS), thus providing an explanation for the reduced PDH and α–KGDH activities characteristic of this syndrome. The impaired energy production results in lactic acidosis, encephalopathy[192] and early death.

4. Summary

The utilization of complex Fe cofactors in biology requires a tightly controlled process of cofactor assembly and of delivery to the correct apoprotein partner. Numerous ailments, some of which outlined within this review, result when there is a breakdown in the assembly process or in delivery of the cofactor. The development of treatment strategies for these disorders will require a more advanced molecular understanding of each protein malfunction outlined above. Therefore cooperation of bioinorganic chemists with the cell biologists alike will be essential to provide the broader understanding of how these complex pathways harness the power of iron in complex cofactors within the biological milieu.

Acknowledgments

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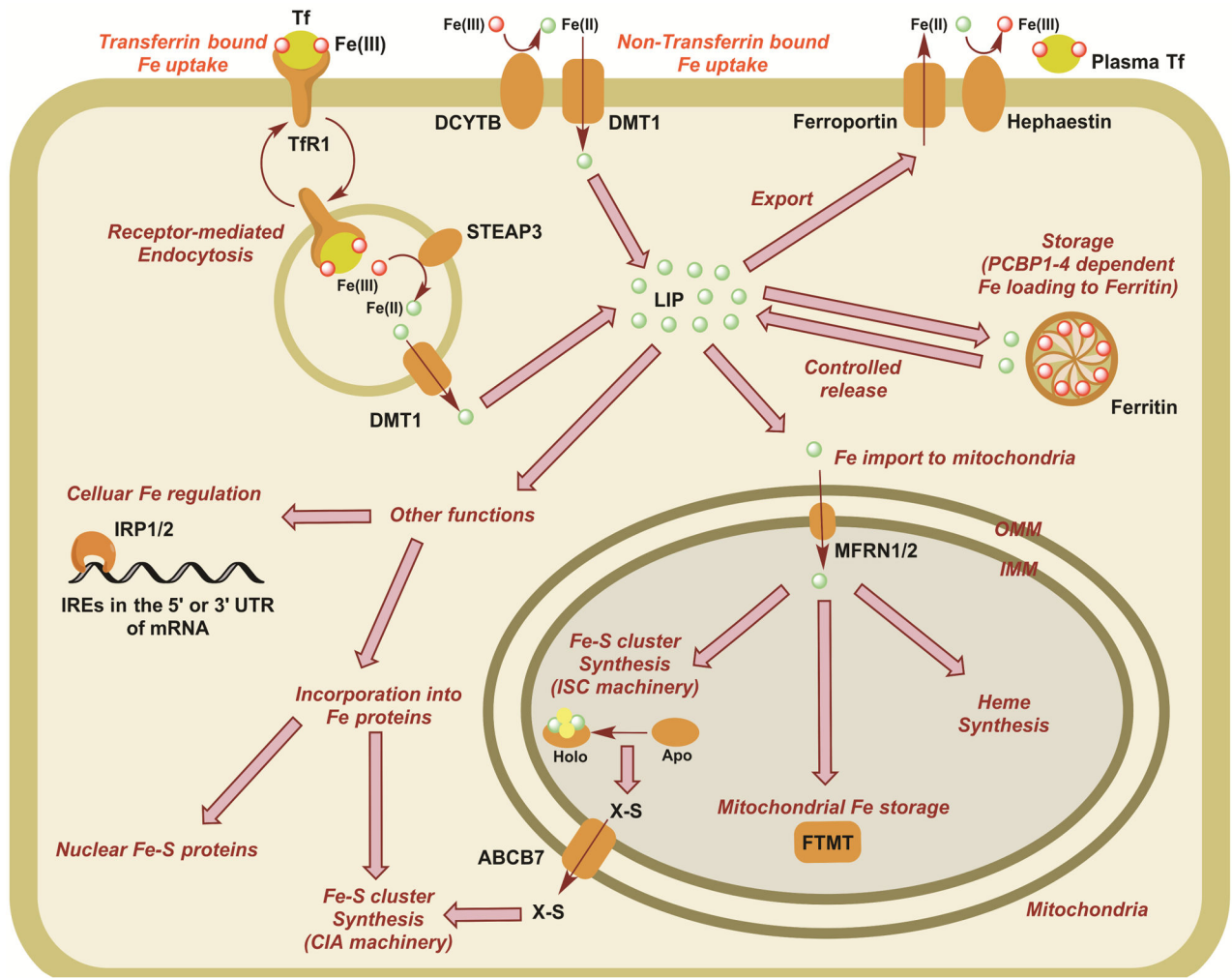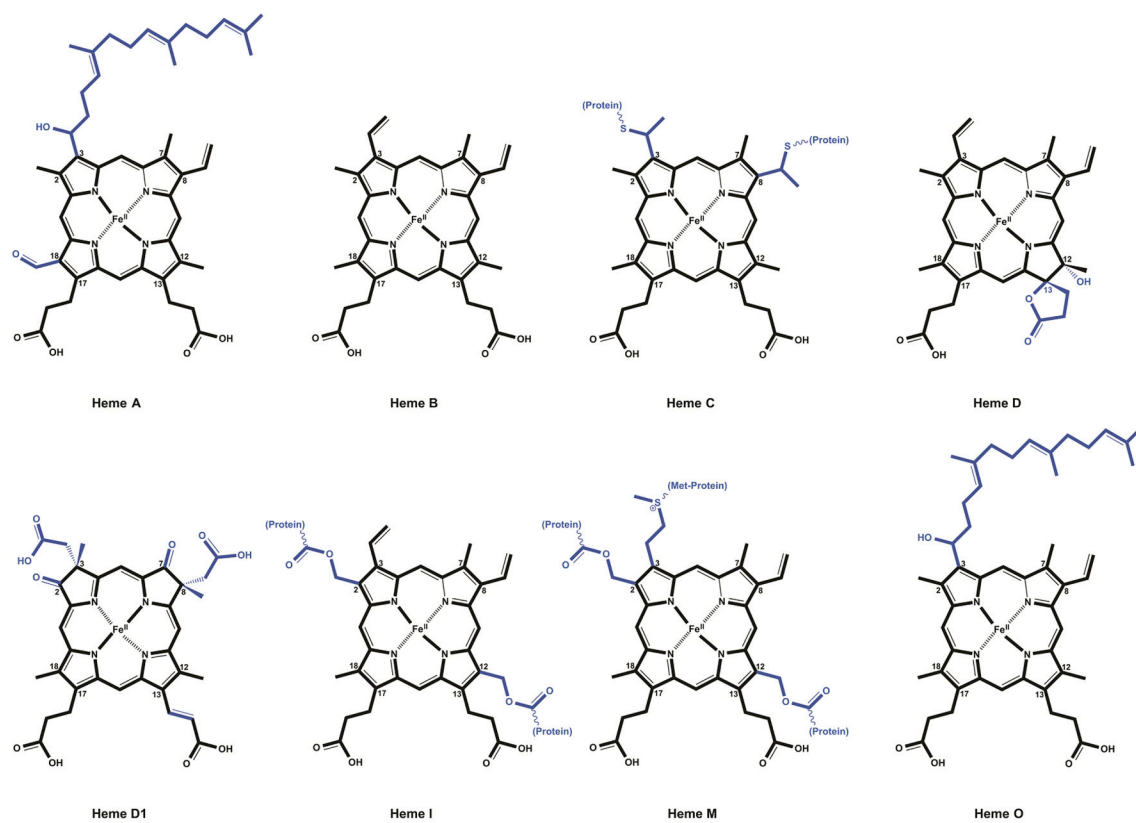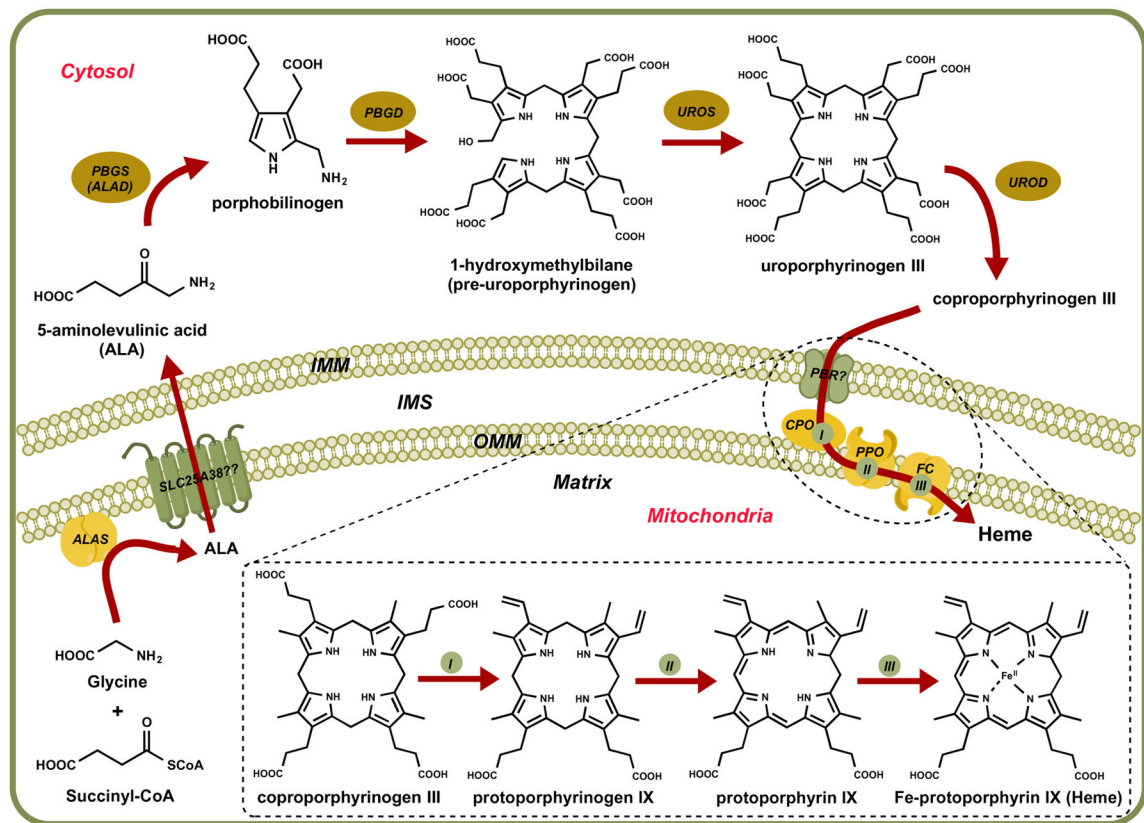
Figure 1.
Major cellular Fe utilization pathways in humans. Under physiological conditions, most Fe is internalized as the Tf-bound form, which undergoes receptor-mediated endocytosis via binding to TfR1. Decrease in endosomal pH releases Fe(III) from Tf, which is then reduced by the endosomal reductase STEAP3 to Fe(II). Fe(II) is then transported to the cytosol via DMT1 to join the LIP. Fe in LIP can be utilized for storage in ferritin, import to mitochondria for storage and heme and Fe-S cluster synthesis, export to the cytosol via ferroportin, cellular Fe regulation via IRPs and incorporation into other Fe proteins in the cytosol. Under non-physiological conditions Fe(III) can enter the cell after being reduced to Fe(II) and imported by DMT1. Tf: Transferrin, TfR1: Transferrin receptor 1, DCYTB: duodenal cytochrome b, DMT1: divalent metal transporter 1, STEAP3: six transmembrane epithelial antigen of the phosphate 3, LIP: labile iron pool, PCBP: poly (rC)-binding proteins, OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane, MFRN: Mitoferrins, FTMT: mitochondrial specific ferritin, ABCB7: transporter for unknown source of sulfur X-S from mitochondria to cytoplasm, IRP: iron regulatory proteins, IRE: iron-responsive elements of mRNA.
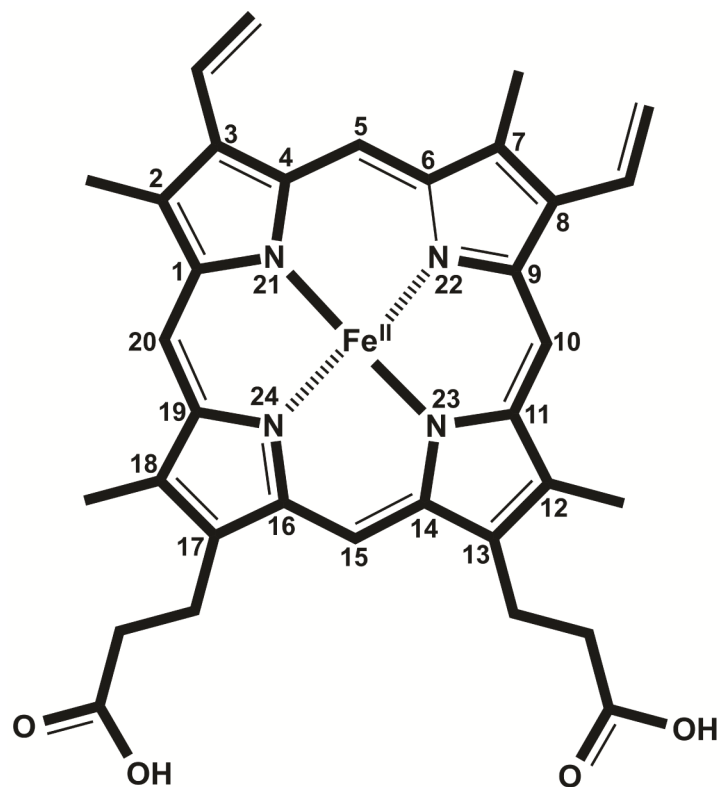
Figure 2.
Structure of heme. Standard 1–24 IUPAC numbering system is used to number the carbon atoms of the tetrapyrrole[183].
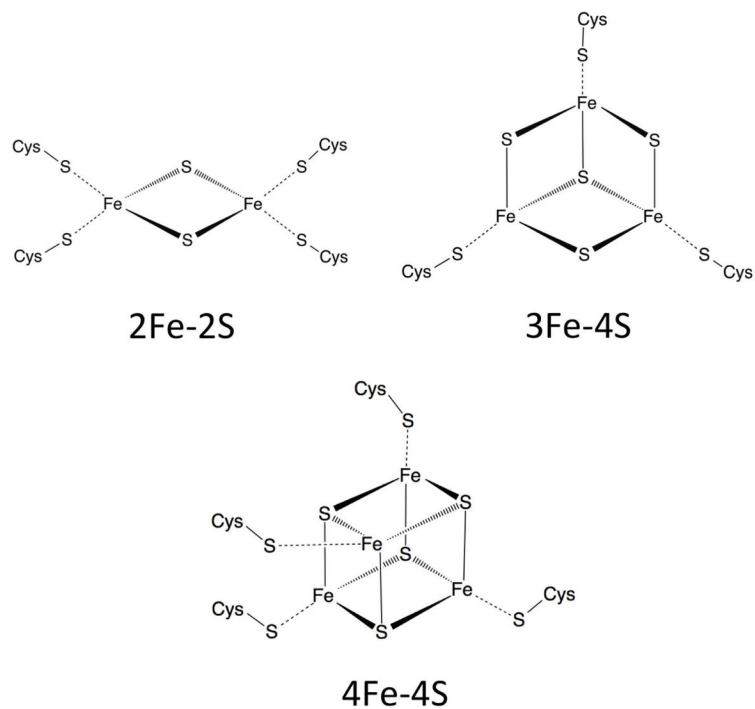
Figure 3.
Important types of heme found in nature. Side chain differences with respect to the parent heme molecule (heme B) are shown in blue.
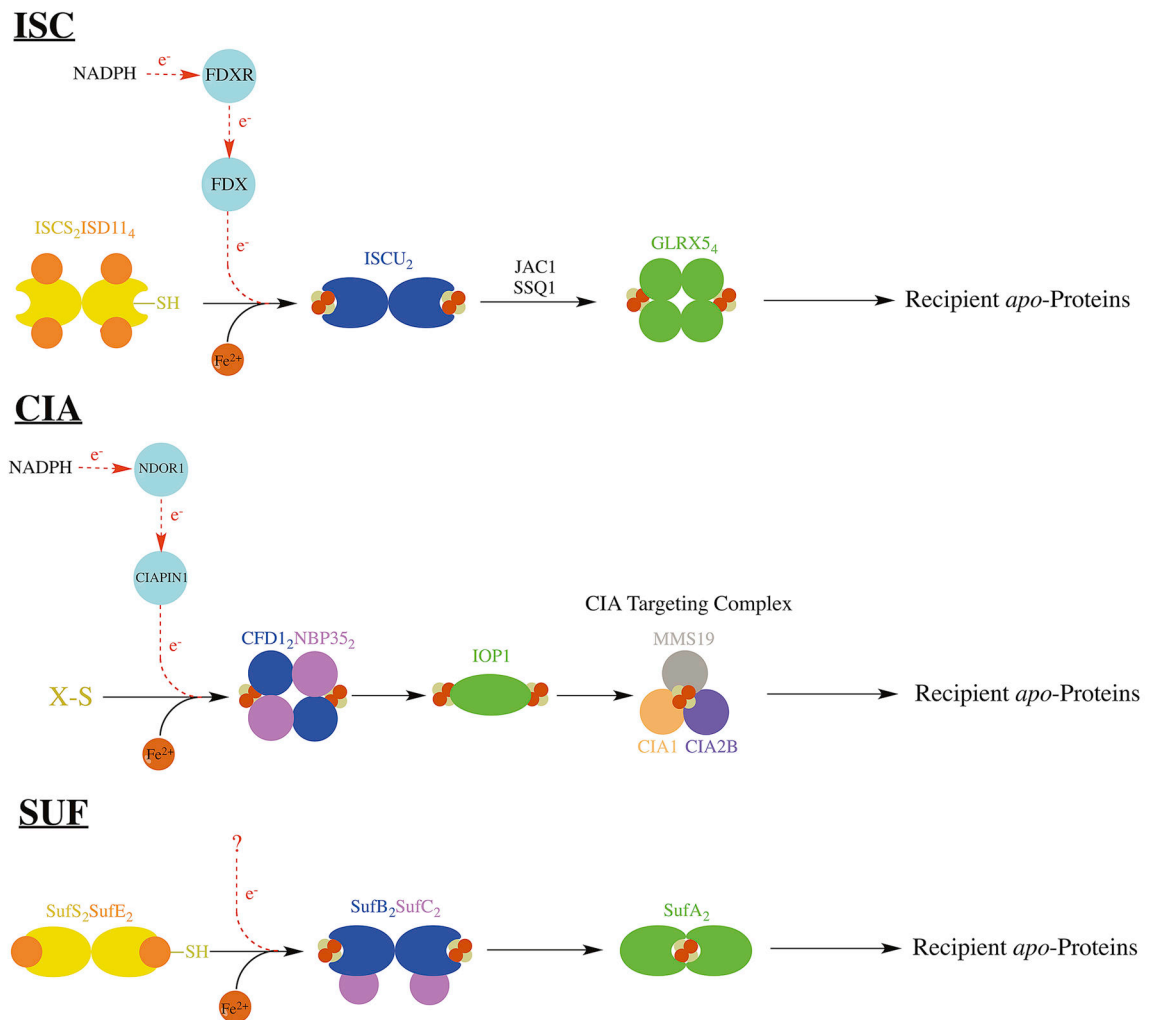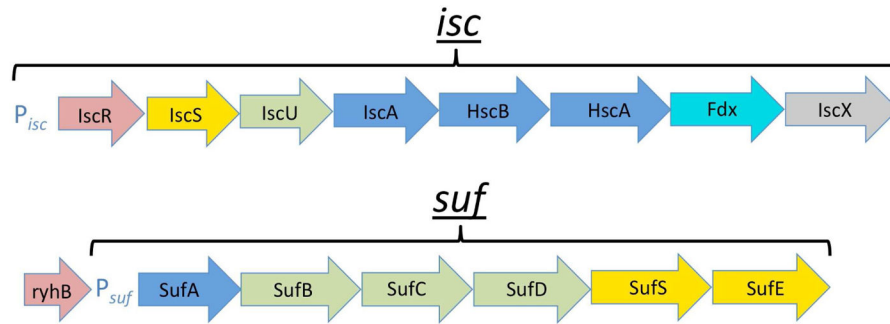
Figure 4.
Heme biosynthesis pathway. ALA: 5-aminolevulinic acid, ALAS: ALA synthase, PBGS: porphobilinogen synthase, ALAD: ALA dehydratase, PBGD: porphobilinogen deaminase, UROS: uroporphyrinogen synthase, UROD: uroporphyrinogen decarboxylase, PBR: peripheral-type benzodiazepine receptor, CPO: coproporphyrinogen III oxidase, PPO: protoporphyrinogen IX oxidase, FC: Ferrochelatase, IMM: inner mitochondrial membrane, IMS: intermembrane space, OMM: outer mitochondrial membrane.

Figure 5.
Structure for the 3 most common forms of Fe-S Cluster: 2Fe-2S, 3Fe-4S, and 4Fe-4S.

Figure 6.
Diagram depicting *de novo* Fe-S cluster formation and the main Fe-S cluster transfer steps in the ISC, SUF, and CIA systems. Protein names used are from the human system for ISC and CIA, and from the bacterial system for SUF.

Figure 7.
Illustration of *suf* and *isc* operons, with corresponding promoters (P*isc* and P*suf*), which play
a central role in regulation of SUF and ISC at the genetic level in bacteria. Operons depicted
are from the *E. coli* model system. Steps are colored based on the encoded protein's function
as follows: red (regulatory), yellow (sulfur delivery), green (primary scaffold), blue
(downstream Fe-S cluster delivery), cyan (electron transfer), and gray (unknown).

Table 1

Comparison of different heme types in nature.

Type	Structure Remarks	Organisms	Proteins	Function	Reference
Heme A	C3= hydroxyethylfarnesyl group C18= formyl group Conversion of heme to heme A is catalyzed by Heme A synthase.	Bacteria, archaea, plants and animals.	Cytochrome a containing heme-Cu oxidases. Ex: Cytochrome c oxidase/ Complex IV in mammalian mitochondria.	Terminal reaction of aerobic respiration. Reduction of oxygen to water.	193
Heme B	Parent heme molecule also known as protoheme IX, Fe-protoporphyrin IX or heme. Binds to apoproteins non-covalently.	In a variety of organisms.	Cytochromes b and hemoglobins.	Diverse array of functions associated with heme b such as electron transport and oxygen carrier.	
Heme C	C3 and C8= thioether bonds with cysteine residues. Binds to apoproteins covalently.	In a variety of organisms.	Cytochromes c Ex: bacterial alcohol dehydrogenase, bacterial caa3 and cbb3 oxidases, mitochondrial and bacterial bc1 complex, and cytochrome cd1 nitrite reductase.	Serve as electron carriers to perform single electron transfers in energy transduction processes such as photosynthesis, respiration and N and S cycles. Other functions include apoptosis, detoxification of ROS.	194
Heme D	C12= hydroxylated, in a *trans* conformation to the γ-spirolactone. C13= hydroxylated, propionyl group forms a γ-spirolactone with the hydroxyl group.	In diverse bacteria including *Azotobacter, Proteus, Acetobacter, Salmonella, Bacillus, Pseudomonas, Haemophilus* and *E. coli.*	Terminal respiratory oxidases. Ex: Cytochrome d oxidase.	Terminal reaction of aerobic respiration. Reduction of oxygen to water at low oxygen levels.	195–197
Heme D1	C3 and C8= an acetyl group and a methyl group on each carbon. C2 and C7= keto groups on each carbon. C13 propionyl side chain has reduced to incorporate a vinyl group between second and third carbons.	In *Pseudomonas perfectomarinus, Alcaligenes faecalis, Paracoccus denitrificans, Thiobacillus denitrificans,* and *Thiosphaera pantotropa.*	Nitrite reductase that functions in dissimilatory nitrogen metabolism. Ex: Cytochrome cd1 nitrite reductase.	Catalyzes the one-electron reduction of nitrite to nitric oxide and fourelectron reduction of oxygen to water.	198,199
Heme I	C2 and C12= hydroxymethyl groups, they form ester bonds with carboxyl side chains of amino acids. Binds to apoproteins covalently.	In mammalian secretory fluids.	Peroxidases such as bovine milk enzyme lactoperoxidase, eosinophil peroxidase and thyroid peroxidase	Peroxide-driven oxidation of halide and pseudohalide ions as a nonspecific antimicrobial defense mechanism for the protection of mucosal surfaces.	200
Heme M	C2 and C12= hydroxymethyl groups, they form ester bonds with carboxyl side chains of amino acids. C3= vinyl group on C3 forms a sulfonium ion linkage with the sulfur of a methionyl residue.	In mammalian neutrophils	Myeloperoxidase	Peroxide-driven oxidation of chloride and bromide ions as an antimicrobial defense mechanism.	11,200
Heme O	C3= hydroxyethylfarnesyl group.	Bacteria such as *E. coli.*	Cytochrome o containing quinol oxidases. Ex: Cytochrome bo$_3$	Terminal reaction of aerobic respiration. Reduction of oxygen to water.	201,202