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COMMENTARY

CONCEPTS OF HEME DISTRIBUTION WITHIN HEPATOCYTES

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Heme is a molecule essential to life. Since its site of synthesis is usually distant from the site of its function within the cell, heme must be channeled across the interior of the cell, possibly bound to special carrier proteins. Heme is very lipophilic and aggregates in aqueous solutions, but this is normally prevented by its association with proteins. Little is known about the mechanism(s) by which heme is translocated intracellularly. The regulation of such translocation is particularly important in the liver which contains a major part of the body's heme, second in magnitude to that in the bone marrow, in the form of short-lived microsomal P-450 isozymes. These enzymes are part of the drug-metabolizing system and contribute greatly to the rapid turnover of heme in hepatocytes. Up to 70% of the total heme synthesized in the liver can be incorporated into the P-450 isozymes when the latter are chemically induced [1].

It is the aim of this brief commentary to discuss the various possible pathways of heme translocation within hepatocytes, to speculate on the different mechanisms by which heme can be translocated, and to describe characteristics putative heme carriers may exhibit.

Heme turnover

In the hepatocyte, heme transport from its site of synthesis in the mitochrondria to the various locations of heme utilization in the cell necessitates efficient compartmentalization (Fig. 1). Besides being incorporated locally into mitochondrial cytochromes, heme is incorporated into microsomal P-450 isozymes and cytochrome b_5, and into catalase in the peroxisomes [2]. It also accumulates in and enhances the activity of cytosolic tryptophan pyrrolase [3, 4], while some is even secreted into the circulation [5]. Part of the newly-synthesized heme is rapidly degraded [6], heme oxygenase being the rate-limiting enzyme ([7]; see also Ref. 8 for review). The heme degradation product, bilirubin, is then conjugated and secreted in the bile [9], while its iron is retained for storage in ferritin.

Figure 1 shows that heme also enters hepatocytes from the circulation. There are two major heme-binding serum proteins, albumin and hemopexin [10, 11]. Heme transport to the hepatocyte by hemopexin appears to involve a mediating receptor at the cellular site releasing the protein to be recycled [12]. Subsequent degradation of heme contributes to the body's iron conservation [11, 13].

Whereas most of the daily bilirubin production is derived from senescent erythrocytes engulfed by liver sinusoidal cells [14], experiments with rats have shown that bilirubin is also formed shortly after administration of labeled heme precursors [15, 16]. Schmid [17, 18] suggested that in these experiments the early part of labeled bilirubin may be derived from heme channeled into the degradative pathway during its transport from mitochondria to subcellular sites of hemeprotein assembly. Another part may be derived from heme incorporated into P-450 isozymes. Treatment with agents which accelerate

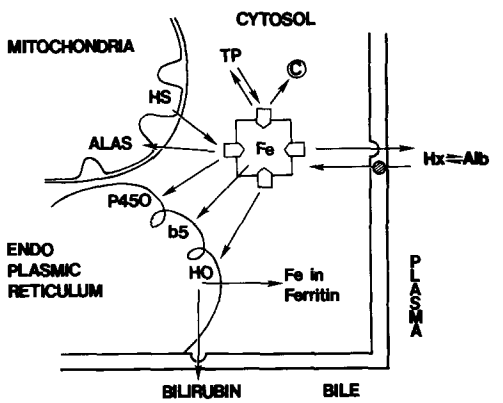

Fig. 1. Heme distribution in the hepatocyte. The first and last steps of heme biosynthesis take place on the inner mitochondrial membrane and are catalyzed by delta-aminolevulinic acid synthase (ALAS) and heme synthase (HS) respectively. Newly-synthesized heme effluxes into the cytoplasm where it mixes with exogenous heme transported from plasma by hemopexin (Hx). In plasma, heme bound to hemopexin is in equilibrium with heme bound to albumin (Alb). In the cytosol heme accumulates in tryptophan pyrrolase (TP). It is incorporated into catalase in the peroxisomes (C), cytochrome c (not shown) in the mitochondria, and cytochromes P-450 and b_5 in the endoplasmic reticulum. Heme inhibits ALAS translocation, and it is degraded sequentially by heme oxygenase (HO) in the endoplasmic reticulum and biliverdin reductase (not shown) in the cytosol to iron (denoted in the figure as Fe) and bilirubin, the latter secreted in the bile and the iron stored in ferritin.

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the turnover of these enzymes increases the magnitude of this part of bilirubin production [19]. In addition, drug metabolism is disturbed when agents are ingested which modify the heme moiety of P-450 ([20]; see also Refs. 21 and 22 for review). Patients with hepatic porphyrias [23–25], conditions which are characterized by partial enzyme deficiencies of the heme biosynthetic pathway, exhibit deficiencies in hepatic drug metabolism [26–28]. They are especially prone to exacerbation of their condition when demands are made on their drug-metabolizing systems. It has been postulated that these diseases are "heme deficiency" states [29] and, indeed, intravenous administration of heme may alleviate exacerbations of the condition [30–32].

It is generally accepted that there is an intracellular "heme pool", fed from endogenous as well as exogenous sources, which contributes to the regulation of heme metabolism. Although no direct evidence for such a heme pool is yet available, indirect evidence for its existence includes the following: there is a similarity in the pattern of initial serum bilirubin production in rats both from endogenous (using the labeled heme precursor delta-aminolevulinate) and from exogenous heme sources [33]; heme injected intravenously into rats [34] or added to chicken embryo liver cells in culture [35–37] inhibits the induction of delta-aminolevulinate synthase and induces heme oxygenase. Delta-aminolevulinate administration is also known to increase hepatic heme oxygenase activity in the mouse and in the rat [38], and it increases bilirubin formation in neonatal rats [39] and in man [40, 41]. These findings imply that heme functions intracellularly: it appears, for instance, to inhibit the formation of delta-aminolevulinate synthase as well as the transfer of pre-delta-aminolevulinate synthase into the mitochondria [42–44] and to induce heme oxygenase ([7, 45]; see also Ref. 8 for review); and it may even be that heme exchanges between different molecular species of microsomally-bound P-450 [46, 47].

A contraction of this heme pool may occur in the hepatic porphyrias and an expansion could occur in either of two states: namely, an increase in heme synthesis above that necessary for hemeprotein production, or an increased influx of heme into the cell—the result of hemolytic anemia or the therapeutic administration of heme (as in the treatment of hepatic porphyrias).

Contraction of the heme pool would result in the induction of delta-aminolevulinate synthase [48]; it might also enhance the synthesis of heme-transporting proteins in an effort to seek heme extracellularly [49] or to shift in some fashion the compartmental heme distribution within the cell.

An expansion of the heme pool would lead to induction of heme oxygenase [50] and to an accumulation of heme in tryptophan pyrrolase [3, 4]. It could lead to production and/or enhanced assembly of cytochromes, although no increase in microsomal P-450 levels has been demonstrated under conditions of accelerated heme production [51]. Incorporation of heme into hemeproteins would prevent the possibility of excess heme causing membrane peroxidation, enhancing protein degradation [52] or inhibiting protein synthesis [53]. Heme injected in small amounts, for instance, increases the synthesis of hemopexin [49], whereas the presence of large amounts of heme—by administration or from severe hemolysis—decreases the production of this protein [49, 54, 55]. Although injections of small amounts of heme can (shortly after administration) replenish drug-induced loss of cytochrome P-450 function [56–58] in the rat, this does not necessarily take place in other species [59]. During chronic hemolysis in mice, hepatic delta-aminolevulate synthase and cytochrome P-450 levels are not decreased despite the marked induction of heme oxygenase, suggesting the development of some adaptive mechanism(s) after chronic exposure to heme [60]. Injection of large amounts of heme into rats [61–63] and rhesus monkeys [64] can cause a loss in total cytochrome P-450 content and in P-450-dependent drug-metabolizing activities. With respect to the latter, two disturbing facts have been encountered recently: (1) heme administered to man in the dose used in the treatment of hepatic porphyrias did not appear in the bile as bilirubin within the first 8 hr after i.v. injection [65], and (2) the same dose of heme injected into rhesus monkeys and rats not only severely depressed drug-metabolizing activities but also failed to cause a rise in microsomal heme oxygenase activity [64, 66].

Clearly, the consequences of disturbed production and utilization of hepatic heme are not fully known and little information is available on the mechanisms by which heme is transported and incorporated into hemeproteins.

Mechanisms of heme transport

Heme efflux from mitochondria. Following its synthesis on the inner mitochondrial membrane, heme has to cross the intermembrane space and outer mitochondrial membrane to reach the cytoplasm. As shown in Fig. 2a, this could take place by one or a combination of three mechanisms. In mechanism 1, heme is shown to diffuse passively through membranes but when in an aqueous environment it is bound to proteins. In mechanism 2, cytosolic heme-binding proteins assume an active role and actually penetrate the outer mitochondrial membrane to obtain heme from the inner membrane. A receptor for the cytosolic heme carrier may or may not be involved. In mechanism 3, there is a transient fusion of the two mitochondrial membranes enabling heme to diffuse onto cytosolic proteins.

The involvement of cytosolic proteins in heme efflux from the mitochondria has been implicated by several *in vitro* studies. (The presence of heme-binding proteins within the mitochondrial intermembrane space has not been studied.) In 1972, Yoda and Israels [67] stated that no heme exits from isolated rat liver mitochondria unless protein is present in the surrounding medium. Moreover, the rate of efflux of a synthetic metalloporphyrin from isolated mitochondria was observed to increase when heme-binding proteins such as hemopexin and myoglobin [68] or ligandin, the major glutathione transferase [69], were added.

Using liposomes as model membranes, we found that heme, which preferentially partitions into

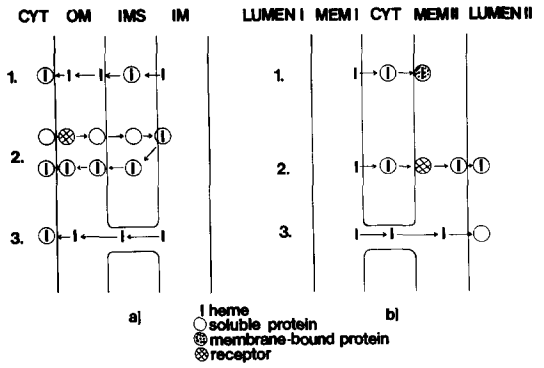

Fig. 2. Possible mechanisms of (a) heme efflux from mitochondria and (b) heme transfer between organelles. Mechanism 1 of scheme (a) involves passive diffusion of heme across the mitochondrial membranes and binding to proteins in the intermembrane space (IMS) and cytoplasm (CYT). In mechanism 2, heme efflux is carrier-mediated. A cytosolic heme-binding protein penetrates the outer mitochondrial membrane (OM) either by binding to a specific receptor or by random internalization and is translocated to the site of heme synthesis on the inner membrane (IM). There it obtains heme molecule(s) and diffuses out into the cytoplasm. In mechanism 3, fusion of the outer and inner mitochondrial membranes allows the heme to diffuse out passively into the cytosol where it is bound by protein. Scheme (b) shows three possible mechanisms for the transfer of heme between two organelles such as the mitochondria and endoplasmic reticulum. Heme is thought to leave the first organelle by any of the mechanisms shown in scheme (a) and, once in the cytoplasm, to be transported by carriers—namely, proteins. The cytosolic protein could deliver the heme molecule to the site of utilization or to the binding site of a membrane-bound apoprotein in the second organelle, if these are exposed to the aqueous environment, as shown in mechanism 1. Alternatively, as shown in mechanism 2, the cytosolic protein could penetrate the membrane of the second organelle (MEM II) to transfer heme to an intermediate protein carrier in MEM II for delivery to its site of utilization in the lumen (LUMEN II). In mechanism 3, there is a transient fusion of the membranes of the two organelles allowing the direct transfer of heme.

liposomes [70], is released in the presence of heme-binding proteins [71]. However, its rate of efflux from these artificial membranes is not dependent on the concentration of protein. The protein may merely serve as a receptacle for heme which could be provided by other substances with high affinity for heme. This finding supports mechanism 1 (Fig. 2a) in which the limiting step of heme efflux from membranes is heme release itself, not its uptake by proteins. Albumin, hemopexin and myoglobin, all three of which have a $K_a \geq 10^7\,M^{-1}$, accept heme with an identical rate constant, $k \sim 2\,sec^{-1}$, when the liposomes are negatively charged. The inclusion into the liposomes of stearylamine (which imparts a positive charge) or of cholesterol (which imparts rigidity to the membrane) lowers the observed rate of heme efflux. Unexpectedly, with each type of liposome used, we found several efflux rates rather than one. Since the rate of heme efflux is not affected by increasing the ionic strength of the medium, by preparing the heme–liposome mixture before or after sonication, or by introducing caffeine (which retains heme in the monomeric state [72]), we suggest that heme may occupy not one but several positions in the liposomes in a complex equilibrium which, in turn, may govern a stable overall rate of heme release.

In support of mechanism 2 for heme efflux (Fig. 2a) we showed in preliminary studies that certain heme-binding proteins can penetrate liposomal membranes. It needs to be stressed, however, that results of investigations with artificial membranes can only serve as guidelines for experiments with biological membranes which are obviously more relevant but may render results difficult to interpret. For instance, there is conflicting evidence as to whether ligandin and Z protein associate with and/or penetrate organelle membranes such as the endoplastic reticulum [73, 74]. An alternative mechanism for heme efflux could be through contact points between the inner and outer mitochondrial membranes [75] as shown in mechanism 3.

These mechanisms will have to be explored singly and in combination for each cytosolic protein suspected of playing a role in heme transport.

Heme transfer and hemeprotein assembly. Heme transfer across subcellular organelles such as the endoplasmic reticulum could take place by mechanisms similar to or different from heme efflux from the mitochondria, as shown in Fig. 2b. In mechanisms 1 and 2, heme is delivered by the cytosolic protein carrier onto the binding site of an apoprotein or a membrane receptor from where it is translocated into the lumen of the recipient organelle. Special mechanisms such as reduction (to be discussed below) may be operating for heme release. In mechanism 3 the aqueous cytoplasm is bypassed because the membranes of the two organelles are fused, as suggested to occur with endoplasmic reticulum [76] and mitochondrial membranes [75].

Mechanism 1 represents a possible route for the assembly of hemeproteins, involving not only heme transport but also the transport of the apoprotein which is synthesized on the endoplasmic reticulum. Two possibilities exist for the location at which heme incorporation into hemeproteins takes place. Heme could be transported out of the mitochondria and become incorporated into the polypeptide chains during translation. The holoprotein may then either insert into the endoplasmic reticulum or translocate to its final destination. Alternatively, the apoprotein could first be embedded into the endoplasmic reticulum to await assembly, or be translocated as such to other organelles to be coupled there with heme into the holoenzyme. The P-450 isozymes are inserted into the endoplasmic reticulum co-translationally [77]; when and how the heme moiety is incorporated into the proteins is not as yet known.

Catalase assembly has been shown to take place in the peroxisomes [2]. The apoprotein traverses the peroxisomal membrane and is combined inside the organelle with heme which has also been imported into that organelle, possibly by mechanism 2 of Fig. 2b. Assembly of the mitochondrial cytochrome *c* seems to be more complicated. Apocytochrome *c* produced on the ribosomes is viewed as interacting

with a receptor on the mitochondrial membrane and then attaching to the inner mitochondrial membrane while heme is inserted [75].

The direct transfer mechanism 3 could be operative during P-450 assembly [78–80]. It is envisioned that complexes exist between mitochondria and rough endoplasmic reticulum in which heme diffuses from its site of production (the mitochondrion) to the site of apo-P-450 synthesis (the ribosome), the holoenzyme being subsequently located in the smooth endoplasmic reticulum. This mechanism is an attractive one since the membranous lipid–lipid contact would provide an optimal hydrophobic environment necessary to prevent heme from aggregating [81]. However, the directional movements of heme could not be accounted for by assuming its passive diffusion; this would lead to a uniform rather than a localized distribution in the model. The entrapment of a heme carrier in the mitochondrial/endoplasmic reticulum complexes has also to be excluded.*

Heme transport within the cytoplasm. Because heme is such a hydrophobic molecule, transport within the cell by simple diffusion is highly unlikely. Rather, intracellular heme is thought to exist bound to a membrane or protein. Indirect evidence for the involvement of proteins in intracellular hepatic heme distribution was provided by Grandchamp *et al.* [82] who constructed a mathematical model for this process for a culture system of primary hepatocytes. Results from dual labeling experiments conducted in our laboratory [83], in which consecutive pulses of [^{3}H]- and [^{14}C]-delta-aminolevulinic acid (ALA) were given to rats, indicate strongly that several proteins are involved in the transport of endogenous heme from the mitochondria to other parts of the cell. Briefly, the experiment was conducted in the following manner: [^{3}H]- and [^{14}C]-ALA were administered intravenously to rats 1 hr apart. At time intervals of up to 3 hr, the livers were fractionated into mitochondria, microsomes and three cytosolic fractions of different molecular weight, and the ratio of [^{14}C]- : [^{3}H]-heme was determined. A fraction containing a high ratio of second [^{14}C] to first [^{3}H] label was taken to indicate that the fraction contained components in which heme turns over particularly rapidly.

The results of this experiment are shown in Fig. 3 taken from Ref. 83. The [^{14}C]- : [^{3}H]-heme ratio in two cytosolic subfractions, one of which contained a protein immunoreactive with an antibody to ligandin, increased for about 30 min after the second pulse at which time the ratio of heme loosely bound to mitochondria had reached unity. Loosely-bound heme could be heme in transit and/or heme non-specifically associated with organelles during subfraction. Heme tightly associated with microsomes, mitochondria and with proteins in a third cytosolic subfraction (containing catalase) reached equilibrium within an hour. A further significant finding was that the [^{14}C]- : [^{3}H]-heme

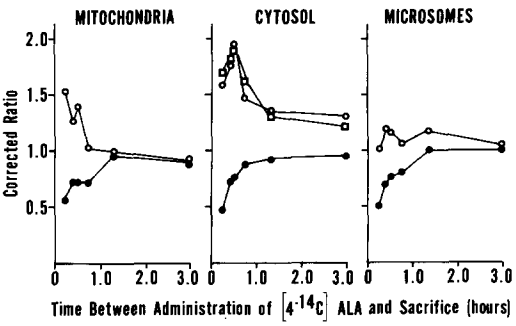

Fig. 3. Incorporation of heme synthesized from ^{3}H- and ^{14}C-labeled precursors into mitochondria, cytosol and microsomes. [^{3}H]-ALA (delta-aminolevulinate) was administered to rats (i.v.) followed by [^{14}C]-ALA 1 hr later. The animals were killed at various intervals, and the mitochondrial, cytosolic and microsomal fractions were obtained by subfractionation of the liver homogenate. Labeled heme was extracted from precipitated protein, purified, and counted for both ^{14}C and ^{3}H. The measured ratio of second to first label in the heme was divided by the ratio of the two isotopes in the administered ALA to give the corrected ratio. In addition, the ratio of labeled ALA incorporated into heme was corrected for the loss of ^{3}H during heme synthesis. The corrected ratio is shown as a function of time after administration of the second label for loosely bound (○) and tightly bound (●) heme in the mitochondria and microsomes and for three different cytosolic fractions obtained by gel filtration: two cytosolic fractions (○ and □), one of which contains ligandin, and another fraction (●) containing catalase. Loosely bound heme was separated from tightly bound heme in mitochondrial and microsomal fractions by treatment with human serum albumin and dithioerythritol [84]. Data are the averages of four experiments for the first time point and two experiments for the remaining points. (Reprinted by permission of *Biochem. J.* [83].)

ratio in the two cytosolic protein fractions suspected to contain proteins involved in heme transport had not reached equilibrium even at 3 hr after administration of the second pulse. This finding indicates that several cytosolic proteins exist which contain heme exhibiting different rates of turnover.

To develop working hypotheses of heme distribution and to explore what kind of non-specific and specific mechanisms of heme transport may exist, we have conducted a number of experiments concerned with heme exchange between soluble heme-binding proteins. For such functional inquiries it was necessary to study the rate of heme transfer by stop-flow kinetics. Albumin, hemopexin and myoglobin served as soluble proteins. Due to the pronounced tendency of heme to aggregate in aqueous media [81], our initial investigation [85] was conducted with a water-soluble heme-like metalloporphyrin, (tetraphenylporphinesulfonato) ferrate (III) (FeTPPS), which has, compared to heme, a relatively low dimerization constant [86]. The interaction of FeTPPS with albumin and hemopexin was found to have a biphasic reaction profile which we interpreted as showing the binding of FeTPPS dimers followed by dissociation of one of the monomer units forming a stable 1 : 1 porphyrin–protein complex. Subsequent studies on the interactions of these proteins with heme showed a monophasic reaction profile for heme–hemopexin

* Meier *et al.* recently reported data excluding a major role of these complexes in the incorporation of heme into apo-P-450 (P. J. Meier, R. Gasser, H-P. Hauri, B. Stieger and U. A. Meyer, *J. biol. Chem.* **259**, 10194 (1984)).

formation but biphasic profile for heme–albumin formation [87]. The much higher affinity of hemopexin for heme apparently monomerizes the heme aggregates instantaneously. As to be expected, the rate of interaction of heme with hemopexin, $k_a = 3.9 \times 10^7\ M^{-1}\ sec^{-1}$ [87], was much greater than that of heme with albumin, $k_a = 1.7 \times 10^5\ M^{-1}\ sec^{-1}$ [88]. Since the transfer of heme from albumin to hemopexin may be enhanced by catalysts, a number of nucleophiles and peptides were added to the reaction mixture. Indeed, the presence of imidazole (in excess of 10 mM)—but not histidine-containing peptides—did facilitate the transfer of heme from albumin to hemopexin,* The tissue concentration of imidazole is probably never as high as 10 mM and we are not implying that it is an *in vivo* catalyst for heme transfer. This finding points to the possibility, however, that proteins providing hydrophobic pockets may effect heme transfer by interprotein contact.

Considering the other alternative, that of a specific mechanism for heme delivery by proteins with very high affinity for this anion, we next explored whether the affinity of the two proteins for heme in which the iron was reduced to the divalent state differs sufficiently to accelerate heme transport. Indeed, the rate of transfer of reduced heme from albumin to hemopexin is $k = 0.3\ sec^{-1}$ which is approximately 60 times faster than that of oxidized heme.* Consequently, in the presence of dithionite and argon (to prevent the reoxidation of heme iron), heme is rapidly transferred from albumin to hemopexin. Deriving from these results a model mechanism, one could envision the co-ordinated presence of a reducing potential, provided enzymatically or nonenzymatically, as heme is passed on from one protein to another. Both proteins could be in solution or one could be anchored in a membrane, the other one either approaching from the cytosol or residing in a neighboring membrane. Such a mechanism would provide orderly transfer of heme strictly depending on the particular binding property of each participating protein.

Characteristics of putative cytosolic heme carriers

At the outset it should be stated that a protein which binds heme is not necessarily involved in its transfer, as it could merely serve as a storage place or be a hemeprotein itself. A protein functioning in heme transport may exhibit either a very low affinity or an exceptionally high affinity for heme. A protein which binds heme with a very high affinity (high k_a, low k_d) can be expected to be a specific heme carrier requiring special mechanisms such as a conformational change to release heme—possibly at a target site in the cell, such as a membrane receptor site. Such putative heme carrier(s) may even transfer the heme to a membrane protein which, acting as an intermediate receptor, passes it on in turn to other heme carriers—protein or possibly even non-protein carriers.

This brings us to a possible function of proteins with very low binding affinity for heme (comparable k_a and k_d). These proteins would simply provide hydrophobic pockets which would transfer heme in a stepwise fashion from one protein (or membrane) to the next, each successive molecule with a slightly higher affinity for heme. The finding that the cytosolic proteins presently known (mentioned below) exhibit a wide range of affinity for heme binding is consistent with this mechanism. Should such a chain mechanism exist, it would explain why several hitherto isolated proteins of the hepatic cytosol bind many anions with similar affinities. These anions would compete for binding sites on the assembly line and specific local conditions would determine their destination points.

Having stated a case for the heme-transport function of cytosolic proteins we must ask which candidates for such a function are known at present. Besides ligandin [89, 90], protein D_v [91] and protein Z [90] of the rat have been shown to bind heme. (Ligandin has also been proposed to function in heme transport in developing erythroid cells [92].) All glutathione transferases of rat [93, 94] and human [95] interact with heme, the binding constants ranging from $K_a = 10^5\ M^{-1}$ to $10^8\ M^{-1}$. The glutathione transferases [93] and protein Z [96], but not D_v [91], also bind bilirubin. Whether any of these or other heme-binding proteins actually possess transport properties is currently under investigation.

In conclusion, this brief account examines the experimental evidence that heme is distributed by mechanisms involving carrier proteins after its production by the mitochondrion and after its uptake from the circulation by the hepatocyte. The actual mechanisms of these processes are not understood. Heme transport into subcellular organelles may take place by similar or totally different mechanisms from those for heme efflux. Few details are available on the mechanisms—and there are several [97, 98]—for heme entry into hepatocytes from the circulation. Whether heme distribution and transport in non-hepatic cells operates in a manner similar to that in hepatocytes is not known. Since there is evidence suggesting that the mode of regulation of heme biosynthesis is different in non-hepatic cells such as erythroid cells, then heme distribution and transport in such cells might also be different. It can be expected that many interdisciplinary efforts will be required before we can begin to understand what prompts a heme molecule to be directed to one particular metabolic pathway rather than another.

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