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Trafficking of Heme and Porphyrins in Metazoa

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1. Introduction

A half century ago, Max Perutz and John Kendrew determined the crystal structure of two heme-containing proteins: hemoglobin and myoglobin, respectively.[1,2] These landmark discoveries created the foundation for a detailed biochemical understanding of how protein structure influences and affects its ability to bind and carry oxygen. Perutz and Kendrew were awarded the Nobel Prize in 1962. Since then, scores of hemoprotein structures have been solved, although the mechanisms and molecules responsible for assembling heme into hemoglobin (Hb) and other hemoproteins remain unknown.[3]

Heme is the prosthetic group of proteins that perform diverse functions such as oxygen transport (globins), xenobiotic detoxification (cytochrome P450s), oxidative metabolism (cytochrome *c* oxidase), gas sensing (soluble guanylate cyclases), input/regulation of the circadian clock (nuclear hormone receptor, Rev-erb α, mPER2), microRNA processing (DGCR8),

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Dr. Iqbal Hamza received his B.Sc. in 1989 and M.Sc. in 1991 in Biochemistry from the University of Bombay, India. In 1991, he joined the Biochemistry Department at the State University of New York at Buffalo to pursue a Ph.D. Under the mentorship of Dr. Mark O'Brian, he discovered the Iron Response Regulator that coordinates heme synthesis with iron transport and availability in bacteria. After receiving his Ph.D. in Nov. 1997, Dr. Hamza continued his studies of biological metals by joining the laboratory of Dr. Jonathan Gitlin at Washington University School of Medicine. As a postdoctoral fellow, he created and characterized transgenic mouse models to identify the genetic basis of copper trafficking by metallochaperones and P-type ATPases. Dr. Hamza joined the University of Maryland, College Park, in Oct. 2002 as an Assistant Professor. Using his research experiences in heme biology, copper trafficking, and multiple genetic model systems, he deliberately set out to tackle the problem of how heme is transported in eukaryotes. He discovered that the roundworm *Caenorhabditis elegans* is a unique animal model to identify heme transport pathways because it does not make heme, but it eats heme to survive and reproduce. Using the worm model, his group identified the first heme importer/transporter in animals. He is currently a tenured Associate Professor. The main thrust of his research group is to identify the genes and elucidate the mechanisms of heme trafficking and transport in eukaryotes using genetic, cell biological, and biochemical approaches.

Dr. Scott Severance was born in Framingham, MA. He graduated from Bob Jones University with a major in chemistry and a minor in biology in 1992. After teaching high school science for several years, he was selected to be a Presidential Fellow by the Interdisciplinary Graduate Program in the Biomedical Sciences at the State University of New York at Buffalo. Scott joined the laboratory of Dr. Daniel J. Kosman to study iron transport in the budding yeast *Saccharomyces cerevisiae*. He determined the orientation and topology of Ftr1p, the high affinity iron transporter in the plasma membrane; characterized a number of amino acid motifs involved in the transport of iron by Ftr1p; and contributed to the understanding of how Ftr1p interacts to form a complex with Fet3p. Before joining the laboratory of Dr. Iqbal Hamza at the University of Maryland, College Park, Scott was a visiting assistant professor of Biochemistry in the Department of Chemistry and Biochemistry at Canisius College in Buffalo, New York. Under Dr. Hamza's guidance, Scott conducted a genome-wide, functional reverse genetics, RNA-mediated interference screen in *C. elegans* to identify genes involved in heme homeostasis. He is currently characterizing novel heme transporter genes. Scott is an NIH NRSA postdoctoral fellow.

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antibactericides/microbicides (myeloperoxidase), and thyroid hormone synthesis (thyroperoxidase).[4−17] Heme is also known to directly regulate processes such as cell differentiation and gene expression.[18−20] Heme's function as an acute cell-signaling molecule has been implicated by high affinity binding and inhibition of both the large-conductance calcium-dependent Slo1 BK channels and the epithelial sodium channels.[21−24]

Hemes are iron-coordinated porphyrins containing four pyrrole rings joined at the α-position by four methine bridges (=CH−). The iron atom at the center of this organic ring can adopt either the ferric (Fe^{3+}) or the ferrous (Fe^{2+}) oxidation state. Many, though not all, naturally occurring porphyrins contain iron, and a significant portion of porphyrin-containing proteins possess heme as a prosthetic group. The most abundant heme, heme *b*, is found in the hemoproteins myoglobin and Hb and contains two propionate, two vinyl, and four methyl side chains (Figure 1A). Oxidation of a methyl side chain to a formyl group and substitution of a vinyl side chain with a 17-carbon isoprenoid side chain converts heme *b* to heme *a*, the prosthetic group of the mitochondrial enzyme cytochrome *c* oxidase. C-type hemoproteins such as cytochrome *c* and the *bc1* complex contain heme *c* in which the two vinyl side chains of heme *b* are covalently attached to the protein.

Although the thermodynamically favored structure of heme is planar, hemes can assume surprisingly distorted nonplanar 3-D structures within proteins.[25] Analyses of more than 400 different hemoprotein crystal structures by Shelnutt and co-workers using the Normal-Coordinate Structural Decomposition program revealed nearly 70 different heme shapes or distortions.[25] Of these, six shapes, named for how the heme appears in the protein—propellering, saddling, doming, ruffling, and waving in the *x*- or *y*-axis—were determined to be important for heme function (Figure 1B). Only a small amount of energy is required to distort the heme shape. Heme distortions can affect the chelated iron spin-state as well as its absorption properties, fluorescence yields, and reduction potentials.[26] More importantly, heme shapes often correlate with hemoprotein function, i.e., dissimilar proteins with "conserved" heme shapes may perform similar functions.

This review focuses on the mechanisms of heme trafficking and transport with specific emphasis on metazoans. Although current knowledge of heme transport pathways in metazoans is limited (Figure 2), we will derive models of heme trafficking pathways based on recent findings in membrane trafficking and interorganellar transfer of metabolites. We will also draw parallels with paradigms for bacterial heme transport, which has been extensively characterized at the genetic and biochemical levels. The models are provided purely as a conceptual framework to infuse new ideas into the field and permit broad generalization of the principles of heme trafficking from single-cell organisms to complex eukaryotes. Finally, we will discuss genetically tractable, emerging model systems that will permit exploration, identification, and validation of the evolutionary conservation of heme transport and trafficking pathways across metazoa.

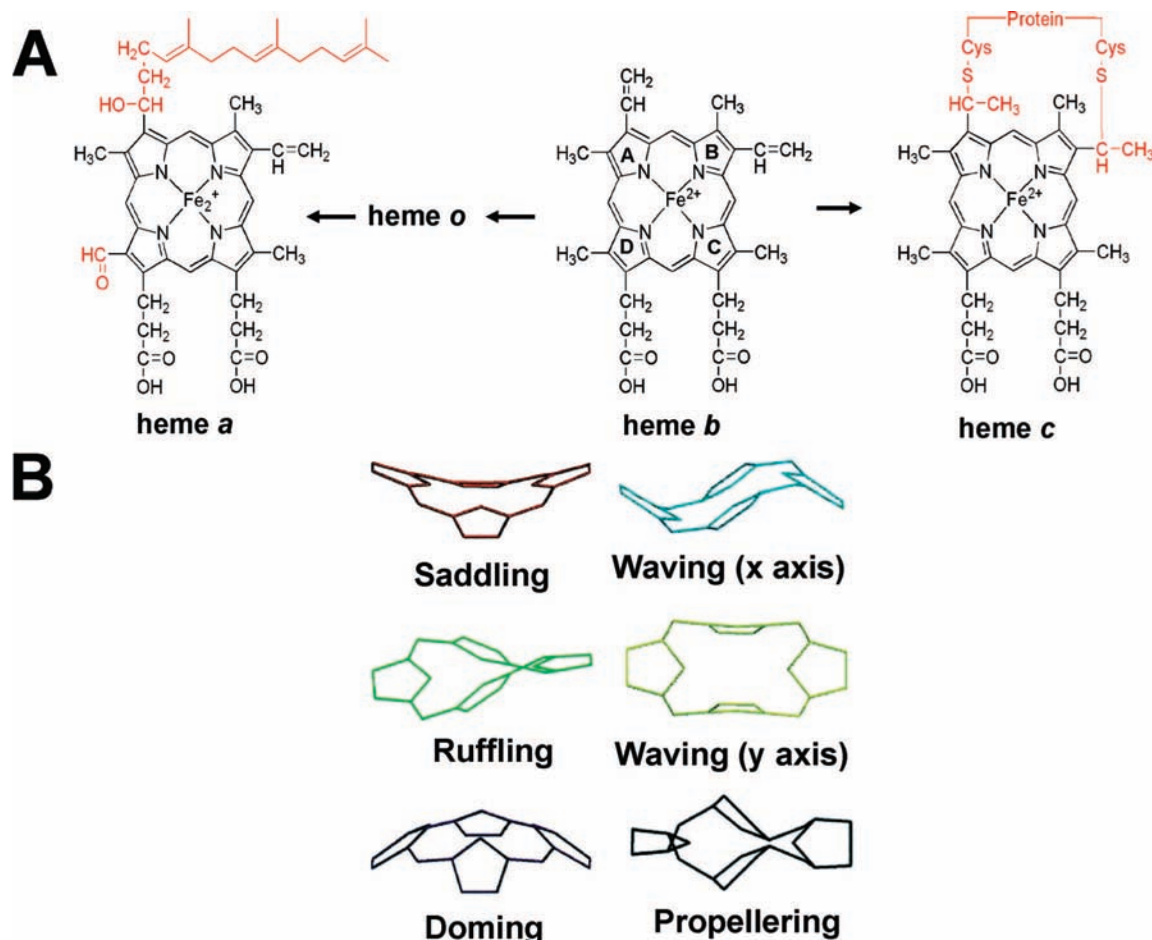

Figure 1. Heme types and shapes. (A) Structures for heme *b* with the pyrrole rings lettered using the Hans Fischer system. Hemes *a* and *c* are synthesized from heme *b* via side chain modifications (red). (B) Six most frequent nonplanar distortions of the porphyrin macrocycle. Reprinted with permission from ref 25. Copyright 1998 Elsevier.

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2. Heme Synthesis

2.1. Overview

Although the focus of this review is the transport of porphyrins and heme, the heme biosynthesis pathway is briefly presented to provide a framework for how heme intermediates and heme might be shuttled between the well-characterized enzymes responsible for synthesizing heme. (For a comprehensive review of heme synthesis, see reference 27). In most metazoans, fungi, and the alpha proteobacteria, heme is synthesized via a highly conserved, eight-step process known as the Shemin pathway.[28,29] All eight genes in the heme synthesis pathway have been cloned, and the associated enzymes have been crystallized from a number of organisms.[30] In metazoans, the first and the last three conversions take place in the mitochondria, while all remaining steps occur in the cytosol (Figure 3A). By contrast, only the last two reactions occur in the mitochondria in the budding yeast.[31,32] Though slight variations occur, heme synthesis pathways convert the universal precursor δ-aminolevulinic acid (ALA) into iron-protophorphyrin IX (heme).

2.2. δ-Aminolevulinic Acid

The first step in heme synthesis involves the condensation of glycine with succinyl-coenzyme A (succinyl-CoA), which results in the formation of δ-aminolevulinic acid (ALA). In most prokaryotes and all higher plants, ALA is synthesized via an alternative method—the glutamate C-5 pathway.[33−36] In all organisms that produce heme, aminolevulinic acid synthase (ALAS) catalyzes the production of ALA. In most vertebrates, there are two isoforms of ALAS: ALAS1 and ALAS2.[37−39] ALAS1 is expressed in all tissues, while ALAS2 is expressed only in erythroid cells.[40,41] The activity of ALAS1 is downregulated by heme, but the activity of ALAS2 is not repressed by heme.[42,43] Instead, the presence of iron stabilizes the ALAS2 messenger RNA (mRNA) transcript via an iron-response element in the 3′ untranslated region.[44] ALAS utilizes the active form of vitamin B_6, pyridoxal 5-phosphate, as a cofactor. Recently, it was demonstrated that patients with a form of autosomal recessive nonsyndromic congenital sideroblastic anemia harbored mutations in *SLC25A38*.[45] It was postulated that, at least in erythroid cells, SLC25A38 facilitates glycine import for ALA synthesis or exchanges glycine for ALA across the mitochondrial inner membrane. How nonerythroid cells import glycine is still unanswered. Following synthesis, ALA is exported from the mitochondria by an unknown mechanism.

2.3. Porphobilinogen

Once in the cytosol, two molecules of ALA are converted to porphobilinogen (PBG) in a condensation

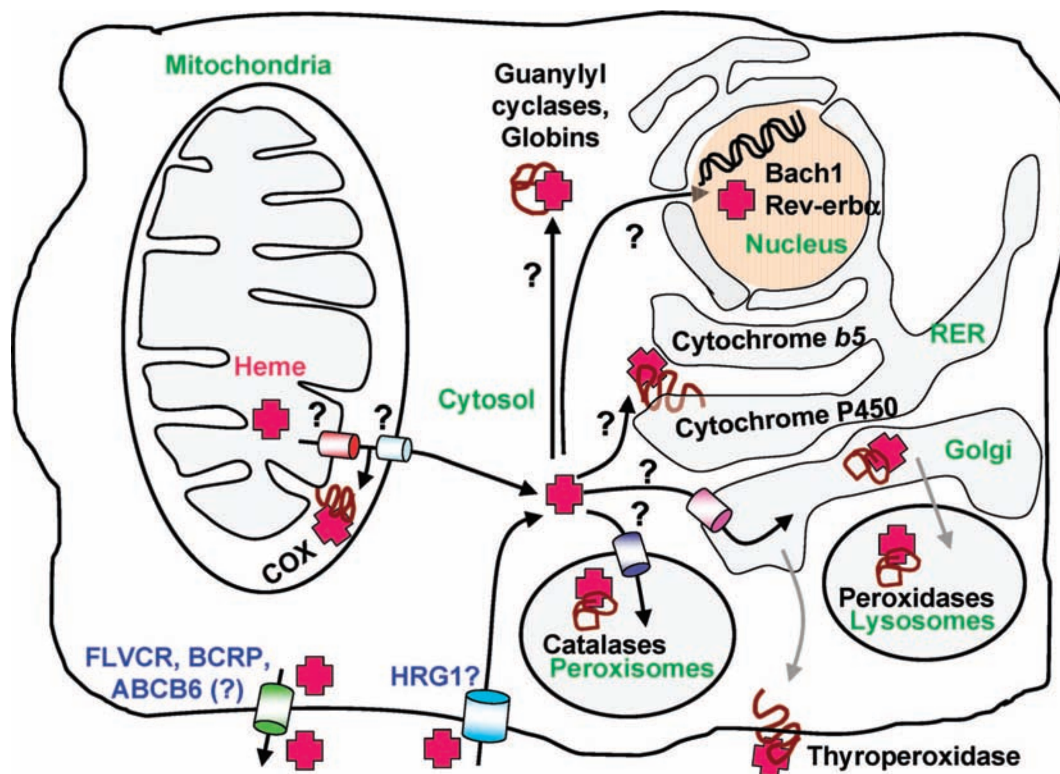

Figure 2. Schematic model of intracellular heme trafficking. Presumptive heme pathways (black arrows) that are currently unknown are marked with a (?). In eukaryotic cells, the final step of heme synthesis occurs in mitochondrial matrix. The nascent heme moiety is somehow transported through mitochondrial membranes and incorporated into a multitude of hemoproteins found in different cellular compartments (green). This process is presumably mediated by hemochaperones and transporters. Heme transport proteins that have been already identified are highlighted in blue.

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reaction catalyzed by aminolevulinic acid dehydratase (ALAD). Most, if not all, ALAD enyzmes are metalloenzymes. Curiously, even though ALAD enzymes from all organisms are highly similar in sequence, a variety of metal cofactors is utilized. In animals, PBG synthesis is dependent on the availability of zinc because zinc is required for ALAD function.[46] Its inhibition in humans by lead substitution is one of the most sensitive indicators of accumulation of lead in the blood, a measurement frequently used to detect recent lead exposure.[47] In contrast, ALAD from plants and many bacteria requires magnesium instead of zinc.[48] ALAD from yeast and *E. coli* is composed of four homodimers, with one active site per dimer.[49−51] Each active site binds two ALA molecules. One ALA molecule is the source of the propionate side chain and the pyrrole nitrogen, and the other molecule contributes the acetate and aminomethyl group of PBG.[52] PBG is the pyrrole precursor utilized by all living systems for the biosynthesis of tetrapyrroles, including hemes, chlorophylls, and corrins.[53]

2.4. Hydroxymethylbilane

The next two steps in the synthesis pathway combine four molecules of the monopyrrole PBG to form a cyclic tetrapyrrole. The first of these two steps is catalyzed by porphobilinogen deaminase (PBGD), which catalyzes the formation of a linear tetrapyrrole—hydroxymethylbilane (HMB)—from four molecules of PBG. HMB is released after a hexapyrrole intermediate is generated and the four pyrroles distal to the active site of PBGD are cleaved. PBGD retains the two proximal pyrroles to be used as a dipyrrole cofactor in subsequent reactions.[54]

2.5. Uroporphyrinogen III

HMB released from PBGD is unstable and spontaneously forms uroporphyrinogen I, a tetrapyrrole that cannot be converted to heme. Thus, HMB must be quickly converted to uroporphyrinogen III (UROgenIII) by uroporphyrinogen III synthase (UROS). UROS catalyzes ring closure to form an asymmetrical macrocycle. UROgenIII can enter different synthesis pathways. UROgenIII can enter the heme biosynthetic pathway or the corrin synthesis pathway.[55] In photosynthetic organisms, UROgenIII also serves as a precursor for chlorophyll synthesis.[30] Higher plants synthesize four major tetrapyrrole compounds via UROgenIII—heme, siroheme, chlorophyll, and phytochromobilin.[56,57] Methylation of UROgenIII and the subsequent insertion of iron (Fe^{2+}) occurs during formation of siroheme—an iron-containing isobacteriochlorin essential for nitrite and sulfite reduction reactions.[58,59]

2.6. Coproporphyrinogen III

The next two reactions in the heme synthesis pathway modify the side chains of the tetrapyrrole. UROgenIII is first converted to coproporphyrinogen III (CPgenIII) by the enzyme uroporphyrinogen decarboxylase (UROD)—the last cytoslic enzyme in metazoans. UROD removes the carboxylic acid groups from the four acetic acid side chains of UrogenIII.

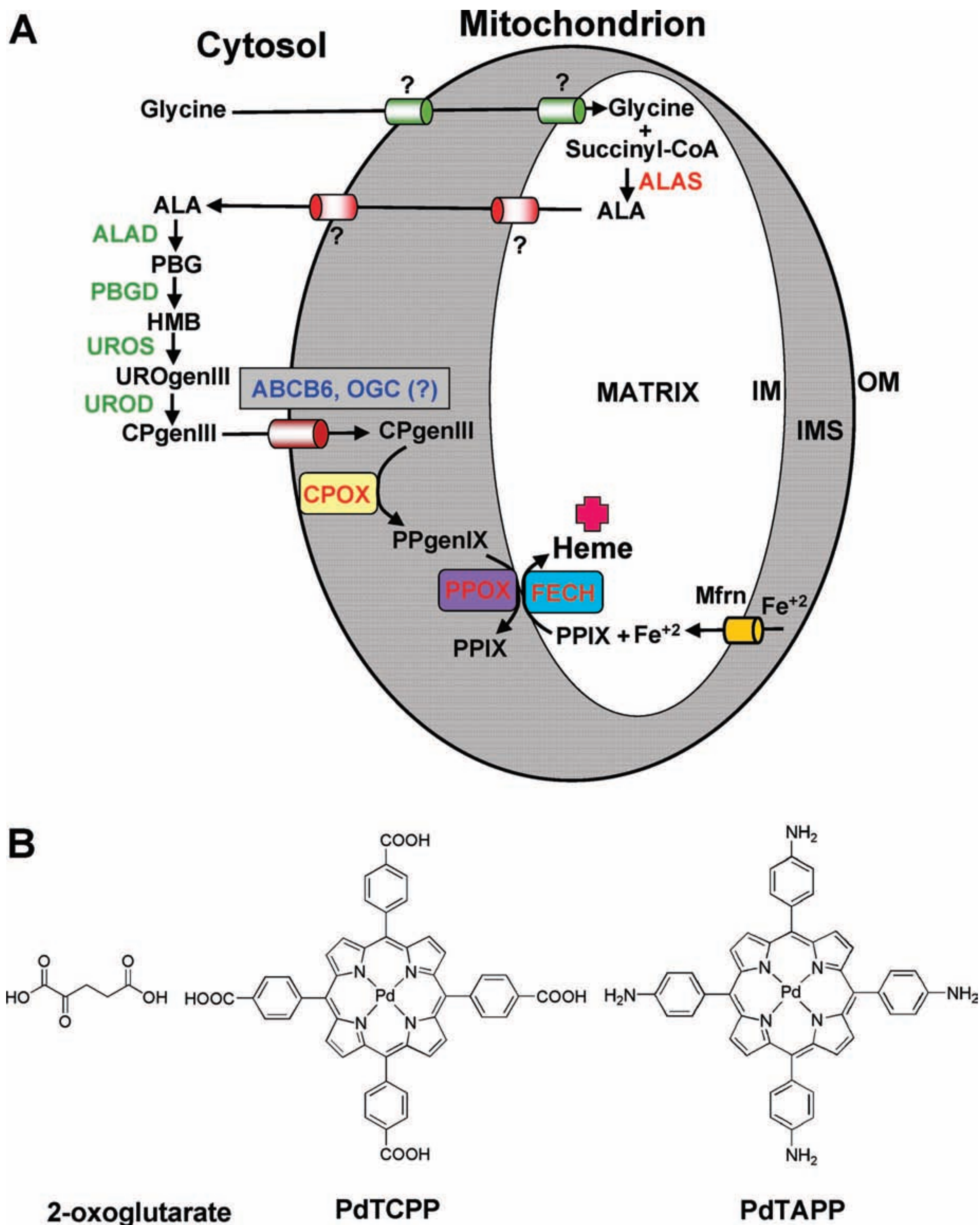

Figure 3. Heme biosynthesis in metazoans. (A) Heme biosynthesis is an eight-step enzymatic pathway that begins with the synthesis of ALA in the mitochondria from the amino acid glycine and succinyl-CoA, derived from the Krebs Cycle. The processes for import of glycine and the export of ALA for heme synthesis are unknown. ALA is then transported from the mitochondria into the cytosol where, in mammals, the subsequent four steps occur. The intermediate CPgenIII is transported back into the mitochondria for the final three steps. Researchers have demonstrated that ABCB6 or OGC may import CPgenIII into the mitochondria. The final step is the insertion of ferrous iron, transported by Mitoferrin (Mfrn), into the protoporphyrin IX (PPIX) ring, and this reaction is catalyzed by ferrochelatase (FECH). Mitochondrial enzymes are highlighted in red and cytosolic enzymes in green. Although the active site of FECH faces the mitochondrial inner membrane and heme is most likely released toward the membrane, for the sake of clarity, heme is depicted as being released into the matrix in this figure. See Figure 4(B) for a more accurate portrayal of the release of heme from the active site of FECH. [IM: mitochondrial inner membrane; IMS: mitochondrial intermembrane space; OM: mitochondrial outer membrane] (B) Structures of 2-oxoglutarate and the fluorescent porphyrin derivatives palladium *meso*-tetra(4-carboxyphenyl)porphyrin and palladium *meso*-tetra(4-aminophenyl)porphyrin. Compare Figure 3B with the structure of heme *b* in Figure 1A.

2.7. Protoporphyrinogen IX

The subsequent side chain modification is catalyzed by coproporphyrinogen oxidase (CPOX), which is located on the mitochondrial outer membrane (OM) facing the intermembrane space (IMS) (Figure 3).[60] CPOX has been reported as the rate-limiting enzyme in heme synthesis during

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erythroid differentiation.[61] In order for CPOX to metabolize its substrate, CPgenIII must be imported into the mitochondria where the propionate side chains of pyrroles A and B are converted to vinyl groups (Figure 1A). This oxidative decarboxylation of the propionate groups is catalyzed by CPOX and results in the formation of protoporphyrinogen IX (PPgenIX).

2.8. Protoporphyrin IX

The penultimate step of the pathway is the six-electron oxidation of PPgenIX by protoporphyrinogen oxidase (PPOX) to form protoporphyrin IX (PPIX). In eukaryotes, CPOX and PPOX are both oxygen-requiring enzymes, while in some bacteria the enzymes that catalyze these steps are oxygen-independent. Oxidation of PPgenIX to PPIX results not only in a fully conjugated macrocycle but also converts a relatively flexible molecule into a rigid, planar ring that has characteristic visible light absorbance. Located on the IMS face of the mitochondrial inner membrane (IM), PPOX functions as a homodimer (Figure 3).[62] PPOX is the last enzyme that the heme and chlorophyll biosynthesis pathways have in common.[63]

2.9. Heme

The final step of heme biosynthesis is the addition of iron into PPIX to form protoheme or heme *b*. The reaction is catalyzed by ferrochelatase (FECH), which is located on the matrix side of the IM (Figure 3A). Since the active site of FECH faces into the IM, protoheme is released toward the membrane and not into the matrix. In plants, Mg-chelatase, an enzyme unrelated to FECH, inserts a Mg^{2+} ion in an ATP-dependent reaction to yield Mg-protoporphyrin IX for the synthesis of chlorophyll.[35]

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3. Transport of Heme Synthesis Intermediates and Heme

Despite the fact that heme biosynthesis and its regulation have been extensively studied, very little is known about the intracellular trafficking of heme or its intermediates. Once heme is synthesized, it has to be translocated from the mitochondrial matrix for incorporation into cytochromes present in the mitochondrial intermembrane space (Figure 2). Heme must also be transported out of the mitochondria to be incorporated into hemoproteins found in the cytosol, nucleus, endoplasmic reticulum (ER), and other organelles. Free heme is hydrophobic and cytotoxic due to its inherent peroxidase activity.[64] Thus, a *prima facie* argument can be made that specific molecules and pathways transport heme from the site of synthesis to other cellular compartments where hemoproteins are assembled.

3.1. Heme Intermediates and Heme Traverse Membranes Multiple Times

In order for heme synthesis to occur, a number of logistical difficulties associated with substrate trafficking must be overcome. First, there are four points during heme synthesis that a heme intermediate or heme must cross at least one mitochondrial membrane. It stands to reason that this movement is accomplished by protein transporters. Second, heme intermediates must be shuttled between transporters or enzymes through an aqueous environment as many as eight times during the heme synthesis process. The accumulation of heme precursors causes toxicity manifested in patients as porphyrias.[65] Porphyrias are associated with every enzyme in the heme synthesis pathway except for the first. In order to prevent heme precursors from going any place except for exactly where they need to go, the products of each reaction must be efficiently directed to the next enzyme in the pathway. Potentially, this could be accomplished by "chaperones" or guided delivery of intermediates to the subsequent enzyme in the pathway. How heme intermediates are shuttled between the enzymes of the heme biosynthesis pathway has yet to be determined.

3.1.1. Movement of δ-Aminolevulinic Acid from the Matrix to the Cytosol

The first instance a membrane is traversed occurs when ALA must be transported from the matrix to the cytosol across both the mitochondrial IM and OM (at least in metazoans). It can be surmised that the heme synthesis pathway involves as few proteins as possible and that ALAS is associated with the mitochondrial IM. If ALAS is a peripheral protein, then the newly synthesized ALA could be transported directly across the membranes by a single protein that interacts with ALAS.

In *E. coli*, a periplasmic dipeptide permease has been shown to transport ALA into the cell.[66] A genetic screen of a *hemA E. coli* mutant strain was performed to isolate mutants that were completely dependent on exogenous ALA for aerobic and anaerobic growth. This screen identified a class of mutants in which exogenous ALA uptake was completely abrogated. These mutations were designated *alu* for ALA uptake. Expression of a recombinant plasmid containing the *dpp* operon, which encodes a dipeptide permease transport system, drastically increased the amount of ALA transported by the *hemA alu* double mutant strains. Addition of high concentrations of Pro-Gly to the uptake experiment abolished import of ALA.[66] Additionally, it was demonstrated that the purified dipeptide periplasmic protein (Dpp permease) bound heme in vitro.[67] The Dpp permease seemingly acts in a redundant fashion (for both dipeptide and heme binding) in the several types of bacteria that contain both a heme transport system and the Dpp permease. It was hypothesized that the Dpp permease acts as a heme permease in bacteria lacking a heme transport system to allow bacteria to utilize exogenous heme.[67] The fact that the Dpp permease in *E. coli* transports dipeptides, ALA, and heme distinguishes it from other bacterial heme permeases that selectively transport only heme.[68]

3.1.2. Coproporphyrinogen III is Transported from the Cytosol to the Intermembrane Space

The second time a heme intermediate crosses a membrane is after CPgenIII is synthesized in the cytoplasm and must be transported across the OM (Figure 3A). CPOX is associated with the OM facing the IMS in mammals; hence, CPgenIII import is not likely to occur via the reverse of ALA export. There are at least two potential pathways for transporting CPgenIII into the mitochondria. First, a specific CPgenIII transporter spanning the OM transports CPgenIII from the cytosol directly to CPOX. This scenario will be discussed below. A second possible mechanism posits that CPOX is accessible from the cytosolic side of the OM. In this case, CPOX could directly interact with UROD or with

a CPgenIII-bound cytosolic chaperone. The latter hypothesis can be reconciled by direct protein−protein interactions between CPOX and PPOX for the transport of PPgenIX;[69] however, direct experimental support for CPgenIII transport by such a mechanism is pending.

It has been established that ATP-binding cassette (ABC) proteins transport heme or heme bound to a protein carrier in many bacterial species.[70,71] The mammalian ABC transporter located on the OM, ABCB6, was shown to interact with heme and porphyrins and also to transport coproporphyrin III (CPIII) from the cytoplasm into the mitochondria.[72] The authors of this work extrapolated their findings to suggest that ABCB6 transports CPgenIII, an intermediate in the heme biosynthetic pathway, from the cytoplasm into the mitochondria.[72] The studies were conducted with a nonphysiological substrate CPIII, which is a planar, oxidized porphyrin, rather than the nonplanar, reduced CPgenIII.[72] This raises doubt whether CPgenIII is a *bona fide* ABCB6 substrate.

Mammalian ABCB6 is able to functionally complement the effects of iron overaccumulation in the yeast *atm1−1* mutant, which lacks a mitochondrial ABC transporter that shows closest homology to mammalian ABCB7.[73] It has been proposed that the function of ABCB6 is similar to that of ABCB7, a mitochondrial iron transporter important in both Fe−S cluster biogenesis and hematopoeisis.[74] It is thus unclear that these proteins are functional orthologues since ABCB6 localizes to the OM, while ABCB7 and Atm1p localize to the IM.

To further muddy the waters, two groups have reported that ABCB6 is also localized to the plasma membrane and the Golgi.[75,76] One group observed two different forms of ABCB6, with the lower molecular weight form being localized predominately to the OM. The major higher molecular weight species, found on the plasma membrane, was capable of transporting iodoarylazido prazosin and pheophorbide A—a porphyrin previously shown to be transported by ABCG2 (also known as BCRP, discussed in section 6.3 of this review).[77] In contrast to mitochondrial ABCB6, plasma membrane-localized ABCB6 not only transported hemin poorly but was incapable of transporting CPIII.[75] It was reasoned that the Golgi-localized ABCB6 lacks an amino-terminal presequence found in mitochondria-localized ABCB6.[78] While these studies do not rule out the possibility that ABCB6 can transport hemes or porphyrins, the authors contest that it does so in an ER-derived secretory organelle, where the majority of ABC transporters function.[76] In summary, the role of ABCB6 in the transport of porphyrin intermediates remains unclear. It may be true that ABCB6 localizes to and functions in different regions of the cell, but more experiments are needed to definitely show that ABCB6 transports the physiological heme intermediate CPgenIII.

It has been proposed that the 2-oxoglutarate carrier (OGC) plays a compensatory role and transports enough CPgenIII to mask a phenotype of a loss-of-function CPgenIII transporter (Figure 3A). In this regard, one study using HeLa, HepG2, and A172 (glioma) human cell lines showed that two fluorescent, nonphysiological porphyrin derivatives—palladium *meso*-tetra(4-carboxyphenyl)porphyrin (PdTCPP) and palladium *meso*-tetra(4-aminophenyl)porphyrin (PdTAPP)—accumulated in the mitochondria (Figure 3B).[79] Various techniques were used to show that this porphyrin derivative bound to OGC on the mitochondrial IM. Transport of 2-oxoglutarate was also shown to be inhibited by different porphyrin derivatives including PPIX, CPIII, and hemin.[79] A major caveat in this study is that the work was performed using synthetic porphyrin derivatives that have no physiological or structural resemblance to CPgenIII. Moreover, it is unexpected that a protein designed to transport 2-oxoglutarate would be able to transport the large cyclic porphyrin derivatives used in the study (Figure 3B).

3.1.3. Protoporphyrin IX is Shuttled from the Intermembrane Space into the Inner Mitochondrial Membrane

The third time during heme synthesis that a membrane must be crossed is when PPIX, produced by PPOX, is delivered to FECH. There must be a mechanism for PPIX to translocate from the IMS side to the matrix side of the IM where FECH is present. This requires either a transporter to shuttle PPIX across the mitochondrial IM or direct transfer of PPIX from PPOX to FECH by protein−protein interaction. When the crystal structure of PPOX was solved, it was suggested that one of the three domains served as a membrane binding domain and reported that the protein purified as a homodimer.[63] It has been hypothesized, based on experiments performed using solubilized mitochondrial membrane-bound and vesicle-reconstituted enzymes from mouse, that PPOX and FECH form a complex to protect the light-sensitive PPIX.[62,69] This prediction is supported by results from *in vitro* and *in vivo* bacterial experiments that were consistent with the existence of a complex between PPOX and FECH, but not CPOX.[80]

This neat and tidy portrayal of PPIX transfer across a membrane from PPOX to FECH may hold true in animal cells and in Gram-negative bacteria, but, in Gram-positive bacteria, these enzymes exist as soluble, nonmembrane associated proteins. Moreover, in photosynthetic bacteria and plants that synthesize chlorophyll, the majority of PPIX is diverted to the synthesis of chlorophyll by Mg-chelatase.[81,82] Thus, the coordination of heme and chlorophyll synthesis, and the distribution of PPIX between the two pathways in plants, is a complex process requiring a paradigm shift from the canonical heme synthesis pathway found in animals.[59,83] Tetrapyrrole synthesis is particularly complex for plants where ALA is exclusively synthesized via the C5 (glutamate) pathway in plastids (of which the chloroplasts are a specialized type).[84] In addition to chlorophyll synthesis, plastids are also the major site of heme synthesis in plants.[85] A small amount of heme is synthesized in plant mitochondria, however, which harbor mitochondria-specific PPOX and FECH isozymes.[86,87] It follows, then, that plants possess plastid-localized transporters that function as PPgenIX and heme exporters; little is presently known about these exporters. Plant mitochondria, therefore, must contain machinery for import of PPgenIX like yeast,[32] and potentially also for import of heme like *C. elegans*, a heme auxotroph.[88] The identity of such transporters remains to be determined.

3.1.4. Heme Must Exit the Mitochondria after Synthesis

After synthesis, heme must be trafficked from the matrix side of the IM to other parts of the cell where many hemoproteins reside. Not surprisingly, even this step is not as simple as it seems. Some heme-containing proteins—e.g., cytochrome *c* oxidases—function in the IMS (Figure 2). This raises a number of questions. Is all newly synthesized heme

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Table 1. Putative Heme/Porphyrin Transporters

protein name	proposed function	location of function	reference
HRG-1 (SLC48A1)	transport heme	vesicles	Rajagopal et al.
FLVCR	export heme	RBC plasma membrane	Quigley et al. 2004
PCFT (formerly HCP-1)	import heme?	plasma membrane	Shayeghi et al., Qiu et al.
ABCG2 (BCRP)	export heme	RBC plasma membrane	Krishnamurthy et al. 2004
ABCB7 & Mfrn	transport Fe for heme synthesis	inner mitochondrial membrane	Shaw et al., Taketani et al. 2003
ABCB6	transport coproporphyrinogen III?	outer mitochondrial membrane	Krishnamurthy et al. 2006
OGC	transport coproporphyrinogen III?	outer mitochondrial membrane	Kabe et al.
ABC-me (M-ABC2/ABCB10)	transport heme intermediates	inner mitochondrial membrane	Shirihai et al., Zhang et al.

transported to the cytoplasm, with a small amount returned to the IMS? Or is some of the newly synthesized heme "siphoned off" in the IMS as the heme is transported through two membranes to the cytoplasm (Figure 2)? Once in the cytoplasm, where and how does the heme move? The latter issue will be covered in more detail in sections 3.5−3.7.

3.2. Movement of Heme Intermediates Between Cytosolic Enzymes

An excess of heme derivatives is deleterious to cells. Hemes, and the porphyrins from which they are made, are hydrophobic. A strong case can be made that the products of each enzyme in the heme synthesis pathway must be delivered to the next enzyme in the pathway in a timely and efficient manner. How ALA is shuttled from the mitochondrial matrix to the first cytosolic enzyme, ALAD is not known; nor is it known how each successive product is delivered to the subsequent three cytosolic enzymes.

3.2.1. Argument for Substrate Channeling

Since ALAD is a complex consisting of eight identical subunits with a combined relative molecular mass of 250 000−300 000,[27,49,89] this octamer could serve as a scaffold to which the other three cytosolic heme synthesis enzymes dock and facilitate the direct transfer of heme intermediates. Substrate channeling, the process in which the product of one enzyme is passed directly to another enzyme without being released into solution, eliminates the necessity of chaperones by providing an efficient and protected transfer of reagents between enzymes.[90−95] The necessity of such channeling in heme synthesis can be illustrated by the fact that HMB can form toxic uroporphyrinogen I nonenzymatically.[27] In the presence of UROS, however, HMB is converted to UROgenIII. In bacteria, the genes encoding ALAD and UROS are often organized in an operon.[96,97] It has been suggested that this might ensure that a complex of both enzymes is formed in order to prevent solvent exposure and spontaneous cyclization of the unstable intermediate HMB to UrogenIII.[27] This implies that PBGD is part of the same complex. In this regard, the PBGD of *Plasmodium falciparum* has UROS activity,[98] which is consistent with the multienzyme complex hypothesis.

3.2.2. Argument against Substrate Channeling

The argument against an uninterrupted substrate channel between all four cytosolic heme enzymes is based on the observations that, in plants,[99,100] yeast,[101] and bacteria,[102] some UROgenIII must be removed from the heme synthesis pathway in order to produce siroheme. Additional UROgenIII is needed for conversion to vitamin B_{12} in bacteria that synthesize this cofactor[103] and for conversion to coenzyme F_{430} in methanogenic bacteria.[104−106] These observations raise the possibility that the proposed multienzyme complex is dynamically associating and dissociating.

3.3. Movement of Protoporphyrinogen IX through the Intermembrane Space

CPOX, located on the IMS side of the OM, converts CPgenIII into PPgenIX. PPOX, located on the IMS side of the IM, subsequently oxidizes PPgenIX to PPIX. PPgenIX must therefore cross the IMS that separates the OM and the IM. There are at least two possibilities as to how this occurs. A chaperone or chaperones could relay the newly formed PPgenIX to PPOX, where PPgenIX is converted to PPIX. Since the bilayers are in close proximity, it seems that the chaperone could even be a membrane-bound protein like CcmE or CcsBA (discussed in section 3.6). Alternately, due to the spatial arrangement of the final three enzymes and the chemical reactivity of intermediates, it has been suggested that CPOX, PPOX, and FECH may form a transient complex to facilitate the terminal steps of heme synthesis.[62,107] This complex may also include mitoferrin, a mitochondrial iron transporter located on the IM that is essential for heme formation.[108] Currently, there is no direct experimental evidence to support this hypothesis. How this complex might form is discussed in section 3.7.2.

3.4. Possible Role of ABC Proteins in the Transport of Heme Intermediates

ABC transporters have been implicated in mediating transport of components required for heme biosynthesis and hematopoiesis, although the ligands of these transporters remain unknown (Table 1). Two recent publications have demonstrated that the mitochondrial transporter ABCB7 interacts with FECH, suggesting that this ABC transporter was somehow involved in the expression of FECH.[109] Follow-up studies showed that the *Abcb7* knockout was embryonic lethal in mice.[110] ABCB7 appears essential for hematopoiesis. Partial loss-of-function mutations in *Abcb7* resulted in X-linked sideroblastic anemia with ataxia.[110] Loss of function of *Abcb7* may alter the availability of reduced iron for heme synthesis.[110] While providing iron to FECH for incorporation into PPIX is essential for heme synthesis, ABCB7 does not appear to transport either PPIX or heme. ABCB7 participates in cytosolic iron−sulfur cluster biogenesis.[111] ABCB7 is a functional orthologue of yeast Atm1p, a mitochondrial ABC transporter involved in transporting Fe/S clusters from the mitochondria to the cytosol. The growth, mitochondrial cytochromes, and cytosolic iron levels of a Δ*atm1* mutant yeast strain were restored to wild-type levels when transformed with human ABCB7.[112]

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Table 2. Putative Heme Binding Proteins

protein name	proposed function	reference
L-FABP	bind heme/tetrapyrroles in cytosol	Vincent and Müller-Eberhard
GST	bind heme/tetrapyrroles in cytosol	Ketley et al.
HBP23	bind heme/tetrapyrroles in cytosol	Iwahara et al.
SOUL/p22HBP family	bind heme/tetrapyrroles in cytosol	Taketani et al. 1998, Zylka and Reppert
haptoglobin	bind extracellular Hb in blood	Wejman et al.
hemopexin	bind extracellular heme in blood	Hrkal et al.
HDL and LDL	bind extracellular heme in blood	Miller and Shaklai
human serum albumin	bind extracellular heme in blood	Adams and Berman

A second transporter implicated in the transport of heme pathway intermediates is ABC mitochondrial erythroid (ABC-me). This transporter was identified using a subtractive analysis in G1E cells (an erythroid line from GATA-1-deficient murine embryonic stem cells) to identify genes regulated by GATA-1.[113] ABC-me localizes to the IM and is induced during erythroid maturation. Overexpression of ABC-me induces Hb synthesis in erythroleukemia cells. Because of the expression pattern, mitochondrial localization, and overexpression effects of ABC-me, the authors proposed that ABC-me may mediate the transport of one or more intermediates of the heme biosynthesis pathway.[113] The human homologue was named M-ABC2 (mitochondrial ABC2) since it was the second mitochondrial ABC transporter to be identified.[114] Since M-ABC2, also known as ABCB10, is expressed at the highest levels in the bone marrow and is upregulated during erythropoiesis, it was postulated that M-ABC2 is involved in hematopoiesis.[115] Functional proof for heme transport activity of M-ABC2 remains to be determined.

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3.5. Cytosolic Heme Binding Proteins

Evidence exists to show that cytosolic heme/porphyrin binding proteins may be important players in the heme biosynthesis pathway. These include the liver fatty acid binding protein (L-FABP),[116] the abundant yet poorly understood glutathione S-transferases,[117−120] and the better characterized heme-binding proteins (HBPs)[121,122] (Table 2).

The HBPs are classified according to their molecular mass. HBP23 (a 23 kDa protein belonging to the antioxidant family of peroxiredoxins) is present in the cytosol of liver, kidney, spleen, small intestine, and heart, with the liver showing the highest content.[121] HBP23 mRNA levels were increased in rat primary hepatocytes in response to heme and PPIX as well as Sn-, Co-, and Zn-protoporphyrin.[123] This could be an indication that HBP23 is involved in heme and tetrapyrrole scavenging instead of specifically binding heme.

The protein p22HBP was purified from mouse liver and was so named because the purified protein ran slightly above the 20 kDa marker on a denaturing polyacrylamide gel and bound PPIX, CPIII, and bilirubin. It contains 190 amino acids and was found to be expressed in numerous tissues.[122] After a more thorough characterization of both the mouse and human proteins, it was suggested that this p22HBP was not a specific heme binding protein but rather a protein that binds tetrapyrroles indiscriminately.[124] In spite of its crystal structure being determined and its identification in murine erythyroleukemia cells as part of a protein complex that bound exogenous heme, no definite function has been assigned to this protein.[125,126]

SOUL is a protein with 44% amino acid sequence similarity to p22HBP and is expressed exclusively in the retina and pineal gland.[127,128] Human and zebrafish proteins with homology to SOUL and p22HBP are categorized into the SOUL/p22HBP family, even though their heme binding mechanisms and tissue expression patterns differ.[128] Six members of this family have been identified in *Arabidopsis*, and heme binding of two representatives was demonstrated to be reversible in vitro.[129] Furthermore, four SOUL family members in *Arabidopsis* were highly upregulated by phytochromes—light sensory photoreceptor proteins that are covalently linked with a linear tetrapyrrole bilin chromophore.[130,131] Each of the four HBPs was shown to function in a different plant tissue, seemingly indicating that these proteins would make suitable intracellular heme transporters/chaperones. It remains to be seen what role, if any, HBPs play in the trafficking of heme and tetrapyrroles.

3.6. Heme Chaperones

After heme is exported from the mitochondria, it must be incorporated into a multitude of hemoproteins that organisms and cells produce. Surprisingly, little is known about when and where heme is incorporated into proteins, which could occur either cotranslationally or post-translationally. Post-translational incorporation of copper has been demonstrated for cuproproteins.[132,133] Whether designated chaperones exist for heme incorporation into apo-proteins within membrane compartments remains unknown (Figures 2 and 4A). It is feasible that multiple pathways are utilized for heme insertion, and these are specific to target hemoproteins or the organelle in which the proteins reside.

In several strains of bacteria and in *Arabidopsis*, CcmE, a protein essential for cytochrome *c* protein maturation, is a known heme chaperone. It is a membrane-bound protein that covalently binds heme to a single histidine residue 29 amino acids from its carboxyl terminus.[134−136] CcmE functions to deliver heme to apo-cytochrome *c*, and organisms in which the heme-binding residue in CcmE is mutated fail to produce holo-cytochrome *c*.[137] CcmE retains its heme-binding capabilities even when expressed in a soluble form.[137]

While wild-type CcmE protein is membrane-bound, a number of soluble, periplasmic, bacterial heme-binding proteins have been genetically identified from pathogenic strains, and all are 30−90% identical at the amino acid level.[138] It has been proposed that these periplasmic heme-transport proteins—as has been shown for PhuT, a periplasmic heme transport protein in *Pseudomonas* spp.—bind heme and deliver it to an ABC transporter responsible for transporting heme across the cytoplasmic membrane.[139] It remains to be seen whether all the identified proteins will have heme-binding and/or heme-delivery properties.[140]

Another bacterial protein, CcsBA, which contains 10 transmembrane domains and is a member of a superfamily of integral membrane proteins containing a tryptophan-rich domain, was shown to function in cytochrome *c* biosynthesis in the periplasm.[141,142] Using purified recombinant CcsBA,

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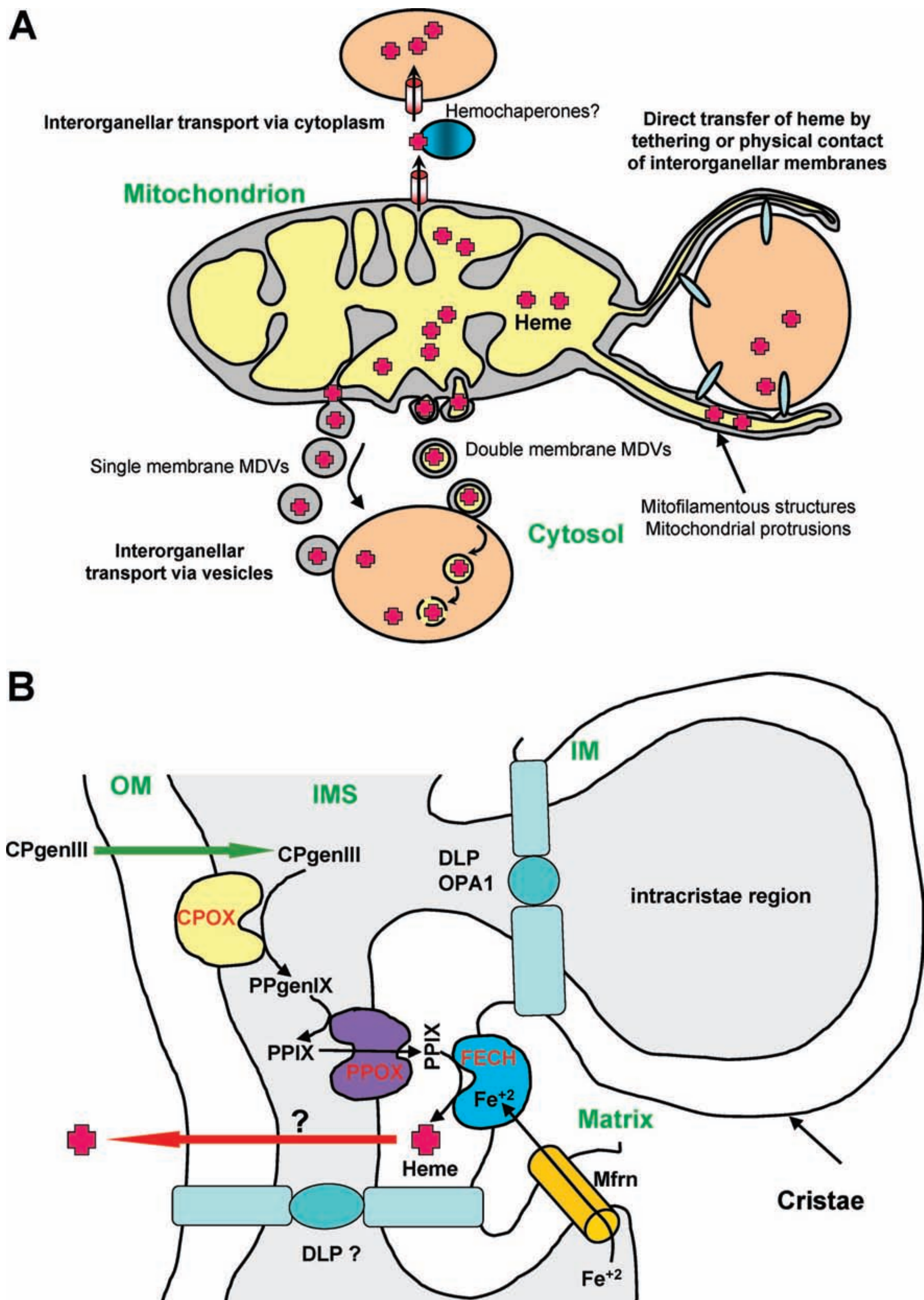

Figure 4. Model for heme transfer from the mitochondria to intracellular organelles. (A) On the basis of models for transfer of other metabolites, it is possible that heme can be directly transported by either membrane-bound transporters and chaperones via the cytoplasm (top) or by physical contact between the mitochondria and organelle (e.g., endoplasmic reticulum) using membrane tethering proteins (right). Interorganellar heme transfer could also be mediated by either single- or double-membrane mitochondrial-derived vesicles (MDVs) to deliver mitochondrial cargo (bottom) by membrane fusion. (B) Transport of heme synthesis intermediates in the mitochondria. Conversion of CPgenIII to heme is catalyzed by three membrane-associated enzymes: CPOX located on the OM facing the IMS, PPOX on the IM facing the IMS, and FECH located on the matrix side of the IM facing the IMS. A possible mechanism for transfer of heme intermediates between the three enzymes would be to bring them in close proximity using tethering proteins. For instance, the dynamin-like protein (DLP) OPA1 resides on the IM to form tight cristae and intracristae regions that contain proteases and cytochrome *c*. A similar process might also take place to tether the OM and IM to form junctions for metabolite transfer during heme synthesis. Heme synthesized in the IM could be transported out of the mitochondria to organelles by either membrane-associated chaperones, transmembrane transporters, or direct transfer via the OM by interorganellar membrane tethering.

it was demonstrated that two pairs of highly conserved histidines were essential for forming ligands with the heme on the cytosolic side and for protecting the heme from being oxidized as it passes through the membrane from the cytoplasm to the periplasm.[141]

Internalized heme in the bacterial cytoplasm can be sequestered by cytosolic heme binding proteins such as the *Shigella* heme uptake protein S (ShuS) or *Pseudomonas* heme uptake protein S (PhuS). Both ShuS and PhuS, as well as HemS from *Yersinia enterolitica*, possess the ability to bind and sequester heme.[143−145] While many heme-binding proteins share a common motif such as CXXCH or CXXC, it takes but a single residue in the right environment to bind heme, as evidenced by the heme chaperone CcmE.[146] This makes it difficult in silico to identify heme binding proteins using linear protein sequences. In addition to binding heme, PhuS has been shown to be able to deliver heme to a protein with heme oxygenase function.[147] Originally thought to be a heme oxygenase, HmuS from *Y. pestis* is now thought to function as a heme storage protein since mutant strains with inactivated *hmuS* were able to utilize heme as an iron source.[139,148]

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3.7. Proposed "Mechanisms" for Interorganellar Heme Transfer

The examples of bacterial heme transfer pathways detailed in the previous section serve to underscore the existence of specific proteins that exist to temporarily sequester and safely transport heme. In exiting the mitochondria in eukaryotes, heme might bind a protein functionally equivalent to CcmE or PhuS and subsequently dock with an apo-hemoprotein or be chaperoned to one of a number of subcellular destinations where it would be delivered to an apo-hemoprotein (Figure 2). It is necessary to emphasize, however, that scientists have very little information about where heme is inserted into proteins in metazoans. It is unknown whether this occurs in the ER, the Golgi, or in the organelles where the holo-protein functions (e.g., catalase in the peroxisome). Some insight as to where heme can be inserted into apo-proteins in the secretory pathway was gained by work performed using myeloperoxidase and Brefeldin A (BFA). Myeloperoxidase is a heme-containing enzyme that manufactures hypochlorous acid in the lysosomes of neutrophils, and BFA is a fungal metabolite that interferes with the transport of proteins from the ER to the Golgi apparatus. In BFA-treated cells, heme was inserted into myeloperoxidase, but myeloperoxidase was not properly processed and did not traffic to the lysosomes, indicating that myeloperoxidase receives its heme in the ER.[149] In spite of all the work currently being done to understand the function of heme-containing proteins, it has not been determined how the heme is inserted into apo-hemo-proteins.

While it is possible that chaperones may transport heme from the mitochondria to organelles, it is also possible that other or additional mechanisms exist to deliver endogenous heme from the mitochondria to its target organelle. Several of these are explained below. It is important to keep in mind that, although these cellular processes have been determined experimentally, whether heme utilizes any of these processes has not been demonstrated.

3.7.1. Role of Mitofusin in Mitochondrial Remodeling

Long thought to be static organelles, a growing body of work has shown that mitochondria are dynamic, capable of changing morphology and joining with and splitting from other mitochondria.[150−152] Mutations in genes involved in these processes have been demonstrated to cause neuropathies.[153,154] One of the many proteins involved in fusion and fission of mitochondria is mitofusin 2, a large dynamin-like GTPase that spans the OM.[155] Mitofusin 2 has recently been shown to be involved in fusing the ER membrane with the mitochondria.[156,157] Although the authors of the studies were interested in this interorganellar connection because of the ramifications in Ca^{2+} transfer and signaling, we find this melding of membranes intriguing because of its implication for heme transfer. Could heme be passed directly from the mitochondria to the ER, much like Ca^{2+} transfer during IP_3 mediated signaling (Figure 4A and B)? Since mitofusin 2 is normally located on the mitochondrial OM and the ER, heme would still need to cross the mitochondrial IM.

3.7.2. Role of Mitofilin in Morphology of Cristae

Other data has brought to light that the dynamin-like protein (DLP) mitofilin/optic atrophy 1 (OPA1) is essential for the maintenance of the proper morphology of the cristae—folds of the IM.[158] It is feasible that concerted action of an OM protein, an IMS protein,[159] and an IM protein could control the flow of heme from the matrix to the ER. Conversely, if DLPs tether the IM and OM at specific points in the mitochondria, similar to OPA1 tethering cristae to form intracristae regions for storage of cytochrome *c*, then CPOX, PPOX, and FECH would be placed in close enough proximity to each other for rapid transfer of porphyrin intermediates for heme synthesis (Figure 4B).

3.7.3. ERMES-Facilitated Transfer of Heme

In *Saccharomyces cerevisiae*, Mmm1, Mdm10, Mdm12, and Mdm34 have been identified as the components of a complex that forms a molecular tether between the ER and mitochondria.[160] It was demonstrated that this complex, referred to as the ER−mitochondria encounter structure (ERMES), functions as a molecular zipper between the ER and the mitochondria. Mutation of a single component disrupts normal assembly of the complex. A synthetic biology screen was conducted by analyzing the interaction patterns of 1 493 genes in >700 000 double mutants. These studies resulted in the identification of two unknown genes, *GEM1* and *PSD1*, which showed strong correlation to every ERMES gene. On the basis of this and other[161] work, the ERMES complex is proposed to be part of a large macromolecular assembly responsible for recruiting other proteins that facilitate the exchange of phospholipids and other materials (for example, heme) between the ER and the mitochondria.

3.7.4. Mitochondrial-Derived Vesicles

Mitochondria have been shown to give rise to single-membrane and double-membrane bound mitochondrial-derived vesicles (MDVs) that are 70−100 nm in diameter and often contain mitochondria-anchored protein ligase (MAPL). MDVs can fuse with peroxisomes, and a subset of peroxisomes also possess MDV markers.[162] Interestingly, MDVs that contain MAPL lack the OM protein TOM20, while MDVs that contain TOM20 lack MAPL. These

observations suggest that MDVs are able to discriminate between types of cargo loaded within.[162,163] If part of the OM were contiguous with the secretory pathway, it is easy to envisage that many types of mitochondrial components could be transported by cargo vesicles between compartments (Figure 4A). Transfer of heme via such an intermediate compartment (e.g., vesicle) would not only minimize release of cytotoxic heme-iron but also facilitate regulated movement of sequestered heme and instant availability for specific cellular processes that require intracellular signaling (Figure 4A).

3.7.5. Interorganellar Stromules

In the early 1960s, several papers from Wildman and colleagues demonstrated that the chloroplasts of wild-type plants can develop tubular structures and protrusions.[164−166] These findings were largely forgotten until recently.[167,168] The term "stromules" was coined to described these stroma-filled tubules.[168] Stromules can form between and connect plastids, and both fluorescently tagged chloroplast protein complexes and green fluorescent protein have been observed to move through stromules from one plastid to another.[169] In the stroma, proteins traffic by diffusion as well as by an active process of directional travel, whose mechanism is unknown. It is thought that, besides functioning to aid in the exchange of materials between plastids, stromules play a role in facilitating exchange of materials between plastids and other organelles.[170,171]

All six of the above mechanisms allow for the possibility of heme to be transported between organelles such as the mitochondria and the ER. These scenarios would be sufficient to explain how heme could be incorporated into only proteins that are processed in the secretory pathway, i.e., proteins that are synthesized in the ER, are processed in the Golgi, and function either outside the cell or in the lumen of an intracellular organelle. These proposed mechanisms do not explain how heme is incorporated into soluble proteins that function in the cytoplasm, e.g., globins and soluble guanylate cyclases. These soluble proteins could acquire heme in the cytoplasm either via an intermediate vesicular storage compartment derived from the secretory pathway, or directly from the mitochondria, the source of endogenous heme. Adding another layer of complexity to heme acquisition by proteins is whether insertion of heme is cotranslational or post-translational.

4. Heme Uptake in Gram-Negative Bacteria

Heme uptake and transport have been well-characterized in Gram-negative bacteria, many of which are pathogenic.[172,173] In order to cause infection in the host, pathogenic bacteria must have adequate iron levels. To defend against pathogens, the host often restricts access to iron and heme. This poses a serious problem that the bacteria must overcome to survive, and it has been shown that virulence of pathogenic bacteria can be affected by targeting heme uptake pathways. Mutations in heme-iron uptake genes in *Staphylococcus aureus* reduced its pathogenicity in the invertebrate animal model, *C. elegans*.[174] In *Bordatella pertussis*, the bacterial agent responsible for whooping cough in humans, heme utilization by the bacteria has been shown to contribute to bacterial pathogenesis in the mouse infection model.[175] The general mechanisms of heme acquisition in pathogenic Gram-negative bacteria are demonstrated in Figure 5. Such bacteria can be classified into the following two groups: those that directly bind heme or hemoproteins by specific outer membrane receptors and those that secrete hemophores that bind heme and deliver it to specific receptors.[176]

4.1. Pathogenic Gram-Negative Bacteria That Bind Heme or Hemoproteins

There are 29 outer membrane heme receptors that have been identified from different species of Gram-negative bacteria. Although these receptors share between 21% and 90% similarity with one another, all share the highly conserved amino acid motif FRAP(10X)H(XX)NPL(2X)E.[138] The outer membrane receptors can be divided into subgroups (Figure 5A). The first subgroup of outer membrane receptors is not substrate-specific and binds either heme or heme-containing proteins. Such receptors are found in *Yersinia enterocolitica*, *Escherichia coli*, and *Shigella*.[176] Outer membrane receptors such as HemR bind heme and actively transport it across the outer membrane into the bacterial periplasm. The second subgroup of receptors is substrate-specific and has high affinity binding sites for one or two particular heme sources such as Hb and haptoglobin−hemoglobin complexes.[176]

4.2. Pathogenic Gram-Negative Bacteria That Secrete and Bind Hemophores

Several Gram-negative bacteria have ABC transporters that secrete small extracellular proteins called hemophores.[177] Hemophores bind heme and deliver it to specific outer membrane receptors. One of the best-studied examples is the heme acquisition system (HasA-HasR) in *Serratia marcescens*. Hemophores usually bind free heme or Hb. However, the hemophore secreted by *S. marcescens*, HasA, has been shown to extract heme from hemoproteins. The free heme is then delivered to the outer membrane receptor HasR.[178,179] HasR can transport either free heme or heme from Hb, but the presence of HasA promotes heme uptake to a greater extent. The mechanism of heme transfer from Hb to HasA was not understood until recently. It was previously thought that Hb lost its heme moiety in solution, and the free heme was then bound by HasA.[180] The crystal structures proved that HasA docks to HasR, and that heme is induced to be transferred to a lower affinity site in the receptor by a combination of exergonic complex formation and steric displacement.[181] Similar hemophore-mediated heme acquisition systems have been identified in *Porphyromonas gingivalis* and the human pathogenic strain of *Escherichia coli*.[182,183]

4.3. Transport of Heme from the Outer Membrane Receptor to the Cytoplasm in Pathogenic Gram-Negative Bacteria

Once bound by the receptor, the movement of heme into the bacteria is similar and is not dependent on the type of receptor that bound it. An intact Ton system, with the cytoplasmic membrane proteins ExbB and ExbD and membrane-anchored protein TonB, is required to provide energy for heme transport across the outer membrane (Figure 5B). The TonB system uses a proton-motive force for the passage of heme into the periplasm. Heme is bound

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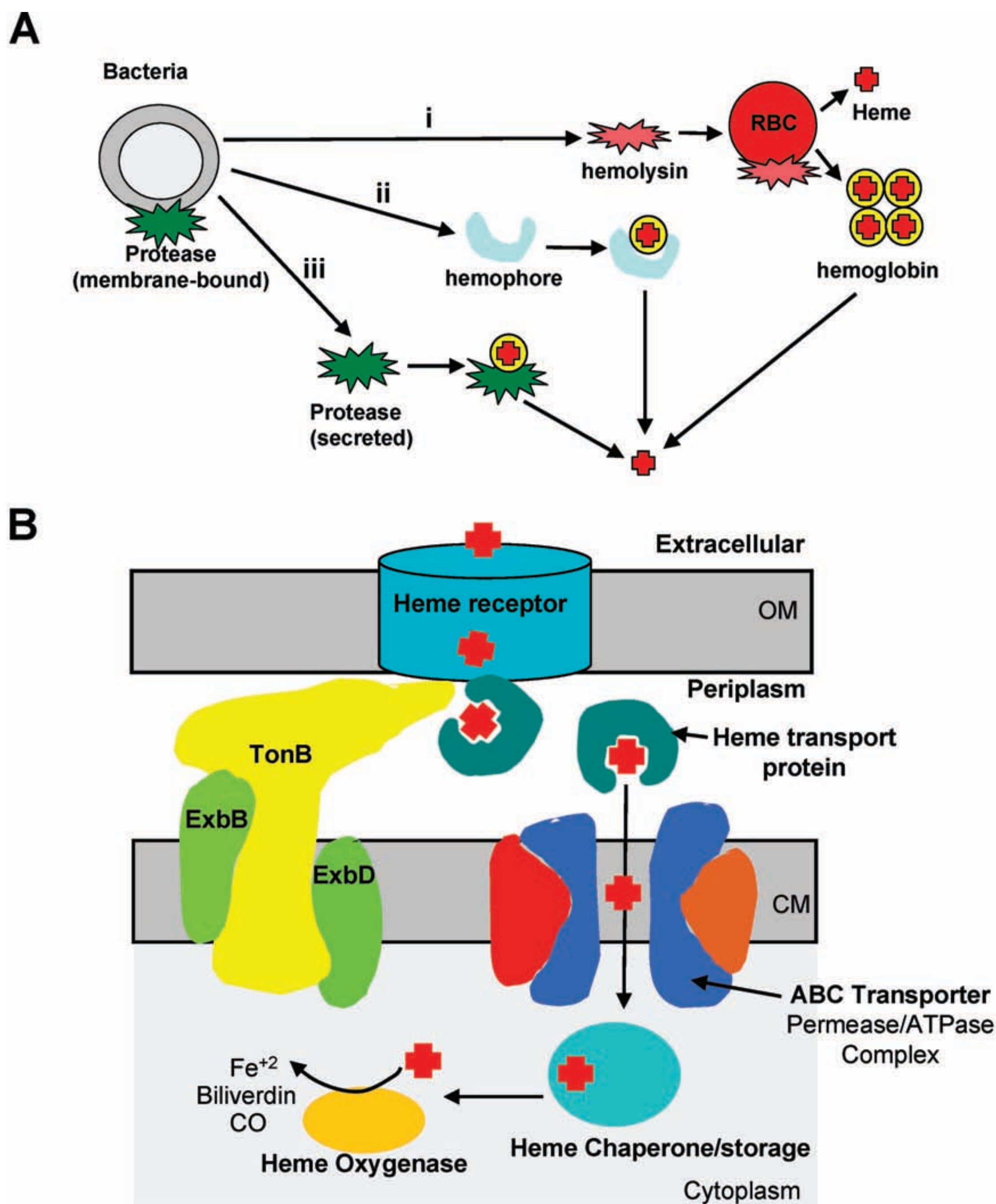

Figure 5. Heme acquisition by gram-negative bacteria. (A) Hemolysin secreted by bacteria degrades red blood cells (RBCs), releasing hemoglobin and heme. Once released, heme may be transported into the bacterial cell by different mechanisms: (i) direct binding of hemoglobin or heme to a specific TonB-dependent outer membrane receptor, which results in transport of heme into the periplasm; (ii) capture of hemoproteins such as hemoglobin or hemopexin by hemophores, which delivers these hemoproteins to a specific TonB-dependent outer membrane receptor, and (iii) degradation of hemoproteins by either membrane-bound or secreted bacterial proteases to release heme. (B) Heme derived from hemoproteins binds to specific outer membrane (OM) receptors. The energy for heme transport is provided by the energy transducing complex TonB in association with helper proteins ExbB and ExbD. Internalized heme in the periplasm is bound to periplasmic heme transport protein, which ferries heme to the cytoplasmic membrane (CM) ABC transporter complex, a system composed of a membrane-associated permease and an ATPase. Heme translocated to the cytoplasm is either degraded by heme oxygenases or sequestered by heme storage proteins or chaperones.

by a periplasmic heme-binding protein such as HemT and then delivered to permeases on the cytoplasmic membrane, like HemU and HemV.[184] Transport of heme across the cytoplasmic membrane requires expenditure of ATP. In the cytoplasm, HemS and HemO proteins process heme to release iron.

5. Heme Uptake in *Candida albicans* and *Saccharomyces cerevisiae*

While several bacterial heme uptake and transport systems have been well-characterized, only recently have proteins involved in heme uptake been identified in eukaryotes. In

the pathogenic yeast *Candida albicans*, *RBT5* and *RBT51* have been identified as heme uptake genes. Rbt5p and Rbt51p are mannosylated proteins, and Rbt5p is highly induced upon iron starvation.[185] Despite 70% homology between the two genes in *C. albicans*, *RBT5* played a more important role than RBT51 in the utilization of heme as an iron source. Disruptions in these genes did not reduce the virulence of *C. albicans* in murine models.[186] *C. albicans* possesses a heme-oxygenase enzyme (*CaHMX1*) that shares 25% identity with the human heme oxygenase, and CaHMX1 has been shown to be positively regulated by both heme and Hb.[187] Whether heme-iron utilization is essential for pathogenicity could be best addressed by using double or triple mutants in RBT and HMX1.[188]

Unlike pathogenic yeast, the budding yeast *Saccharomyces cerevisiae* utilize exogenous heme very poorly, and it has been reported that heme uptake is induced under conditions of heme starvation.[189] In these organisms, heme synthesis requires oxygen. A microarray analysis of cells grown under reduced oxygen tension or reduced intracellular heme conditions revealed that overexpression of protoporphyrin uptake gene 1 (PUG1) resulted in reduced utilization of exogenous heme but increased utilization of PPIX by a heme-deficient strain.[189] Pug1p was localized to the plasma membrane by indirect immunofluorescence and subcellular fractionation. Strains overexpressing PUG1 exhibited decreased accumulation of [^{55}Fe]hemin but increased accumulation of PPIX compared to the wild-type strain.[189] These data seem to indicate that Pug1p plays a role in PPIX influx and heme efflux.

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6. Mammalian Heme Transport

6.1. Heme and Nutritional Iron Deficiency

6.1.1. Prevalence of Iron Deficiency

Iron is a critical micronutrient because it readily participates in a wide variety of biochemical oxidation−reduction reactions. Iron is one of the most abundant transition metals present on Earth, yet iron deficiency is a global diet-related health problem.[190] Iron deficiency is characterized by diminished iron stores such that there is insufficient iron to maintain the normal physiological function of tissues and is often determined by measuring serum ferritin levels.[191] Iron deficiency impairs physical and mental development in children worldwide.[192] It is the only nutrient deficiency which is significantly prevalent in industrialized countries.

6.1.2. Prevalence and Causes of Iron Deficiency Anemia

Approximately one-third of the world's population is anemic, a condition in which the body's iron levels are depleted to the point that they are insufficient to maintain normal levels of hemoglobin. Many of these two billion people are anemic due to iron deficiency. In resource-poor areas, iron deficiency anemia is frequently exacerbated by infectious diseases. In developing countries, complications from nutritional iron deficiency are compounded by malaria and hookworm infestations.[193−195] Hookworms induce anemia by feeding on blood from lacerated capillaries in the intestinal mucosa. The malarial parasite contributes to the etiology of anemia through direct destruction of parasitized red blood cells and sequestration of iron by the parasite. As a result, therapeutic regimens for treating both hookworm infections and malaria include iron supplements.[196−199] Major health consequences of iron deficiency anemia include poor pregnancy outcome, impaired physical and cognitive development, increased risk of morbidity in children, and reduced work productivity in adults. Anemia contributes to ∼20% of all maternal deaths.[200]

6.1.3. Intestinal Absorption of Iron and Heme

There are two major reasons that iron deficiency anemia is so prevalent. First, elemental Fe(II) readily oxidizes to Fe(III) under aerobic conditions at near neutral pH to form insoluble ferric hydroxide or hydroxyl−iron dimers that are poorly absorbed by the small intestine. Second, plant compounds, such as tannins and phytates, can chelate free iron to form insoluble complexes that prevent iron absorption.[201−203] In contrast, heme, or iron−protoporphyrin IX, is absorbed more readily than inorganic iron. By some estimates, heme uptake is responsible for two-thirds of body iron despite the fact that heme constitutes only one-third of total dietary iron.[204,205] This can be attributed to the fact that heme is readily soluble at the pH of the small intestine where most absorption occurs. Moreover, heme uptake is not negatively influenced by plant compounds that interfere with iron absorption.[206] Despite the importance of heme as a crucial source of dietary iron, the mechanism by which heme absorption occurs through the intestinal cells is poorly understood. Biochemical studies have shown that heme is absorbed via the brush border present on the enterocytes; however, the proteins responsible for heme uptake remain unidentified.[207−209]

It is thought that heme uptake from the enterocytes is mediated by a receptor-mediated endocytic pathway. An early study showed the existence of a heme-binding protein on the microvilli of the upper small intestine of pigs,[210] and evidence for such an intestinal receptor has been reported in humans.[211] It has also been demonstrated that this high affinity binding is lost when a trypsin digestion is performed, indicating the existence of a receptor for heme.[212] Electron microscopy studies conducted after administration of heme or Hb into the duodenum from rats or dogs reported the presence of heme on the microvilli and the accumulation of heme in vesicles in the microvilli that were identified as secondary lysosomes.[209] Heme was absent from these vesicles approximately three hours after the initial dose, and heme was not observed in the basal region of the cells. These results provided strong evidence that heme uptake occurs via endocytosis, but the heme-binding receptor has yet to be identified.[209]

6.2. Heme Import

6.2.1. HCP-1

SLC46A1 was initially identified as a heme carrier protein and named HCP-1.[213] *Hcp-1* was isolated from mouse duodenum using a suppression subtractive hybridization screen using hypotransferrinemic mice.[214] *Hcp-1* encodes a protein of ∼50 kDa that localized to the plasma membrane. Functional studies in *Xenopus* oocytes expressing *Hcp-1* revealed a 2−3 fold increase in heme uptake with an apparent K_m of 125 μM. Uptake of radiolabeled heme from duodenal tissue was inhibited upon incubation with HCP-1 antibodies. However, more recent studies have shown that SLC46A1 is, in fact, a folate/proton symporter and transports folic acid

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with a K_m of 1.3 μM, resulting in the renaming of HCP-1 to the proton-coupled folate transporter (PCFT).[215] RNA interference assays of *PCFT* in CaCo-2 cells reduced pH-dependent folate uptake by 60–80%. Importantly, human patients with hereditary folate malabsorption carry a missense mutation in *PCFT* that leads to the formation of a nonfunctional protein.[215] Thus, PCFT has a low affinity heme transport activity in vitro but may not have any physiologic relevance.

6.2.2. HRG-1

The first bona fide heme importer to be identified was heme-responsive gene-1 (*hrg-1*). HRG-1 is the sole member of the solute carrier family 48, member 1 (SLC48A1), permease heme transporters. HRG-1 was initially identified using a genomic screen in *C. elegans*. Worms have four *hrg-1* paralogues, while a single homologue exists in humans.[216] In HEK293 cells, both the worm and human Hrg-1 colocalized to the late endosomal and lysosomal compartments; worm Hrg-4, a paralogue of Hrg-1, was present on the plasma membrane. It stands to reason that, given that worms do not make heme, they have redundant heme acquisition pathways via multiple Hrg-1 paralogues. Heterologous expression of *hrg-1* in *Xenopus* oocytes results in significant heme-induced inward currents, indicating heme-dependent transport across the plasma membrane. Genetic studies using morpholino knockdown in zebrafish *hrg-1* resulted in severe anemia, hydrocephalus, and a curved body with shortened yolk tube. All these defects were fully rescued with *C. elegans hrg-1*, which is ~20% identical to the zebrafish *hrg-1* at the amino acid level.[216] How HRG-1 functions at a molecular level and the cellular relevance for the fate of heme once it enters the cytoplasm are unclear.

6.3. Heme Export

Three heme exporters have been reported in erythroid cells. The first, feline leukemia virus receptor, was initially identified as a cell surface protein for feline leukemia virus subgroup C.[217] Infection of cats with this virus resulted in aplastic anemia, a type of anemia in which erythroid progenitor cells failed to mature from burst-forming units to the colony-forming units-erythroid cells. The genetic determinant of this virus-induced anemia was mapped to the surface envelope glycoprotein region 1 of the virus, which binds to the host receptor—feline leukemia virus subgroup C receptor or FLVCR.[218,219] FLVCR, a 60 kDa protein, is a member of the major facilitator superfamily (MFSD7B). FLVCR was expressed in different hematopoietic cells and showed weak expression in the fetal liver, pancreas, and kidney.[217] FLVCR mediated efflux of zinc mesoporphyrin in rat renal epithelial and hematopoietic K562 cells, and heme efflux mediated by FLVCR was important for erythroid differentiation.[220] FLVCR-null mice failed to undergo erythropoiesis and died at midgestation.[221] These mice also exhibited craniofacial and limb deformities reminiscent of patients with Diamond-Blackfan anemia, a severe but rare congenital erythroid anemia that presents in infancy. By using heme oxygenase and ferritin as reporters for heme and iron status in macrophages, the authors provided evidence that heme exported by FLVCR was essential during phagocytosis of senescent red blood cells by macrophages. These observations support earlier studies which demonstrated that not all heme is broken down in the macrophage.[222]

A second potential heme exporter in red blood cells, ABCG2, was initially identified as a protein that confers drug resistance in breast cancer cells (BCRP).[223] The putative role of ABCG2 in heme transport was discovered when ABCG2-null mice fed a modified diet developed skin photosensitivity.[224] This was due to the accumulation of pheophorbide a, a degradation product of chlorophyll with structural similarity to PPIX, suggesting a role for ABCG2 as an exporter of protoporphyrin compounds. Expression of ABCG2 is regulated by hypoxia-inducible factor 1, and it has been shown that ABCG2 transported porphyrins under hypoxic conditions.[225] An issue with the conclusions of this study is that the mice lacking the putative heme exporter ABCG2 did not have any phenotypes associated with defects in heme-dependent pathways other than the mild photosensitivity associated with accumulation of pheophorbide a.

It is hard to imagine that red blood cells (RBCs), which need to synthesize large amounts of heme for Hb synthesis, contain multiple heme or porphyrin exporters. Yet, as was described earlier in section 3.1.2 of this review, the high molecular weight isoform of ABCB6 localized to the plasma membrane of RBCs. Thus, three putative heme transporters on the cell surface—FLVCR, ABCG2, and ABCB6—would be exporting heme out of RBCs. It remains to be determined whether there is genetic redundancy or a hierarchy among these transporters for heme export.

6.4. Intercellular Heme Transport

Heme, complexed to Hb, is released from mammalian cells into the bloodstream, often in response to disease, inflammation, intravascular red blood cell hemolysis, or planned release from erythroid cells. In addition to having intrinsic peroxidase activity, heme is lipophilic and intercalates readily into and disrupts the lipid bilayers of cell membranes. The iron in heme can generate reactive oxygen species. Therefore, it is imperative that heme, as well as Hb, is not allowed to exist in a "free" or unbound state. Indeed, the concentration of "free", unbound heme is thought to be between 10^{-7} to 10^{-8} M.[226] Several molecules are able to bind heme or Hb with high affinity outside of cells.

6.4.1. Haptoglobin Binds Hemoglobin

Haptoglobin (*haptein* = "to bind"), a blood glycoprotein synthesized primarily by hepatocytes, is the only known molecule that binds Hb. There are three major subtypes of haptoglobin, which are the products of two closely related genes.[227] The three subtypes range in size from 100 to 500 kDa, but all bind Hb dimers with a $K_d \approx 10^{-12}$ M.[139,228,229] Haptoglobin–Hb complexes bind to the CD163 receptor on the surface of monocytes and macrophages, and these complexes are subsequently endocytosed.[230] The iron is freed by heme degradation and transported by plasma transferrin to the bone marrow for synthesis of new Hb.[231,232]

6.4.2. Hemopexin Binds Free Heme

Several serum proteins are able to bind free heme, plausibly because, in addition to free heme being reactive, it is also a potential source of iron for pathogens.[233,234] Hemopexin (HPX) is a blood plasma protein with a $K_d \approx 10^{-13}$ M for free heme.[235] It is present in the blood after red blood cell lysis, export of cellular heme, or release of heme from damaged tissue(s).[235,236] HPX–heme complexes are

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endocytosed in response to their binding the low-density lipoprotein (LDL) receptor-related protein, alias CD91 (LRP/CD91) in a variety of cells including hepatocytes, macrophages, and syncytiotrophoblasts.[237] The liver removes the heme moiety and recycles the apo-HPX protein and LRP/CD91 receptor.[237,238]

6.4.3. Other Serum Proteins That Bind Free Heme

Although no other molecule has been shown to bind heme with an affinity higher than HPX, three other serum proteins have been shown to bind heme. It is known that human serum albumin (HSA), the most abundant plasma protein, binds a wide variety of proteins as well as binding heme with a $K_d \approx 10^{-8}$ M.[239,240] The crystal structure shows that a hydrophobic cleft in one of its three subdomains binds heme. Three basic residues at the entrance to this cleft form charge-pair interactions with the propionate side chains of heme, and the iron in heme is ligated by a tyrosine residue.[241] How heme is removed from HSA is not yet known. In addition to HSA, both high- and low-density lipoproteins bind heme. Interestingly, they bind heme faster than both HPX and HSA with an affinity that is higher than that of HSA for heme ($K_d \approx 10^{-11}$ M).[242,243] It is thought that this rapid binding is important to prevent damage by heme during the initial release of heme and provides a buffer period for HPX and HSA to steadily but tightly bind heme. Eventually, HPX and HSA remove all but a residual amount of heme from the lipoproteins.[242] Exactly how HPX and HSA reverse the binding of heme to the lipoproteins and where this process occurs have not been determined.

6.5. Heme Transport and Body Iron Recycling

Humans have $\sim 2.5 \times 10^{13}$ RBCs, and >60% of the body's iron is used for Hb production in RBCs. Consequently, iron-deficiency anemia is the first and primary clinical manifestation of iron deficiency. About 33% of the protein in a typical RBC is Hb, which contains more than 1 billion iron atoms in the form of heme.[244] After a life-span of ~120 days, senescent RBCs are cleared from circulation by erythrophagocytosis in macrophages of the reticuloendothelial system (RES), and the heme-iron is recycled. The heme molecule is broken down into biliverdin, carbon monoxide, and iron. The bone marrow recycles this iron to produce heme for repackaging Hb in RBCs. Thus, the majority of iron reutilization by the body is via heme-iron, and genetic defects in the recycling of iron by macrophages result in anemia.[244]

Each day, the RES—comprising the spleen, bone-marrow-derived macrophages, and Kupffer cells of the liver—recycles ~25 mg of iron by ingesting and degrading ~360 billion senescent RBCs (~5 million/s).[232] When RBCs are lysed in the acidic environment of the endophagolysosome, a massive amount of heme is released into the lumen of these cellular compartments. Eventually, this heme must be broken down and the iron utilized, but how this is accomplished is poorly understood. The expression of heme oxygenase-1 increased dramatically in bone marrow-derived macrophages four hours after the phagocytosis of senescent RBCs.[245] A similar increase was observed in the RNA for the iron exporter ferroportin four hours after phagocytosis of RBCs. A full 12 h after phagocytosis of the RBCs, the levels of the mRNA for the iron storage protein, ferritin, were drastically higher. From their expression profiles, it is possible to develop a model of what may occur in the macrophage after feeding. An increased amount of heme oxygenase, the rate-limiting step in heme catabolism in macrophages,[246,247] is needed to degrade the heme. Ferroportin, the only iron exporter identified in mammals,[231] is required to export the iron out of the macrophages. Seemingly, macrophages store the excess iron in ferritin—a globular iron storage protein composed of 24 subunits and capable of sequestering ~4 500 iron atoms. Despite the importance of heme in macrophage-dependent iron recycling, the molecules and the pathways responsible for heme transport across phagolysosomal membranes remain to be identified experimentally.

7. Heme Auxotrophs as Models to Study Heme Transport

A major experimental obstacle in identifying molecules and pathways involved in heme homeostasis has been the inability to dissociate the tightly controlled biosynthesis and degradation of heme from intracellular heme trafficking events. Thus, there is a need to identify facile molecular tools and genetically tractable model systems to study heme homeostasis where synthesis and downstream trafficking pathways can be uncoupled for systematic identification of heme homeostasis genes.

7.1. *C. elegans* and Parasitic Nematodes

Caenorhabiditis elegans, a free-living nematode, is a natural heme auxotroph. This roundworm lacks all eight genes required to synthesize heme and acquires heme from its diet to sustain growth and development[88] (Figure 6). Heme biosynthetic enzyme activity measurements and radioisotope labeling experiments confirmed the genetic analyses that *C. elegans* lack the ability to make endogenous heme.[88] Because worms are heme auxotrophs, it stands to reason that they must have both a heme storage system to store essential but cytotoxic heme and an intercellular heme transport system to mobilize heme from intestinal cells—the site of absorption—to other cell types including neurons, muscles, hypodermis, and developing embryos (Figure 6).

Importantly, the phylogenetically related parasitic nematodes were found to also be heme auxotrophs.[88] Analysis of the genomes of these nematodes, however, indicates that they encode abundant hemoproteins. This suggests that they must acquire heme from their environment. Thus, heme transport pathways in parasites could be a potential therapeutic target for anthelmintics. This is especially relevant because more than two billion people are afflicted by helminthiasis, and global agricultural losses due to plant-parasitic nematodes are estimated to be approximately 125 billion dollars annually.[248−250]

Heme may also play a role in the pathogenicity of parasitic worms. Hookworms, like *C. elegans,* lack the ability to make heme. Hookworm infections due to *Ancylostoma ceylanicum* are prevalent in developing countries and are associated with iron deficiency anemia. The importance of host iron status as a key mediator of hookworm pathogenicity was demonstrated in a study in which hamsters were fed either an iron-replete diet or a diet with severe iron restriction and then subsequently infected with hookworms.[251] Animals fed a low-iron diet had reduced worm burden, possibly due to inadequate amounts of heme for hookworm metabolism.[88,251]

Due to morphological similarities, intestinal heme absorption in *C. elegans* may be comparable to dietary heme uptake

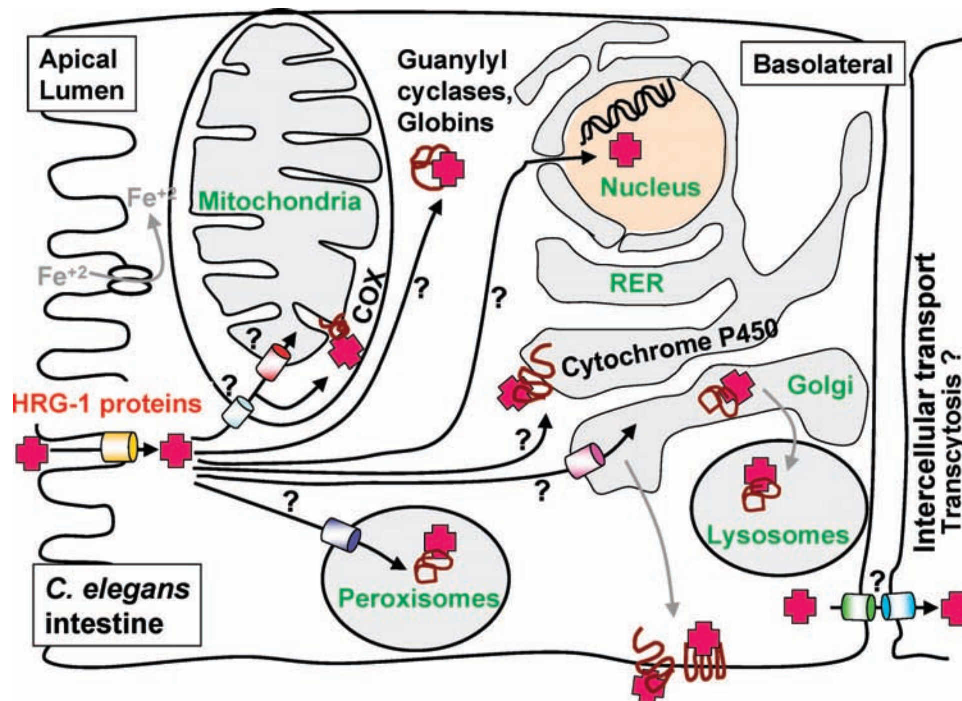

Figure 6. Heme transport pathways in *C. elegans*. *C. elegans* lacks the entire heme biosynthetic pathway and acquires heme *in toto* for incorporation into hemoproteins. Worms absorb heme as a nutrient in the gut via the apical intestinal surface. The heme is either transported directly via membrane-bound transporters (HRG-1) and hemochaperones or transported via intracellular vesicles. Worms must also have an intercellular heme transport system to mobilize heme from the twenty intestinal cells to other cell types including neurons, muscles, and developing embryos.

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by the human intestine. Furthermore, the absence of intracellular heme synthesis in *C. elegans* provides a "clean" genetic background to study organismal heme trafficking pathways. *C. elegans* has been used as a model organism for over four decades.[252] The developmental fate map for every cell has been determined. The genome has been fully sequenced, and a large number of human genes are conserved in *C. elegans*.[253,254] These animals are genetically tractable, optically transparent, and amenable for genetic screens and cell biological studies. Taken together, *C. elegans*, a natural heme auxotroph, may serve as a unique genetic animal model in delineating the cellular pathways and biochemical mechanisms in heme homeostasis.

7.2. Need for Other "Model" Organisms

As attractive of a model as *C. elegans* is, it is imperative that researchers identify additional organisms that can be used to model heme trafficking pathways. This is especially important considering that what is known in one organism is often not applicable to another and that no obvious orthologues of bacterial and yeast heme-binding proteins have been identified in mammals.[138,185,189,255] Using bioinformatic tools to find heme pathway molecules is difficult even in well-characterized systems. This is poignantly illustrated by a recent paper identifying two heme degrading enzymes in *E. coli*.[256] It was necessary to perform a functional screen because, as the authors noted, even in strains known to utilize heme as an iron source, genome BLAST searches fail in identifying orthologues of heme-degrading enzymes in many bacterial species.

Many molecules in diverse organisms have been shown to unexpectedly interact with heme. Vitellogenins—phospholipoglycoproteins that form the major yolk proteins in nonmammalian, egg-laying animals—bind heme in ticks and insects.[257] Human RNA binding protein PCBP1was shown to bind iron and transport it to ferritin when expressed in *S. cerevisiae* engineered to produce the heavy and light chains of human ferritin.[258] HeLP, a lipoprotein in the *Boophilus* tick, binds heme. At least in vitro, HeLP plays an antioxidant role by preventing lysis of red blood cells and preserves the integrity of the heme moiety that needs to be scavenged by the tick from its host.[259,260] In addition to *C. elegans*, several other organisms have been demonstrated to be heme auxotrophs. The cattle tick *Boophilus microplus*,[261] the *Leishmania* spp.,[262] the filarial nematode *Brugia malayi*,[263] and the parasitic hemoflagellate *Trypanosoma cruzi*[264] all lack one or more of the enzymes in the heme biosynthetic pathway. While they might not be as genetically facile as traditional model organisms, these heme auxotrophs may hold clues to the identity of proteins that traffic heme or heme intermediates.

8. Conclusion

It is difficult to write a comprehensive and cohesive review when what is currently known is fragmented and, at times, seemingly contradictory. However, it is because of this that it is such an exciting time to be studying the trafficking of hemes and tetrapyrroles. It is already clear that the molecular mechanisms of heme trafficking are diverse, complex, and incredibly fascinating. Indeed, it seems that each organism has slightly different variations in the general principles for heme synthesis and/or acquisition. At this point, this complexity makes understanding heme transport daunting. However, we can be encouraged in our efforts by the progress made by researchers in the biometals field. Several decades ago, an appreciation for metals in biological systems was growing and an understanding of how many processes in which metals were involved was beginning. At that time, however, it was still thought that metal ions diffused freely through membranes and the cellular milieu. The heme

trafficking field is now working to catch up with the scientists in the biometals field and will benefit from their experiences. An extensive groundwork has been laid, yet more remains to be done. What compels us to push forward toward and to encourage others currently outside the field to get involved in achieving our goal of understanding the trafficking of heme and tetrapyrroles are the significance and ramifications of being successful.

9. List of Abbreviations

ALA	δ-aminolevulinic acid
PBG	porphobilinogen
HMB	hydroxymethylbilane
UROgenIII	uroporphyrinogen III
CPgenIII	coproporphyrinogen III
PPgenIX	protoporphyrinogen IX
PPIX	protoporphyrin IX
CPIII	coproporphyrin III
ALAS	aminolevulinic acid synthase
ALAD	aminolevulinic acid dehydratase
PBGD	porphobilinogen deaminase
UROS	uroporphyrinogen III synthase
UROD	uroporphyrinogen decarboxylase
CPOX	coproporphyrinogen oxidase
PPOX	protoporphyrinogen oxidase
FECH	ferrochelatase
OM	mitochondrial outer membrane
IMS	mitochondrial intermembrane space
IM	mitochondrial inner membrane
ER	endoplasmic reticulum
ABC	ATP-binding cassette
RES	reticuloendothelial system
DLP	dynamin-like protein
MDVs	mitochondrial-derived vesicles
MAPL	mitochondria-anchored protein ligase
Hb	hemoglobin
HPX	hemopexin
HSA	human serum albumin
BFA	Brefeldin A

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