00 Porphyrin and Heme Trafficking in Metazoans

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List of Abbreviations

ABC	ATP-binding cassette
AIP	acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAS	ALA synthase
ALAS-1	housekeeping ALAS
ALAS-2	erythroid ALAS
BH3	Bcl-2 homology 3
CP	carrier protein
CPOX	coproporphyrinogen oxidase
Dcytb	duodenal cytochrome b
DLP	dynamin-like protein
eNOS	endothelial NOS
EPP	erythropoietic protoporphyria
ER	endoplasmic reticulum
ERMES	ER-mitochondria encounter structure
FABP	fatty acid-binding protein
FECH	ferrochelatase
FLVCR	feline leukemia virus subclass-C receptor
GAPDH	glyceraldehyde phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
Hb	hemoglobin
HCP1	heme carrier protein
HeLp	heme lipoprotein

HMB	hydroxymethylbilane
HMBS	hydroxymethylbilane synthase
HO	heme oxygenase
HRG	heme-regulated gene
HRM	heme regulatory motif
HAS	human serum albumin
IM	inner membrane
IMS	intermembrane space
iNOS	inducible NOS
IRE	iron regulatory element
IRP	iron regulatory protein
MAPL	mitochondria-anchored protein ligase
MARE	Maf recognition element
MDV	mitochondrial-derived vesicle
MEL	murine erythroleukemia
mRNA	messenger ribonucleic acid
MTF	mitoferrin
NMR	nuclear magnetic resonance
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
OM	outer membrane
OPA1	optic atrophy
PBG	porphobilinogen
PBGD	PBG deaminase
PBGS	porphobilinogen synthase
Pf-GST	*P. falciparum* GST
PPIX	protoporphyrin IX
PPOX	protoporphyrinogen oxidase
PUG1	protoporphyrin uptake gene 1
RBC	red blood cell
SLC	mitochondrial solute transporter
Steap3	six transmembrane epithelial antigen of the prostate 3
SUCLG1	succinyl CoA synthetase
UROD	uroporphyrinogen decarboxylase
UROS	uroporphyrinogen III synthase
V-ATPase	vacuolar proton ATPase
XLPP	X-linked protoporphyria

I. Introduction

Heme, as a cofactor in a variety of proteins, is widely acknowledged to be essential for gas transport, respiration, xenobiotic detoxification, peroxide production and destruction, fatty acid desaturation, prostaglandin synthesis, and a variety of one-electron transfer reactions.[4,5] Over the past decade, the number of roles identified for heme has grown substantially. In addition to heme's role as a cofactor, it is now also recognized as a regulatory ligand. Among the list of biological processes for which higher eukaryotic heme-binding proteins have now been implicated are regulation of circadian rhythm,[6,7] adipogenesis,[8,9] glucose homeostasis,[8–10] microRNA processing,[11] intracellular proteolysis[12] and regulation of exocrine peptidases,[13] gas sensing,[6,14] control of ion channels,[15] tRNA charging,[16] and intracellular and intercellular signal transduction.[17,18]

Given the number of regulatory networks modulated by heme, it is not surprising that in individuals with significant impairment in the ability to synthesize heme, one finds developmental abnormalities and chronic disease manifestations.[19] Even in single-allele mutation acute porphyrias, it could be imagined that some symptoms of the neurological attack may be attributable to dysregulation of heme-requiring regulatory network systems resulting from altered cellular heme levels. Interestingly, dysfunctional circadian rhythm, whose regulation involves heme-binding transcription factors (see Ref. 12), has been suggested to be associated with psychiatric disorders such as bipolar disorder and schizophrenia,[20,21] and human diseases such as breast cancer,[22] non-Hodgkin's lymphoma,[23] metabolic syndrome,[24] and atherosclerosis.[25] It has even been suggested that heme deficiency may be a factor in the mitochondrial and neuronal decay of aging.[26]

The heme biosynthetic pathway is ancient and, with a few notable exceptions, is highly conserved. The pathway as found in metazoans is depicted in Figure 1. It differs from that found in plants and most bacteria in that the first committed intermediate, 5-aminolevulinic acid (ALA) is synthesized by ALA synthase (ALAS) using succinyl-CoA and glycine as substrates.[27] In plants, *Archaea* and most prokaryotes, ALA is formed from glutamyl-tRNA by two enzymes, glutamyl-tRNA reductase and glutamate-1-semialdehyde aminotransferase.[28] For organisms that possess hemoproteins, the inability to synthesize or obtain heme is ultimately lethal. Few organisms do not synthesize heme and among eukaryotes, *Caenorhabditis elegans* and other Helminths[29] and the cattle tick *Boophilus microplus*[30] are the only well-characterized heme-requiring organisms. The observation that these organisms possess hemoproteins in all cells, but yet can only acquire heme from their diet demonstrates that a robust intercellular and intracellular heme trafficking network must exist. Over the past 50 years, all of the enzymes of the eukaryotic heme biosynthetic pathway have

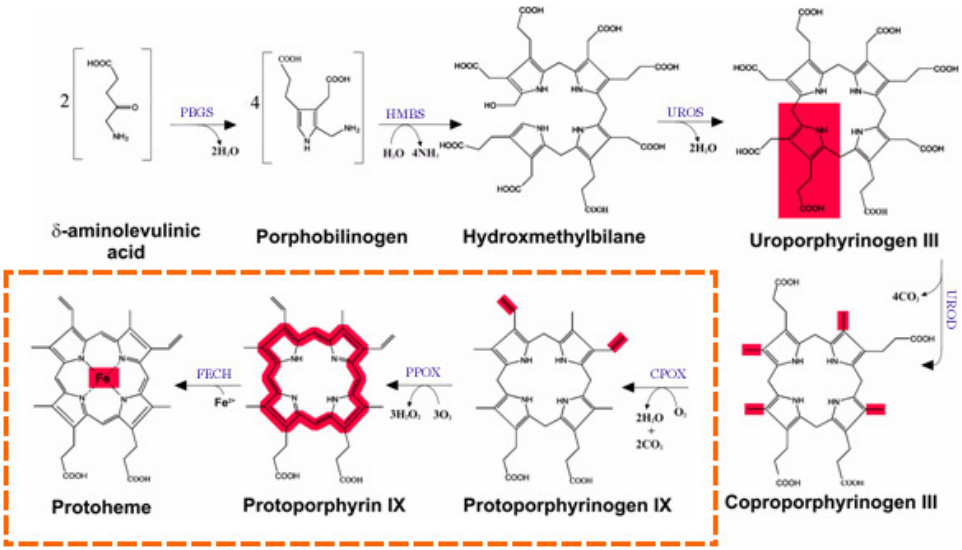

Figure 1. The mammalian heme biosynthetic pathway. The diagram presents the enzymatic steps and structures of intermediates in the pathway from ALA to heme. Steps that occur in the mitochondrion are enclosed in the dashed box and those outside the box are present in the cytosol. Regions highlighted in red show the site of chemical change catalyzed by the enzyme listed. Synthesis of ALA from glycine and succinyl-CoA, which is the first committed step and occurs in the mitochondrion, is not shown. Abbreviations are as in the text. Adapted from Ref. 27.

been identified, purified, cloned, and characterized to varying extents. At least one example of each pathway enzyme has now had its crystal structure determined. In spite of the information gleaned on the individual proteins, little is known about the coordinated regulation of gene expression among diverse cell types, the mechanism of enzymatic catalysis for most enzymes, and the essential elements of potential protein–protein interactions. For heme synthesis, both ferrous iron and protoporphyrin IX (PPIX) are required to form protoheme IX (heme) and because all of these are chemically reactive and potentially toxic to the cell, it is essential to maintain tight controls over the synthesis of heme and tetrapyrrole precursors, iron supply, and heme degradation.

Little is known about intracellular heme and tetrapyrrole trafficking. This is of importance since the last step of heme biosynthesis occurs inside the mitochondrial matrix while target hemoproteins such as guanylyl cyclases, catalases, cytochrome P450, and certain transcription factors are present in extramitochondrial compartments, including the cytoplasm, peroxisomes, the secretory pathway, and the nucleus.[5,31–33] As an iron-containing amphipathic porphyrin, free heme can catalyze the production of reactive oxygen species and intercalate

into lipid bilayers and is, thus, unlikely to diffuse freely within the cell.[34,35] Instead, specific molecules and pathways must exist to facilitate heme delivery to distinct cellular destinations. These issues are of significance since dysfunctional substrate/product handling may lead to ineffective heme synthesis, hemoprotein assembly, and/or toxicity to the cell.

II. Heme Biosynthesis

All of the enzymes of heme biosynthesis are nuclear encoded and cytoplasmically synthesized. The overall reaction to synthesize heme in metazoan cells is: 8 glycine + 8 succinyl CoA + 5 O_2 + Fe^{2+} → Heme + 8 CoA + 4 NH_4 + 14 CO_2 + 3 H_2O_2 + 2 H^+ + 11 H_2O. Of the products, 8 CoA, 8 CO_2, 2 H^+, and heme are generated in the mitochondrial matrix and 2 CO_2 + 3 H_2O_2 + and 2 H_2O are generated in the mitochondrial inner membrane (IM) space. All remaining products are cytosolic.

A. Synthesis of 5-Aminolevulinate

The first committed step in heme biosynthesis is the formation of 5-aminolevulinate (ALA).[3,36–38] In the metazoa, the enzyme ALAS (E.C. 2.3.1.37), which is located in the mitochondrial matrix, catalyzes the condensation of glycine with succinyl CoA to form ALA and CO_2. ALAS is a homodimeric, pyridoxal phosphate-containing enzyme that is a member of the large and well-characterized α-oxoamine synthase family. At present, only the crystal structure of ALAS from the bacterium *Rhodobacter capsulatus* has been determined,[39] but it is clear that the mature eukaryotic ALAS is highly similar to the bacterial enzyme except that it possesses an additional carboxyl-terminal sequence of approximately 25 residues. Much is known about the mammalian enzyme from kinetic and site-directed mutagenesis studies.[37] In vertebrates, two isozymes of ALAS exist, one specific for differentiating erythroid cells (ALAS-2 or ALAS-E) and the other expressed in all other cell types (ALAS-1 or ALAS-N). The genes for ALAS-1 and -2, as for all heme biosynthetic enzymes, are nuclear and synthesized in the cytosol, although the final destination for both ALAS-1 and -2 is the mitochondrion. Both ALAS-1 and -2 possess mitochondrial targeting sequences that are cleaved as part of the translocation of ALAS into the mitochondrial matrix.[40–42]

B. Synthesis of the Monopyrrole, Porphobilinogen

ALA is exported out of the mitochondrial matrix (see below) to reach the second pathway enzyme, porphobilinogen synthase (PBGS) (E.C. 4.2.1.24) (previously

named ALA dehydratase).[43] This enzyme catalyzes the condensation of two molecules of ALA to form one molecule of the monopyrrole porphobilinogen (PBG). The cytosolic homo-octomer can best be described as a tetramer of homodimers with one divalent metal atom per subunit.[36,43] In humans and yeast, this metal is zinc, while in bacteria, one may also find magnesium. Four metal atoms are essential for catalysis and four are involved in stabilization of tertiary structure. These zinc ions may be replaced by lead in lead poisoning resulting in an inactive enzyme. The protein has been crystallized from multiple sources and kinetically characterized (see Refs. 38 and 44). Interestingly, PBGS exists in alternate quaternary structures named morpheeins.[45] One morpheein is the well-characterized octomer which possesses high activity and another is a hexamer that has lower activity. While this change in quaternary structure is proposed to be the basis of allosteric regulation of PBGS, it is something of an enigma, given that ALAS is considered rate-limiting to heme synthesis.

C. Assembly of the Linear Tetrapyrrole

Four molecules of PBG are linked head to tail by the cytosolic enzyme hydroxymethylbilane synthase (HMBS, previously called PBG deaminase or PBGD) (E.C. 2.5.1.61). The reaction results in the formation of the linear tetrapyrrole hydroxymethylbilane (HMB) and releases four molecules of ammonium.[43] Partly because a decrease in HMBS activity leads to the human disease acute intermittent porphyria (AIP),[46,47] this enzyme became the early focus of much attention by researchers. HMBS has been purified from a variety of sources and the crystal structures of the *Escherichia coli*[48] and human[49] enzymes have been determined. The enzyme is a monomer that is synthesized as an apoprotein. In its first complete catalytic cycle, it synthesizes a covalently bound hexameric linear polypyrrole (E-S6). From this, the distal linear tetrapyrrole HMB is cleaved resulting in formation of the holoenzyme HMBS with a covalently bound dipyrromethane which serves as a cofactor for future turnovers and the product HMB.

D. Cyclization of the Tetrapyrrole to Form Uroporphyrinogen

Conversion of HMB to the physiological uroporphyrinogen III isomer requires the action of uroporphyrinogen III synthase (UROS) (E.C. 4.2.1.75).[43] HMB is chemically reactive and will spontaneously cyclize to form uroporphyrinogen I in the absence of the next pathway enzyme. As uroporphyrinogen I cannot be converted into PPIX, it is essential to produce the III isomer. The reaction catalyzed by UROS is the "flipping" or inversion of the final, or D, ring of HMB followed by cyclization to yield the III isomer of uroporphyrinogen. UROS is a monomeric

protein whose structure has been determined for both human[50] and *Thermus thermophilus*.[51] It does not possess a cofactor and has an unusual tertiary structure that is composed of two distinct domains connected by a flexible linker region. Crystal structures have been obtained for a variety of domain orientations suggesting that the molecule is highly flexible in solution.

E. Decarboxylation of Uroporphyrinogen

The final cytosolic enzyme in the pathway is uroporphyrinogen decarboxylase (UROD) (E.C. 4.1.1.37).[28] This homodimeric enzyme has been crystallized[52] and its catalytic mechanism well studied.[53] Each subunit contains an independent active site and the enzyme contains no cofactors or metal ions. UROD catalyzes the decarboxylation of the four pyrrole acetic acid side chains of uroporphyrinogen to yield the four methyl pyrrole side chains of coproporphyrinogen and four molecules of CO_2. UROD will utilize both uroporphyrinogen I and III, and in the presence of high concentrations of substrate, UROD will decarboxylate randomly.[43] However, it is believed that *in situ* the reaction starts with the decarboxylation of the D ring acetate and proceeds sequentially in a clockwise fashion (i.e., D, A, B, C).[52,53]

F. Formation of the Vinyl Groups of Protoporphyrinogen IX

Coproporphyrinogen III produced by UROD is transported into the mitochondrial intermembrane space (IMS) where the antepenultimate enzyme coproporphyrinogen oxidase (CPOX) (E.C. 1.3.3.3) is located.[54,55] This protein is synthesized in the cytosol with an unusually long (~120 amino acids) mitochondrial leader sequence that targets it to the IM space.[40] The mature protein is a homodimer without bound cofactor. The structures of both human[56] and yeast (which is a cytosolic protein)[57] CPOX have been solved and were found to possess unique folds. The reaction catalyzed is an unusual oxidative decarboxylation of the A and B ring propionates to yield the vinyl groups of protoporphyrinogen IX. CPOX will utilize only the coproporphyrinogen III isomer and proceeds in a stepwise fashion that requires two molecules of molecular oxygen and generates two molecules of CO_2. The reaction catalyzed by CPOX has been extensively studied both experimentally and *in silico*, but at present no definitive mechanism has been identified.[58,59]

G. Oxidation of the Porphyrinogen to Protoporphyrin IX

The penultimate step is the oxidation of protoporphyrinogen IX to PPIX. This is catalyzed by protoporphyrinogen oxidase (PPOX) (E.C. 1.3.3.4) which requires

three molecules of molecular oxygen and generates three molecules of hydrogen peroxide.[54,55] Crystal structures of the plant,[1] bacterial,[60,61] and human[62] enzymes have been determined and show that the protein is a homodimer with one non-covalently bound FAD per subunit. PPOX is synthesized in the cytosol in its mature size and is translocated to the outer surface of the inner mitochondrial membrane *via* a mechanism that requires an internal mitochondrial targeting sequence.[40,63,64] Interestingly, the active site is proposed to be situated in the middle of a tunnel that passes through the protein.

H. Insertion of Iron to Form Protoheme IX

The terminal step of heme synthesis is the insertion of ferrous iron into the protoporphyrin macrocycle to produce protoheme IX (heme). This is catalyzed by the enzyme ferrochelatase (FECH) (E.C. 4.99.1.1).[65] In metazoans, this enzyme is synthesized in the cytosol as a preprotein and is translocated to the mitochondrial matrix where it is associated with the inner mitochondrial membrane. The mature, processed protein is a homodimer with each subunit possessing a [2Fe-2S] cluster.[66] Similar [2Fe-2S] clusters are not found in plant FECHs but are found in the actinobacterial enzymes and some Gram-negative bacterial FECHs.[65,67] There is no evidence to suggest that the cluster participates directly in catalysis, but in its absence enzyme activity is significantly reduced leading to speculation that it serves a role as a *de facto* iron sensor *in vivo*. Shah *et al*. have presented evidence suggesting that the cluster itself plays a regulatory role during catalysis *in vivo*.[68] The crystal structures of human FECH with and without substrate and product have been determined and it has been shown that the molecule undergoes considerable active site remodeling during its catalytic cycle.[3,69]

I. Possible Role of Supramolecular Protein Assemblies in Heme Synthesis

Of significance to the current review is the intracellular distribution of heme synthesis pathway enzymes and the manner in which pathway intermediates are moved between enzymes. The chemical intermediates in tetrapyrrole synthesis are relatively reactive and can be cytotoxic. The physiological proof of this statement is amply demonstrated by the fact that a deficiency in any one of the pathway enzymes results in a clinically distinct disease in animals,[47,70,71] and inhibition of some later steps can result in photo-induced death of plants or microorganisms. Other than a defect in ALAS-2 which results in X-linked sideroblastic anemia, the clinical manifestations of these disorders, named porphyrias, are generally not anemia but result from the accumulation of pathway intermediates upstream from

the genetic block. These diseases and their molecular basis have been well studied and frequently reviewed by others (see Refs. 46, 47, 70, and 71).

Given the reactivity of the pathway intermediates, it is highly unlikely that the intracellular concentrations of substrate or products attain the μM concentrations of the measured enzyme K_ms. Thus, it is reasonable to assume that "free intermediates" do not exist in the cell although various porphyrin intermediates are routinely found in the extracellular medium of mammals. This suggested that the pathway was somehow compartmentalized so as to allow direct transfer of product/substrate between enzymes. This topic was first approached experimentally in the 1980s when data were presented in support of the hypothesis that the terminal three pathway enzymes, which are all mitochondrial membrane-associated, form at least a transient complex to facilitate the transfer of intermediates.[55,72,73] Biochemical data clearly demonstrated that while obligate, tight substrate channeling such as occurs in tryptophan synthesis does not exist, under normal circumstances equilibration of products/substrates with the bulk medium does not occur. Data gleaned from PPOX and FECH crystal structures provide good support for possible interactions.[54,69]

Other than data for the terminal, membrane-associated enzymes, there exists little experimental evidence to support multienzyme complexes of earlier pathway enzymes. For the first step, an interaction of ALAS-2 with succinyl-CoA synthetase (SUCLG1) on the inner mitochondrial membrane has been demonstrated by two groups.[74,75] With the identification of mitochondrial solute transporter-25A38 (SLC25A38) as the putative glycine/ALA transporter,[76] it is reasonable to anticipate the existence of at least transient complexes among ALAS, SUCLG1, and SLC25A38 on the mitochondrial IM (Figure 2). The possibility for a stable,

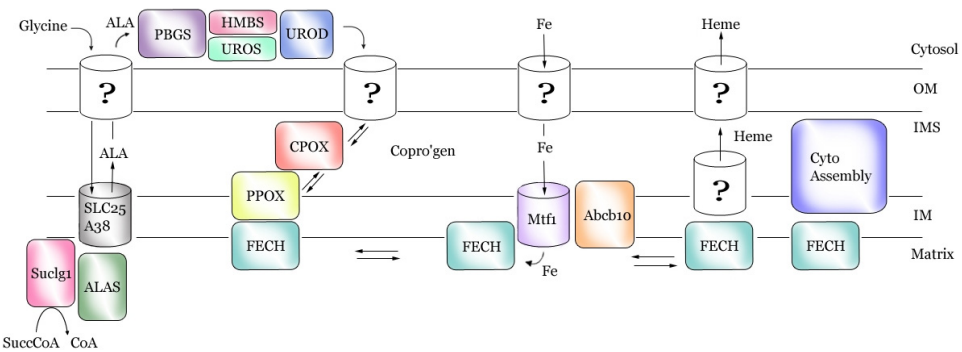

Figure 2. Proposed model for components involved in heme synthesis. Details are discussed in the text and where specific data exist for a particular component, its name is shown. Components that are currently unidentified experimentally are denoted with "?". Abbreviations and conventions are given in the text. Adapted from Ref. 27.

rigid complex for these and most other heme synthesis enzymes seems unlikely given that they possess active site pockets with a single entrance/exit (see Ref. 38). The necessity for substrate(s) and product(s) to enter and exit *via* a single route would require movement of one enzyme between donor and acceptor molecules in the complex, much as cytochrome c physically cycles between electron donors and acceptors. The only enzyme for which this may not be the case is PPOX which appears to have a channel through the protein with the active site located within this feature.[1,60,62]

For the synthesis of uroporphyrinogen III from PBG, it has long been thought that close proximity of HMBS and UROS would be probable, given the chemical reactivity of the linear tetrapyrrolic intermediate, HMB. However, no published data exist to support the presence of a multienzyme complex involving PBGS and HMBS. The possibility that a HMB-binding chaperone exists to move HMB from HMBS to UROS, while possible, also has no supporting data either *in vitro* or *in vivo*. Since ALA must be exported out and coproporphyrinogen imported into the mitochondria, it is reasonable to anticipate that PBGS, HMBS, UROS, and UROD exist in a supramolecular complex that is spatially close to the mitochondrion (Figure 2). To date, high-resolution microscopic studies identifying the intracellular distribution of these enzymes is lacking. Approaches that rely upon cell disruption and physical isolation of complexes or *in vitro* reconstitution of complexes from isolated components requires the strength of protein–protein interaction in solution that may not exist and may not be necessary in the highly concentrated cytosolic milieu. One intriguing possibility is that PBGS, which exists as either an octomer or hexamer, could, because of its size, serve as a scaffold for a multienzyme complex. The observation that PBGS can assume multiple morpheein forms[38,45] may hint at its plastic role as a supramolecular assembly nucleation site. Resolution of these issues will require masterful research, but the results will be illuminating.

The presence and nature of multienzyme complexes involving the terminal three membrane-associated enzymes is considerably advanced over what is known, or not known, about the earlier pathway enzymes but is still extremely rudimentary. Little is known about possible interactions between CPOX and PPOX other than substrate channeling experiments.[73] Radiolabeling experiments employing isolated mitochondrial fragments clearly support transient interactions *in situ*, although reconstitution of this process with purified components was not accomplished, which suggests that additional participants other than just CPOX, PPOX, and FECH are required. *In silico* structural studies show that an interaction across the inner mitochondrial membrane to transport protoporphyrin from PPOX to FECH is feasible and highly likely[1] (Figure 3). Interestingly, in the docked PPOX–FECH complex, the opening of the active site tunnels of the

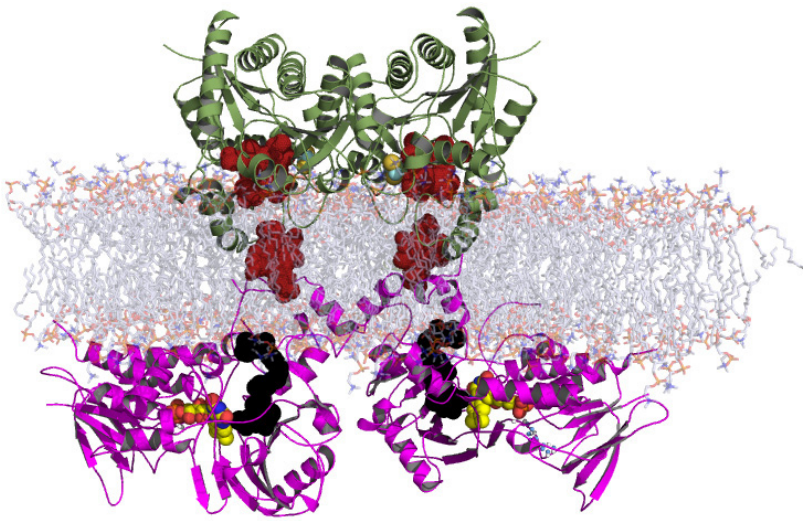

Figure 3. Cartoon of proposed interaction between PPOX and FECH across the inner mitochondrial membrane. The initial PPOX–FECH docking model was published by Koch *et al.*[1] for plant PPOX and human FECH. The current model was derived from PDB files, 3NKS (PPOX) and 2QD1 (FECH). FECH is colored green and PPOX is colored violet. Porphyrin atoms are depicted as red colored solid spheres and the PPOX inhibitor is shown in solid black. Phospholipids are presented as sticks that are colored wheat. Of note is that the crystal structures of FECH with bound porphyrin possess one protoporphyrin bound in the active site and a second one associated with the outer edge of the active site lip. This is proposed to be the entry route of porphyrin into the active site pocket.[2,3] It is proposed that FECH and PPOX interact transiently across the membrane to facilitate transfer of protoporphyrin to FECH. Following this transfer FECH undergoes spatial alterations that are proposed to lower the affinity between FECH and PPOX so that their interaction ceases, thereby, allowing FECH to interact with MTF1 and later a heme-accepting protein. Adapted from Ref. 27.

PPOX dimer not only coincided with the position of the openings to the active site pockets of the dimeric FECH but is also spatially juxtaposed to surface-bound porphyrin molecules observed in some FECH crystal structures. The observation that binding of substrate and product induces changes in the surface contour and charge distribution around the active site pocket opening of FECH[3,69] provide an explanation for how PPOX and heme-accepting proteins recognize the appropriate form of FECH with which to interact. Given the need to acquire coproporphyrinogen from the cytosol and movement of at least some heme out of the mitochondrion, the existence of a complex of an outer membrane (OM) coproporphyrinogen transporter, CPOX, PPOX, the IM iron transporter mitoferrin (MTF1),[77] FECH, and a heme chaperone/OM heme transporter at a mitofilin-mediated junction between outer and inner mitochondrial membranes[64] is an intriguing possibility.

Three significant issues remain unanswered: (i) transport of coproporphyrinogen into the mitochondrion, (ii) mitochondrial supply of iron for heme synthesis, and (iii) transport of heme away from its site of synthesis. A series of studies proposed that ABCB6, a putative mitochondrial OM ATP-dependent transporter, is responsible for coproporphyrinogen transport (see below).[78,79] At present, however, this claim must be viewed with skepticism since only the transport of the fully conjugated, planar macrocycle coproporphyrin, not the physiologically relevant non-planar coproporphyrinogen, has ever been experimentally demonstrated. In addition, and as described below, ABCB6 has been localized to multiple membranes and identified as being involved in non-heme synthesis-related disorders. However, given that ABCB6 has been observed to be one of a relatively few genes induced at high levels during erythropoiesis,[80] it seems likely that it plays some role in erythropoiesis. Given the size of coproporphyrinogen, the possibility that it transits the OM *via* a porin rather than a specific transporter cannot be ruled out by current data.

Although the coordinate regulation of tetrapyrrole synthesis and iron metabolism must exist to prevent their inappropriate accumulation, there have been limited studies that address this issue at either the genetic or molecular level. The role of the iron-responsive element system[62] and the cellular machinery for iron sulfur cluster assembly has garnered significant interest,[81] and our knowledge of whole body iron trafficking is relatively mature.[82] However, there has been significant advancement in identifying participants essential to the iron supply for heme synthesis. This came with the identification of MTF1 as the mitochondrial IM iron transporter that supplies iron to FECH for heme synthesis.[77] MTF1 is responsible for iron transport for heme synthesis during erythropoiesis, but the presence of another IM transport protein, Abcb10, is required to stabilize MTF1.[83] The exact role that Abcb10 plays has yet to be elucidated. MTF1 has been found to form a complex with FECH which might make possible a direct transfer of transported ferrous iron for heme synthesis to FECH,[83] but the actual site of iron entry for FECH remains undefined experimentally. One model proposes that mammalian FECH acquires iron from MTF1 or frataxin *via* the extended π-helix of FECH[84] and enters the active site *via* the main "mouth".[85,86] However, this model is based upon the structure of a single monomer of FECH, not the physiologically significant homodimer, and the putative membrane binding surface of the model is, in fact, the dimer interface. The crystal structures of dimeric human FECH are consistent with a spatial orientation where the π-helix unwinds into the membrane and not back up into the matrix. All available data suggest that the helix extension is related to heme release and in the absence of product there is nothing to stabilize the extended helix. Additionally, the simultaneous exit of heme and entrance of iron *via* the same path is not supported by structural or kinetic studies.[87,88]

Alternatively, structural and site-directed mutagenesis data obtained for human FECH along with its orientation relative to the IM are all consistent with iron entering the active site of FECH *via* a solvent-filled channel whose entrance is on the rear of the enzyme (Medlock *et al*., unpublished data). It will be of interest to learn the molecular details of this process and how it is regulated.

Transit of heme away from its site of synthesis remains an unexplored territory. *In vitro*, the release of heme from the enzyme post-metalation is the rate-limiting step.[88] This probably reflects the absence of the native heme acceptor a cell-free assay. Given that heme is utilized throughout the cell in a variety of compartments, it is clear that multiple and specific heme chaperones may exist. For transit from the mitochondrion to other membranous compartments, it appears possible that transfer occurs at direct contact points rather than by diffusion through the cytoplasm,[31,89] but even that requires first moving heme from FECH to the site of transfer between membranes. As yet unknown is what molecule first accepts heme from FECH and whether there is a single unique protein that interacts with FECH or multiple pathway-specific proteins. Given its location on the IM, it would seem that for respiratory cytochrome maturation direct transfer from FECH to cytochrome assembly machinery is possible, although definitive data are still lacking. For cytoplasmic hemoproteins, including hemoglobin (Hb) and myoglobin, there are no identified players. Putative heme/porphyrin binding/carrier proteins (CPs) have been suggested and even biochemically characterized,[90,91] but none of these is supported by data as *in vivo* participants in heme trafficking (see Sections IV and V).

III. Regulation of Metazoan Heme Biosynthesis

As noted, five pathway proteins possess either organic cofactors or essential metals. To date, no pathway enzymes has been shown to be glycosylated and only mouse liver FECH has been reported to be phosphorylated,[92] albeit on residues that appear to be on inaccessible sites within the protein. Interestingly these investigators also reported that phosphorylated FECH is located on the mitochondrial OM, something never reported by any other group previously.

Regulation of the pathway is tissue- and cell-specific, subject to modulation by a myriad of developmental and environmental pressures through diverse transcriptional factors and is beyond the scope of the current review.[3,71,93] In general, however, synthesis of the first committed intermediate, ALA, is considered rate-limiting. Among red blood cell (RBC)-producing metazoans, there are clear distinctions between regulation of heme synthesis in non-erythroid cells (so-called housekeeping heme synthesis) and in differentiating erythroid precursor cells. The housekeeping ALAS-1 gene is regulated by diverse factors frequently associated

with xenobiotic, hormone and drug metabolism and is subject to tissue-specific regulation.[71,94] ALAS-1 has an estimated half-life of only a few hours and may be significantly induced in some cells.[95,96] ALAS-1 is present in erythroid precursor cells before they begin erythroid differentiation, but the gene is turned off when ALAS-2 is turned on as these cells begin to synthesize heme for Hb. Thus, ALAS-1 cannot substitute for a deficiency of ALAS-2.[97,98]

ALAS-2 is transcriptionally regulated by erythroid-specific factors such as GATA-1 and possesses an iron regulatory element (IRE) located in the messenger ribonucleic acid (mRNA) 5′ untranslated region that allows for translational regulation by cellular "free" iron.[94,99,100] As with similarly regulated systems, such as for ferritin synthesis, the IRE binds the iron-free form of the iron regulatory protein (IRP), thereby, preventing translation of the ALAS-2 message when cellular iron concentrations are insufficient for optimal heme synthesis. Thus, maximal ALAS-2 activity requires gene induction by erythroid-specific transcription factors as well as sufficient cellular iron levels to support heme synthesis. Studies by Schranzhofer *et al*.[101] with differentiating erythroblasts demonstrated that ALAS-2 translational regulation becomes "uncoupled" from that of other IRE-regulated proteins such as transferrin receptors (which are increased, not decreased during erythropoiesis) and ferritin (whose protein levels do not increase). This is reasonable during the later accelerated hemoglobinization phase of erythropoiesis, since under "standard" IRE-IRP regulation, elevated iron would diminish transferrin receptor expression and increase ferritin synthesis, both of which would limit iron availability for heme synthesis. Additionally, it was postulated that during maximal hemoglobinization, ALAS-2 mRNA increases disproportionately to IRP synthesis, thus, circumventing the IRE-IRP regulatory mechanism. The "kiss and run" hypothesis,[102] which proposes direct vesicle-mediated transfer of iron to mitochondria, may offer another possible explanation to this. In this hypothesis, during terminal erythropoiesis, vesicular iron acquired by receptor-mediated endocytosis is targeted directly to the mitochondrion, thereby, bypassing the cytosolic iron pool and the IRE-IRP system.

Multiple studies and reviews have presented data suggesting that pathway enzymes after ALAS are in excess, although relative enzyme amounts vary considerably.[4,47,71] However, these calculations are based on *in vitro* assays of individual enzymes under what are considered optimal conditions for each enzyme and then extrapolated back to intact cell conditions. There are no comprehensive studies that examine the induction of biosynthetic pathway enzymes other than ALAS-1 in non-transformed non-erythroid cells, but the fact that all of the pathway genes possess diverse transcription factor-binding sites is compatible with regulation at multiple sites.[90,97,103–110] Indeed, in cancer cells, it is clear that at least CPOX and FECH are upregulated and downregulated, respectively, and this is the

basis for the effectiveness of photodynamic therapy.[111,112] It has been demonstrated that there is upregulation of all pathway enzymes during erythroid differentiation (see Ref. 111). Even so, current dogma relegates pathway regulation during erythropoiesis to ALAS-2 alone.[3,93] Re-evaluation of this model may be in order with the discovery that some mutations in the carboxyl-terminus of ALAS-2 result in a hyperactive enzyme and this, in turn, causes an accumulation of free protoporphyrin as is found in the disease erythropoietic protoporphyria (EPP),[113] as well as high concentrations of zinc protoporphyrin. The name currently given to this disorder is X-linked protoporphyria (XLPP) to reflect the X chromosome location of ALAS-2. This disorder is, thus, similar, but not identical, to that found when ALAS-2 is overproduced in *Irp2*$^{-/-}$ mice.[114] The observation of protoporphyrin accumulation in XLPP is particularly interesting since levels of FECH are generally assumed to be present in considerable excess in the cell. Indeed, normal EPP occurs only when FECH levels drop to approximately one-fourth of normal.[70] This suggests that either the erythroid pathway is designed so that the amount of FECH and/or iron transport mechanisms for heme synthesis are closely linked to normal, maximal amounts of ALAS-2 activity or that additional regulatory steps or limiting mechanisms exist at the end of the pathway that we currently do not recognize or understand.

For both ALAS-1 and ALAS-2, one finds alternative mRNA splice variants. For ALAS-1 two known splice variants occur in the untranslated region of exon 1.[115] These variants possess altered sensitivity to heme-mediated decay of the message. For human ALAS-2, one splice variant has been described in which exon 4 is absent.[74] This variant represents 35–45% of total ALAS-2 mRNA in the cell and encodes a protein with slightly reduced activity. This variant ALAS-2 is translocated into the mitochondrial matrix where it was shown to interact with SUCLG1, just as the full length enzyme does and contributes to erythroid heme synthesis.

Interestingly, both ALAS-1 and -2 possess three heme regulatory motifs (HRMs) composed of a canonical Cys-Pro sequence.[102] Two HRMs are found in the targeting leader sequence and the third is near the amino terminus of the mature processed ALAS.[40] While such motifs have been shown to serve as a redox sensitive switch for heme oxygenase (HO)-2,[69] there is no evidence to suggest such a role for these motifs in ALAS. Indeed, evidence from multiple groups has shown that heme binds to the ALAS HRMs *in vitro*[41] and *in vivo*,[40,42] and a series of mutagenesis experiments clearly demonstrated the significance of all three ALAS HRMs in the heme responsive translocation of the protein into mitochondria *in vivo*.[40] While such heme regulation of apoprotein translocation into the mitochondria of non-erythroid cells can easily be rationalized, a similar occurrence in differentiating erythroid cells where massive quantities of heme are being

synthesized in a relatively short period of time is less easily justified. Several significant pieces of information that would address this issue are lacking. Among these is an identification of the fate of both cytoplasmic ALAS precursor apoprotein and any heme bound to the HRMs. The possibility that the HRM-containing leader peptide or apoprotein HRM serves as a heme chaperone for globin assembly is intriguing, but no data exist to support or rule out this proposition.

While two distinct genes on separate chromosomes exist for ALAS-1 and -2, only single genes exist for the remaining pathway enzymes. However, for PBGS, HMBS, UROS, and FECH, housekeeping *versus* erythroid-specific promoter regions drive expression that leads to tissue-specific alternate mRNA splicing. Only the HMBS alternate splice variants, however, give rise to isoenzymes of HMBS with alternative amino-terminal segments.[104] Here, the mRNA of the erythroid form of HMBS skips exon 1 and starts with the non-coding exon 2. The translation start site employed for the erythroid HMBS is in exon 3. The housekeeping HMBS mRNA starts with exon 1, which contains an internal start site and coding region and skips the non-coding exon 2. The result is that the housekeeping HMBS has an additional 17 amino acid residues at the amino terminus that are lacking in the erythroid form.

Human PBGS possesses two non-coding exons, 1A and 1B.[105] The translational start site for housekeeping PBGS mRNA is exon 1A, while erythroid PBGS mRNA starts in exon 1B. Thus, an additional 5′ untranslated region is present in the mRNA for the housekeeping variant that is lacking in the erythroid splice variant. Since these variants are in the non-coding region, the housekeeping and erythroid forms of the PBGS enzyme are identical. Splice variants also exist for UROS.[103]

The gene for UROS possesses spatially distinct erythroid and housekeeping promoters with the erythroid promoter elements located in the intron between exons 1 and 2. This promoter drives transcription from an initiation site in exon 2. The housekeeping promoter region is present upstream of exon 1 and drives transcription from an initiation site in exon 1. However, since exon 1 is non-coding, both housekeeping and erythroid UROS proteins are identical. For mouse FECH, there appears to be an alternate splice site that gives rise to additional 3′ untranslated nucleotides.[116]

While the existence of splice variants is not a new observation, any experimentally supported rationale for a physiological role for these variants is lacking. However, studies on the role of RNA in the spatial regulation of protein synthesis[117] and/or in stabilization of multiprotein complexes[118] raise the intriguing possibility that the alternative splicing for mammalian housekeeping *versus* erythroid forms of PBGS, HMBS, and UROS are not random but may serve for sequestration of mRNA primed for function.

It has been proposed that during the course of normal erythropoiesis, heme synthesis in developing erythroid cells overproduces heme to an extent that it is toxic to the cell unless it is exported by the plasma membrane heme transporter feline leukemia virus subtype-C receptor (FLVCR) (see Section IV.D.).[119] While it is clear that disabling mutations in FLVCR result in cell death, presumably due to the toxic effects of excessive heme, it seems counterintuitive that evolution would have designed such intricate regulatory mechanisms for erythroid heme synthesis (i.e., IRE-IRP, erythroid-specific transcription factors, and complex iron supply regulatory schemes) that would permit excessive synthesis of heme necessitating heme export. Indeed, given the "cost" of heme synthesis, it seems more likely that the "excessive" heme produced is a planned synthesis of heme by erythroid cells (which must be considered the ultimate heme synthesizing factories in the body) for orderly export and transit to other organs and cells whose heme synthesizing capabilities may be physiologically limited. The presence of specific heme carriers, such as hemopexin,[120] and the observation that exogenous administration of heme to human porphyria patients downregulates heme synthesis, and is subsequently utilized for hemoproteins by the patient receiving the infusion,[46] provide support for this hypothesis.

IV. Heme Transport and Trafficking

A. Heme Import in Animals

The existence of a receptor-mediated endocytic pathway for heme uptake in duodenal enterocytes has long been suspected. Studies have hinted at the existence of such heme receptors and uptake proteins in the microvilli of the upper small intestine, as well as in culture enterocytes and non-intestinal cells.[121,122] HRG-1 (heme-responsive gene-1; systematically named solute carrier 48A1, SLC48A1) was identified in *Caenorhabditis elegans* in a screen for genes that are transcriptionally regulated by heme (Table 1).[123] *C. elegans* requires heme for growth and reproduction; however, it is unique among metazoans for being a heme auxotroph.[29] This nutritional requirement for heme, coupled with the inability of *C. elegans* to synthesize its own heme, negates several of the confounding variables that have prevented the previous identification of heme transporters, including transport of non-heme porphyrins and feedback regulation in heme biosynthesis. In worms, *hrg-1* and its paralog *hrg-4* are both intestinal transmembrane permeases. Depletion of either of these molecules results in disrupted heme sensing and aberrant responses to heme analogs.[123]

HRG-1 function appears to be conserved across phyla. Transient knockdown of the zebrafish homolog, *hrg-1*, results in erythropoietic defects; ectopic

Table 1. Heme/porphyrin transporters.

Protein name	Proposed function	Location of function	Reference
HRG-1 (SLC48A1)	Transport heme	Vesicles	123
FLVCR (MFSD7B)	Export heme	RBC plasma membrane	131
PCFT (formerly HCP1)	Import heme?	Plasma membrane	137,138
ABCG2 (BCRP)	Export heme	RBC plasma membrane	268
ABCB6	Transport coproporphyrinogen III	Plasma membrane, Mitochondria OM, Golgi	79
ABC-me (M-ABC2/ ABCB10)	Transport heme intermediates	IM	269,270
SLC25A38	Transport glycine and ALA	IM	76
PUG1p	Exports heme and imports PPIX in yeast	Yeast PM	267

expression of human *hrg-1* in murine erythroleukemia (MEL) cells led to increased accumulation of heme analogs. When expressed in *Xenopus* oocytes, both human and worm *hrg-1* result in heme-dependent transport across the plasma membrane.[123] While only a single copy of the *hrg-1* gene is present in humans, the *C. elegans* genome encodes *hrg-1* as well as three additional paralogs.[124] These redundant heme acquisition pathways likely evolved to compensate for the worm's inability to synthesize heme.

Human HRG-1 mRNA is highly expressed in the brain, kidney, heart, and skeletal muscle and moderately expressed in the liver, lung, placenta, and small intestine.[123] When expressed in human embryonic kidney cells, HRG-1 localizes to endolysosomes. Furthermore, the interaction between HRG-1 and heme requires low pH conditions, biochemical evidence that *in vivo* HRG-1 may function in the acidic lysosomal microenvironment.[123] HRG-1 has been shown to interact with the c subunit of the vacuolar proton ATPase (V-ATPase) pump and enhance endosomal acidification.[125] It is very important to note that the directionality of heme import across the plasma membrane is topologically identical to heme import from a membrane-bound cellular compartment; in both cases, the substrate is being carried from the exoplasmic space in the cytoplasm. Mechanistic studies using a yeast system have identified conserved amino acids on the exoplasmic, cytoplasmic, and membrane-spanning regions of HRG-1 that are required for heme transport across membranes.[124]

Also reinforcing the role of HRG-1 in heme metabolism are microarray and ChIP-Seq studies showing that HRG-1 is a target of BACH1.[126] BACH1, a basic leucine zipper transcription factor, heterodimerizes with Maf to bind to Maf recognition elements (MAREs) in the promoters of target genes such a globins and HO-1. The BACH1–Maf complex represses transcription of these genes until heme binding to BACH1 relieves the repression.[127–129] In summary, HRG-1 is (1) capable of

transporting heme in a variety of systems, (2) transcriptionally regulated by heme in worms and mammalian cells, (3) distributed in cell types relevant to heme and iron metabolism, (4) localized to endolysosomes, and (5) required for hematopoiesis in zebrafish. Given these results, HRG-1 is a compelling candidate heme transporter relevant to heme metabolism in intestinal cells, RBCs, and macrophages.

B. Heme Export in Animals

There are two main reasons for conjecture over the existence of a heme exporter: (1) heme efflux is a simple solution to the problem of heme detoxification; (2) cellular heme export would facilitate recycling and intercellular transfer of heme. In support of this, studies in macrophages have indicated that, following the phagocytosis of senescent RBCs and degradation of Hb, a portion of the resulting iron in indeed exported *in toto*.[130]

One transporter implicated in heme efflux is the major facilitator superfamily protein, FLVCR1. Cats infected with feline leukemia virus, subgroup-C (FeLV-C) develop pure red cell aplasia in which erythroid progenitor cells fail to mature from burst-forming units into the colony-forming units of erythroid cells. Suppression of FLVCR expression by the virus in embryonic fibroblasts resulted in significantly increased cellular heme content, while ectopic expression of FLVCR in renal epithelial cells reduced intracellular heme levels.[131] These results were further confirmed by heme export assays using a fluorescent heme analog and ^{55}Fe-heme in renal epithelial and K562 cells. In mammals, FLVCR is highly expressed in hematopoietic cells, and heme efflux mediated by FLVCR is essential for erythroid differentiation.[131,132] Knockout mice died at mid-gestation, with no erythropoiesis observed.[133] Deletion of FLVCR also perturbed the heme efflux from macrophages, blocking the recycling of heme iron from senescent RBCs.[133] Heme export by FLVCR is facilitated by hemopexin which binds to a 69 amino acid peptide (residues 132–201) residing between two exoplasmic loops and two transmembrane domains.[61]

It has been reported that FLVCR2, a homolog of FLVCR1, is capable of importing heme in mammalian cells and that the basis for the vascular defect in Fowler Syndrome may result from defective FLCVR2 function.[134] In yeast assays, alongside HRG proteins, FLVCR2 was not observed to import heme.[124] However, given the high level of homology between FLVCR1 and FLVCR1 (including similar membrane topology and several conserved heme-binding ligands in cytoplasmic and exoplasmic loops), it is possible that these proteins have conserved functional mechanisms. It is also possible that FLVCR2 may be exporting heme *via* intracellular compartments or require a cofactor for optimal function.

C. Intracellular Heme Transport

Whether heme is synthesized within a cell or imported from outside the cell, heme must be transported across membrane for storage, sequestration, or insertion into hemoproteins. This aspect of heme metabolism still remains poorly understood. The ATP-binding cassette (ABC) transporter ABCB6 has been proposed to play a role in heme movement between the cytoplasm and mitochondria. ABCB6 was initially identified as a mammalian ortholog for yeast ATM1, a mitochondrial iron transporter important for Fe-S cluster biogenesis.[135] However, in ABCB6-expressing cells, ^{55}Fe-heme was transported into the mitochondria in an energy-dependent manner. Another tetrapyrrole compound, coproporphyrin III, competed with ABCB6 for heme binding and inhibited heme uptake into mitochondria. These results lead Krishnamurthy *et al*. to propose that ABCB6 was more likely involved in porphyrin/heme transport into mitochondria, than in Fe-S cluster biogenesis.[79] This hypothesis raises a number of questions. First, it is puzzling that an ABCB6 homolog is present in the *C. elegans* genome, given that worms do not synthesize heme. Second and most importantly, if mitochondria are the terminal site of heme biosynthesis, what is the physiological relevance of a mitochondrial heme importer? Given the broad substrate specificity of ABC transporters, the true physiological substrate for ABCB6 remains unclear.

Notably, two distinct molecular weight forms of ABCB6 have been identified.[136] Using specific ABCB6 antibodies, it was shown that while the light form (79 kDa) localized to the mitochondrial OM, the heavy form (104 kDa) predominantly resided on the plasma membrane. Overexpression of the plasma membrane form of ABCB6 reduced the cellular accumulation of another tetrapyrrolic compound (pheophorbide A), but did not affect heme levels. It is possible that the two ABCB6 forms have distinct functions at different subcellular locations, but further studies are required to pinpoint their precise physiological roles in the cell.

D. Contradictions

Taking advantage of the gradient of heme absorption along the intestine, suppression substractive hybridization between ileal and duodenal cDNA samples was used to initially identify heme carrier protein (HCP1).[137] Expression of this membrane-bound protein in *Xenopus* oocytes resulted in a two- to three-fold increase in heme uptake with an apparent K_m of 125 μM.[137] Further studies, however, demonstrated that HCP1 was mutated in patients with congenital folate deficiency. This genetic evidence, coupled with direct measurements indicating that HCP1 transports folate with high affinity (K_m ~1.3 μM at pH 5.5), points to folate, rather

than heme, as the primary endogenous substrate for HCP1.[138] Whether HCP1 also contributes to the absorption of heme in the intestine is uncertain at this time.[139]

The ABC transporter, ABCG2 (also known as BCRP), was originally identified as a drug resistance protein in breast cancer cells, but evidence pointed to a role for ABCG2 in heme biology. ABCG2-null mice had 10 times higher levels of PPIX in erythrocytes than did wild-type mice.[140] This suggested that ABCG2 may be involved in exporting porphyrins compounds. Krishnamurthy *et al.*[268] used hemin–agarose pull-down assays to demonstrate that ABCG2 interacts with heme; however, no direct evidence has confirmed that heme is a physiological substrate for ABCG2. ABCG2 has been also identified as the mutated gene resulting in the Junior blood system group.[141] Members of this blood group (i.e., ABCG2-null humans) have no apparent clinical phenotype, aside from transfusion incompatibility.

Likewise, humans with null mutations in ABCB6, the putative mitochondrial heme importer, have no readily apparent defects in erythropoiesis or heme metabolism, but instead constitute a distinct blood group system named Langereis.[141] While this suggests that, at minimum, a portion of ABCB6 resides on the plasma membrane of RBCs, the lack of erythropoietic defects in these people contradicts the hypothesis that ABCB6 plays a significant role in human heme biology. The only known human genetic defect associated with ABCB6 is the L811V allele, which causes ocular coloboma, a defect in the closure of the optic fissure.[142]

Genetic experiments in mice, indeed, reveal a hematological defect consistent with a role for FLVCR1 as a heme transporter. A mitochondrial isoform of FLVCR1 (termed FLVCRb) has been identified that may mediate heme export from the mitochondria into the cytoplasm. The original FLVCR knockout mouse generated by Keel *et al*. resulted in the deletion of both isoforms.[133] Knockout mice which lacked FLVCR1, yet retained expression of FLVCRb, died as embryos, showing impaired skeletal development and vasculature formation.[143] Surprisingly, these mice did not show defects in erythropoiesis. This suggested that the FLVCR null phenotype may be due in part to cytosolic heme deficiency rather than increased cellular heme levels and that FLVCRb may function to export heme from the mitochondria.

E. Heme Transfer between Organelles

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.[144–146] One of the many proteins involved in fusion and fission of mitochondria is mitofusin 2, a large dynamin-like GTPase that spans the mitochondrial OM.[147] Mitofusin 2 has been shown to be involved in

fusing the ER membrane with the mitochondria.[148,149] Although the authors of the studies were interested in this interorganellar connection because of the ramifications in Ca^{2+} transfer and signaling, this melding of membranes was intriguing because of its implication for heme transfer. It is feasible for heme to be passed directly from the mitochondria to the ER much like Ca^{+2} is transferred during IP_3-mediated signaling. Since mitofusin 2 is on the mitochondrial OM, heme would still need to cross the mitochondrial IM.

Other data have 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 inner mitochondrial membrane.[150] The concerted action of an OM protein, an IMS protein,[151] and an IM protein could be able to control the flow of heme from the matrix to the endoplasmic reticulum (ER). Conversely, if DLPs tether the IM and OM at specific points in the mitochondria, similar to OPA1 tethering cristae to form intracristae region for storage of cytochrome c, then CPOX, PPOX, and FECH could be placed in close proximity to each other for rapid transfer of porphyrin intermediates for heme synthesis.

It is possible that cytosolic heme chaperones are not required for delivery of newly synthesized heme from a mitochondrial exporter to cytosolic target proteins. Using RBCs, endosome-mediated transfer of iron-loaded transferrin to the mitochondria has been demonstrated.[152] If part of the OM of the mitochondria 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. Transfer of heme *via* such an intermediate compartment (e.g., vesicle) would not only minimize release of cytotoxic heme-iron but also facilitate highly regulated movement of sequestered heme required for specific cellular processes.

In *Saccharomyces cerevisiae*, Mmm1, Mdm10, Mdm12, and Mdm34 have been identified as the components of a protein complex that forms a molecular tether between ER and mitochondria.[153] 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 and that mutation of a single component disrupts normal assembly of the complex. A synthetic biology screen was conducted by analyzing the interaction patterns of 1493 genes in over 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. Based on this work and others,[154] 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 (e.g., heme) between the ER and the mitochondria.

Additionally, the 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 also fuse with peroxisomes, and a subset of peroxisomes also possess MDV markers.[155,156] Interestingly, MDVs that contain MAPL lack the outer mitochondrial membrane protein TOM20, while MDVs that contain TOM20 lack MAPL. The observations suggest that MDVs are able to discriminate the cargo loaded within.[155,157] Indeed, steady-state MDVs were shown to carry selected cargos to the lysosomes as an early response to oxidative stress but distinct from mitophagy.[158]

In the early 1960s, several papers from Wildman *et al.* and Spencer and Wildman demonstrated that the chloroplasts of wild-type plants can develop tubular structures and protrusions.[159–161] These findings were largely forgotten until Kohler *et al.* "rediscovered" these structures and named them stromules, for stroma-filled tubules.[162,163] 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.[164] In the stroma, proteins traffic by diffusion as well as by an active process of directional travel, whose mechanism remains 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.[165–167]

F. Heme Reductases

Metalloreductases have been demonstrated to play a critical role in the transport of metals. Six transmembrane epithelial antigen of the prostate 3 (Steap3) and duodenal cytochrome b (Dcytb), for example, are ferric reductases required for efficient iron import into cells.[168–170] Dcytb and other Steap family proteins have also been shown to serve cupric reductases.[171,172]

Studies have indicated that before it is covalently linked to apocytochrome c, hemin (heme with an oxidized iron atom) must be reduced.[173,174] Evidence indicates that the protein Cyc2p can perform this function in yeast. Using NAD(P)H as cofactor, Cyc2p reduces hemin to heme in the IMS of the yeast mitochondria.[175,176] Cyc2p functions in concert with cytochrome heme lyases for covalent attachment of heme to apocytochromes c and c_1. Given that heme synthesis occurs within the mitochondrial matrix and FECH produces reduced heme, the model for heme reduction in the IMS by Cyc2p implies that heme is oxidized in that compartment by an unknown mechanism. Other proposed heme reductases include the cytochrome c synthetase, CcmF, of Gram-negative bacteria, which may function as a quinol:heme

Table 2. Heme-binding proteins.

Protein name	Proposed function	Location of function	Reference
HRG-3	Bind heme	Extracellular in *C. elegans*	225
HRG-2	Bind heme	Hypodermis in *C. elegans*	177
FABP	Bind heme/tetrapyrroles	Cytosol	205
GST	Bind heme/tetrapyrroles	Cytosol	181
HBP23	Bind heme/tetrapyrroles	Cytosol	194
SOUL/p22HBP family	Bind heme/tetrapyrroles	Cytosol	192,271
Haptoglobin	Bind extracellular Hb	Blood	209
Hemopexin	Bind extracellular heme	Blood	217
HDL and LDL	Bind extracellular heme	Blood	224
HeLp	Bind extracellular heme	Hemolymph in insects	251,252
Vitellogenin	Bind heme for heme transfer	Extracellular in insects	246,254,258
GAPDH	Heme insertion into iNOS	Cytosol	200
HSP90	Heme insertion into iNOS/nNOS	Cytosol	202,203
Cyc2p	Heme reductase	Mitochondria IMS	175,176
Dap1p/PGRMC1	Heme transfer to Cytochrome P450	ER but also detected in cytosol, PM and secretory pathway	178,179,272
Dcytb	Ferric reductase	Enterocytes	169
HSA	Bind extracellular heme	Blood	221

oxidoreductase, and the lipocalin α1-microglobulin, which can reduce hemin in cytochrome c and methemoglobin.[174] However, a plasma membrane-associated hemin reductase that plays a role in heme transport has yet to be found.

Another possible candidate heme reductase is HRG-2, a type Ia membrane protein, which localizes to the ER in the *C. elegans* hypodermis (Table 2).[177] Worms deficient in HRG-2 show abnormal distribution of soluble hemoproteins and altered expression of several cytochrome genes. Direct evidence for a reductase activity was not demonstrated because of the inability to isolate purified protein. Nevertheless, the topology of HRG-2 is identical to that of microsomal cytochrome P450s and is reminiscent of the heme-binding protein Dap1p in yeast and its human ortholog PGRMC1 which can interact with cytochrome P450s and increase their activities by an uncharacterized heme delivery system.[178,179] This conserved topology, along with the presence of a thioredoxin-like fold, implies that HRG-2 may function as a reductase necessary for efficient utilization of heme.

V. Cytoplasmic Heme-binding Proteins

Glutathione S-transferases (GSTs) are a class of enzymes that conjugate glutathione to a variety of electrophilic substrates during the process of xenobiotic

detoxification. GSTs have long been known for their ability to bind a wide variety of ligands in the cytoplasm; GSTs were first identified in rate liver as ligandins that selectively bind bilirubin, steroids, and organic anions.[180] Subsequent studies showed that GSTs can interact with heme and porphyrins.[181–183] GSTs are highly abundant in the cytoplasm of malaria parasites and helminthes. A *P. falciparum* GST (Pf-GST) interacts with heme and was later shown to contain both high- and low-affinity heme binding sites.[184,185] Heme but no PPIX can inhibit GST function in the rodent malaria parasite, *Plasmodium berghei*, and an inverse correlation between heme levels and GST activities has been shown both *in vitro* and in *P. berghei*.[186,187] Both a novel *Haemonchus contortus* GST (Hc-GST-1) and a homologous protein in *Ancylostoma caninum*, AC-GST-1, have been shown to interact with heme.[188,189] GSTs have been postulated to play a critical role in the transport and sequestering of heme in these organisms.[184,188,190]

The ubiquitously expressed murine protein, p22HBP, was identified as a cytosolic heme-binding protein.[90] This protein is highly expressed in the liver and is induced during erythroid differentiation in MEL cells; knockdown of p22HBP in MEL cells reduced heme content of these cells. Mouse and human forms of p22HBP bind heme and other porphyrins, including PPIX, *via* a hydrophobic cleft.[90,91,109] Another murine protein, SOUL, has 27% sequence identity to p22HBP and early studies indicated that it binds heme, forming a hexamer in the presence of heme and a dimer in the absence of heme.[191,192] However, evidence from surface plasmon resonance, crystal structure, and NMR studies clearly demonstrates that SOUL is a monomer that does not bind heme. SOUL also possesses a Bcl-2 homology 3 (BH3) domain and likely functions in apoptosis and/or oxidative stress rather than heme trafficking. The *Arabidopsis thaliana* encodes two p22HBP homologs, cHBP1 and cHBP2, which also bind tetrapyrroles reversibly *in vitro*.[193] While cHBP1 is highly expressed in leaves, cHBPs is expressed in roots, suggesting that these two HBPs may have similar functions in different plant tissues.[193]

HBP23, also known as mouse stress-inducible 23 kDa protein or proliferation-associated gene product, was originally purified on hemin–agarose using chromatography. HBP23 belongs to the peroxiredoxin family of peroxidases and was shown to have a high binding affinity for heme.[194] HBP23 is highly expressed in the cytosol of the liver, and is also present in the kidney, spleen, small intestine, and heart.[195] Its expression is upregulated by heme and other metalloporphyrins in primary rat hepatocytes; furthermore, incubation of rat liver HBP23 with heme inhibited its antioxidant activity.[196] Two hydrophobic regions, both containing histidine residues, on the surface of HBP23 may be responsible for heme binding.[197]

Another class of soluble hemoproteins includes the nitric oxide synthases (NOS), which play a role in cellular signaling by enzymatically converting the

amino acid L-arginine to citrulline and nitric oxide (NO). Mammals have three forms of NOS — endothelial (eNOS), inducible (iNOS), and neuronal (nNOS) — and NOS function depends on heme insertion into the active homodimeric form of the protein. Studies from the Stuehr group have found that, in an intriguing feedback mechanism, NO blocks heme insertion into extramitochondrial hemoproteins (including NOS) without changes in intracellular heme levels.[198,199]

Interestingly, the glycolytic enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) has been implicated in mediating heme insertion into iNOS.[200] Though traditionally viewed as a cytoplasmic housekeeping protein, potential new roles have emerged for GAPDH in DNA repair, membrane fusion, cytoskeletal remodeling, and cell survival.[201] A role for GAPDH in heme biology is, thus, less surprising. Alternatively, it has been reported that heme insertion into NOS is facilitated by the stress response protein, HSP90; HSP90 ATPase activity in the presence of a currently unidentified heme chaperone (postulated to be GAPDH) is required for insertion of heme into iNOS and nNOS.[202,203] It must be cautioned, however, that HSP90 is a highly abundance protein localized to many cellular compartments (nucleus, mitochondria, and extracellular matrix) and is known to associate with a host proteins with exposed hydrophobic surfaces or low stability.[204]

One other class of cytosolic proteins known to be capable of binding heme are fatty acid-binding proteins (FABPs).[205,206] However, the *in vivo* function of heme-binding proteins remains unclear.

VI. Extracellular Heme-binding Proteins

During physiological events, including the destruction of senescent RBCs and the enucleation of erythroblasts, free heme and Hb can be released into the plasma. More severe hemolysis is also induced during pathological conditions such as hemoglobinopathies, physical trauma, and infection. In order to protect tissues from the toxicity associated with free heme, to prevent heme iron from being utilized by pathogenic microorganisms, and to enable the recycling of heme iron, a variety of heme- and Hb-binding proteins are secreted into the circulation.

Haptoglobin (from the Greek *hapto* — "bind to") is a plasma glycoprotein secreted primarily from hepatocyte that is capable of binding Hb. There are three major subtypes of haptoglobin, which can all form soluble complexes with Hb dimers (K_d ~10^{-12} M) in an equimolar ratio.[207–209] The CD163 receptor on the surface of monocytes and macrophages recognizes the haptoglobin–Hb complex, which is subsequently endocytosed.[210] Receptors for the haptoglobin–Hb complex also exist on the membrane of hepatocytes and hepatoma cell lines.[211,212] Upon endocytosis, the protein complex is degraded in lysosomes, and the unbound heme

is degraded to free iron.[213–215] Thus, haptoglobin-binding serves a physiological role which results in the conservation and recycling of Hb and heme iron.[215,216]

Another plasma protein involved in heme scavenging and recycling is hemopexin, which directly binds heme with high affinity (K_d ~10^{-13} M).[217] Hemopexin–heme complexes are recognized by LRP/CD91 (LDL receptor-related protein, also termed CD91) on the surface a variety of cells, including hepatocytes, macrophages, and syncytiotrophoblasts.[218] Following recognition and binding, the hemopexin–heme complex is endocytosed. Heme is subsequently released in the lysosome for degradation or possibly recycling, while hemopexin (unlike haptoglobin) may be recycled back to the circulation.[218,219] In hemopexin-null mice, increased oxidative stress and altered regulation of ferritin and HO-1 is observed, supporting a vital role for hemopexin in heme detoxification.[220]

Human serum albumin (HSA), the most abundant protein in human plasma, is a 66 kDa protein that is known to bind an assortment of proteins, including heme (K_d ~10^{-8} M).[221,222] The crystal structure, as determined by Zunszain *et al.*[223] reveals how heme is coordinated by HSA. Three basic residues at the entrance to a hydrophobic cleft form charge pair interactions with the propionate side chains of heme. Inside the cleft, the iron atom within the heme molecule is coordinated by a tyrosine residue.[223] Two other serum proteins known to bind heme are high- and low-density lipoproteins (HDL and LDL). Both HDL and LDL bind heme faster than hemopexin or HSA, with an affinity that is higher than even that of HSA (K_d ~10^{-11} M).[221,224] It is thought that this rapid binding is critical to prevent damage by heme during the initial release of heme and provides a buffer period for hemopexin and HSA to steadily but tightly bind heme. Eventually, hemopexin and HSA remove all but a residual amount of heme from the lipoproteins.[224]

VII. Intercellular Heme Transport

Microarrays in *C. elegans* identified over 200 HRGs. As mentioned, the membrane-bound permeases HRG-1 and HRG-4 are responsible for importing dietary heme into the cytosol of the worm intestine.[123,124] However, as *C. elegans* cannot synthesize heme, there remains a need for transporters and chaperones to deliver heme to extraintestinal tissues, including neurons, muscle, hypodermal cells, and to develop germ cells and fertilized embryos. HRG-3, an 8 kDa peptide secreted from the maternal intestine, delivers heme to developing oocytes, ensuring that sufficient heme is available to support embryonic development.[225] HRG-3 mutant worms die during embryogenesis or developmentally arrest post-hatching — phenotypes that can be rescued by maternal but not zygotic *hrg-3* expression, as well as by supplementation with dietary heme. HRG-3 was shown to bind both ferrous and ferric heme with a stoichiometry of two HRG-3 monomers to a single heme

molecule.[225] While these data suggest that HRG-3 functions as an intercellular heme chaperone, a number of questions persist: (1) How is heme loaded into the complex before secretion? (2) What is the nature of recognition and internalization of the HRG-3–heme complex into oocytes and other extraintestinal tissues? (3) How is heme released from the complex when it reaches its target tissue?

While a system for intercellular heme transport is required in an animal unable to synthesize heme, experimental evidence suggests that such pathways might also exist in vertebrates. First, studies in human colon-derived Caco-2 cells and mouse macrophage suggest that a portion of heme derived from dietary sources or senescent RBCs is released into the blood as an intact metalloporphyrin.[130,226] Second, when human patients with acute attacks of porphyrias are treated therapeutically with intravenously administered heme, heme-dependent enzyme activities in liver increase, indicating that heme infused into the bloodstream can be redistributed and used *in to* by peripheral tissues.[47,227] Third, loss of function mutations in heme synthesis is embryonically lethal in mice; however, null embryos survive at least until E3.5. This hints at the existence of heme stores that can support embryonic development for a short period of time.[228,229] Similarly, deletion of heme synthesis is lethal to zebrafish, but mutant embryos can survive from 10 to 25 days post-fertilization. These embryos may either contain maternal-derived mRNA for heme synthesis enzymes or, intriguingly, stores of deposited maternal heme.[13,230] Consistent with this notion, FLVCR1 and HRG-1 may function to redistribute heme during organogenesis and development. Indeed, FLVCR1 is expressed substantially in the yolk sac and placenta, perhaps to mobilize maternal heme for fetal development.[133,231] Together, these results lead us to propose the existence of a functional intercellular pathway to facilitate the targeted delivery and redistribution of heme during vertebrate development. We deem it worthwhile to look at trafficking and redistribution of metals such as iron or copper as heuristic models of micronutrient homeostasis.

VIII. Parasitic Worms and Heme

Parasitic worms a major cause of chronic infections in humans, livestock, and crops. More than one-fourth of the world's population is infected with one or more of 20 common parasites, including *Ascaris*, *Ancylostoma*, *Trichuris*, *Onchocerca*, and *Schistosome*.[232,233] In addition, animal parasites and plant parasites cause enormous economic damage due to loss of agricultural production each year. As traditional anthelmintics become less effective due to a rise in drug-resistant worms, new drug targets will become increasingly important.[234,235]

The growth, reproduction, and pathogenicity of parasitic worms are all dependent on heme and iron. Iron supplementation stimulates the growth of the fluke *Schistosoma mansoni in vitro*, and in its animal hosts, *S. mansoni* ingests

large quantities of blood.[236] Because the availability of iron in the host's circulation is highly regulated, it is possible that *S. mansoni* acquires the bulk of its nutritional iron in the form of heme.[237] Similarly, host iron status has been shown to mediate pathogenicity in the hookworm, *Ancylostoma ceylanicum.* Animals fed with an iron-restricted diet and then challenged with this hookworm showed a significant reduction in intestinal worm load compared to control animals.[238]

Mechanisms used by parasitic worms to release heme or iron from ingested blood would be appropriate targets for anthelmintic drugs. The canine hookworm, *A. caninum*, breaks down Hb *via* a cascade of degradation reactions involving aspartic proteases, cysteine proteases, and metalloproteases.[239] Vaccination of dogs with APR-1, an *A. caninum* aspartic protease, resulted in the generation of neutralizing antibodies which actively reduced hookworm loads, fecal egg counts, and host blood loss in response to challenge with *A. caninum* larvae.[189]

A number of parasitic worms, including *Strongyloides*, *Ancylostoma*, *Haemonchus*, *Trichuris*, and *Ascaris*, have been shown to lack the heme biosynthetic pathway.[29] The genomes of these worms encode a number of hemoproteins (including globins, cytochrome P450s, catalases, and guanylate cyclases) and thus likely require heme for survival. Like *C. elegans*, these worms must, therefore, have evolved an efficient system for intestinal absorption of nutritional heme, as well as storage and distribution of this heme to extraintestinal tissues. Thus, worm-specific molecules involved in heme homeostasis, especially in heme uptake, could be potential drug targets for helminthic control.

IX. Heme Transport in Insects

Hematophagous insects ingest massive amounts of blood in a single meal.[240] In the mosquito *Aedes aegypti*, heme is the source of over 98% of iron in the insect body and eggs.[241] Accordingly, Hb is undoubtedly the major source of iron for blood-feeding arthropods. To minimize the toxicity of free heme released by Hb digestion, these insects have evolved efficient strategies to excrete, transport, and sequester heme.

Insects' first line of defense occurs at the level of heme absorption in the gut. First, insects have unique mechanisms to sequester and detoxify heme in the intestinal lumen. Ferrous heme can be oxidized to ferric heme, which aggregates into an insoluble crystal compound called hemozoin.[242] Hemozoin has been detected in the malaria parasite *Plasmodium falciparum*, the parasitic worm *Schistosoma mansoni*, the parasitic protozoan *Haemoproteus columbae*, and the kissing bug *Rhodnius prolixus*.[242–244] Inhibition of hemozoin formation by chloroquine leads to increased levels of free heme and increased lipid peroxidation.[245] *In vitro* studies

further confirmed that, compared with free heme, hemozoin generated fewer free radicals, caused less lipid peroxidation, and did not lead to the lysis of RBCs.[246,247]

The mosquito *A. aegypti* does not form hemozoin and relies on alternate mechanism for protection from heme toxicity. A study in *A. aegypti* showed that 87% of total ingested heme was excreted before the end of the first gonotrophic cycle, indicating that only a small percentage of ingested heme is actually absorbed in the intestine.[248] *A. aegypti* has a layer of peritrophic matrix covering the intestinal epithelium that separates intestinal cells from the food. This matrix has been shown to be associated with high levels of heme after feeding and contains a heme-binding protein, *A. aegypti* intestinal mucin 1, that is likely a major heme sequestration molecule.[249]

The pathways of heme uptake have been well characterized using heme analogs and fluorescently labeled Hb in the cattle tick *Boophilus microplus.* While most eukaryotes synthesize their own heme, *B. microplus* has been shown to lack the heme biosynthetic pathway.[30] Hb is absorbed *via* receptor-mediated endocytosis into specialized digestive cells and transported to primary lysosomes, where it is degraded[250] The released heme is sequestered in specialized intracellular organelles called hemosomes, where it is sequestered and prevented from forming free radicals.[240,250]

Heme is transported from the intestine to target organs *via* the hemolymph-filled open circulatory system — the hemocoel. Contained in the hemolymph are a variety of heme-binding proteins involved in sequestration and delivery of heme. Heme lipoprotein (HeLp) is a major component of *B. microplus* hemolymph that is expressed in male and female insects after host-attachment and blood feeding.[251] HeLp is likely to play a role in delivering heme to tissues. It has been shown to be bound to two heme moieties, and is capable of binding six more. Additionally, injection of HeLp labeled with ^{55}Fe-heme resulted in a decrease of radioactivity in the hemolymph and a concomitant increase of radioactivity within oocytes, indicating that heme bound to HeLp can ultimately be transported to a target tissue.[252] The HeLp homolog, hemolymph CP, has also been characterized as a major hemolymph protein involved in heme sequestration in the American dog tick, *Dermacentor variabilis*.[251,253,254] *In vitro* assays indicated that phospholipids incurred less oxidative damage in the presence of HeLp/CP-bound heme when compared to free heme, further implicating a role for these proteins in minimizing heme toxicity.[255]

Besides HeLp/CP, other proteins, such as *Rhodnius* heme-binding protein, may bind heme and decrease toxicity of free heme in insect hemolymph.[256,257] Similarly, in ticks and other insects, the major yolk protein, vitellogenin, is capable of binding heme. The binding of heme by vitellogenin strongly inhibited lipid peroxidation induced by free heme.[246] Insect vitellogenin also likely plays a role

in transferring heme to eggs. Its expression is induced in the fat body and midgut of female ticks after blood feeding, after which it transferred to developing oocytes.[254,258] To this end, an important role has been found for endopeptidases such as vitellin-degrading cysteine endopeptidase, *Boophilus* yolk cathepsin, and tick heme-binding aspartic proteinase, which are all involved in releasing bound heme in the developing eggs.[259,260]

X. Heme Transport in Yeast

Pathogenic fungi must extract a number of nutrients from their hosts and have been shown to be adept at utilizing both nutritional iron and heme. Weissman *et al.* first revealed that *Candida albicans* can use a pathway distinct from iron uptake pathways to utilize nutritional heme and Hb.[261] Studies of heme uptake kinetics in *C. albicans* showed a fast early binding phase followed by a slow gradual uptake phase.[262] A genome-wide screen in *C. albicans* revealed a Rbt5 and Rbt51 to be candidate genes involved in heme uptake.[263] Expression of *RBT5* is highly upregulated in *C. albicans* under iron-limited conditions, and deletion of this gene results in impaired heme utilization. Both Rbt5 and its close homolog, Rbt51, are glycosylphosphatidylinositol (GPI)-anchored proteins transiently located on the plasma membrane to serve as receptors for heme and Hb; upon binding to heme and Hb, the complex is endocytosed and delivered to the vacuole, where heme can be degraded by HO.[264] The HO enzyme *CaHMX1* of *C. albicans* is positively regulated by heme and Hb and has been characterized both *in vivo* and *in vitro*.[262,265]

In contrast with fungal pathogens, the budding yeast *Saccharomyces cerevisiae* lacks high-affinity heme transport systems and, thus, utilizes exogenous heme very poorly. However, an energy-dependent pathway for heme uptake in *S. cerevisiae* has been detected specifically under conditions of heme starvation or hypoxia.[266] Microarray analysis of *S. cerevisiae* grown under heme-poor conditions showed transcriptional upregulation of *protoporphyrin uptake gene 1* (*PUG1*).[267] Overexpression of PUG1 in both wild type and heme synthesis-deficient strains resulted in increased cellular PPIX levels and increased heme content, suggesting that PUG1 is involved in PPIX import and heme export. However, heme uptake is not altered in a *PUG1*Δ strain.[267] The mechanism and components of the *S. cerevisiae* heme uptake system remain unknown.

XI. Conclusions and Future Directions

Because of its vivid color and near ubiquitous presence in biology, heme has long been an object of scientific inquiry. For almost a century, the enzymes of heme

biosynthesis have been identified and characterized, yet knowledge of heme transport, trafficking, and assembly into hemoproteins has clearly lagged. With the availability of advanced genetic, microscopic, and biochemical tools, as well as paradigms set by researchers studying the transport of biologically relevant nutrients, such as metals and sterols, it is hoped that the players involved in these processes will be identified and unified into a holistic model for porphyrins and heme metabolism.

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