Chapter 10
Erythroid Iron Metabolism

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Keywords Erythrocyte • Erythropoiesis • Globin • Heme • Hemoglobin • Red blood cell • Reticulocyte

1 Introduction

Iron is indispensable for the proper functioning of virtually all cells in the body. However, red blood cells, which contain approximately 80% of organismal iron, have a particularly intimate relationship with this precious metal. It is safe to say that the iron content of erythroid progenitors (e.g., BFU-Es; please see below) is infinitesimal compared to the amount of iron in mature erythrocytes that contain approximately 12×10^8 atoms per cell [1]; hence, the circulating red blood cells hold heme iron in a 20 mM "concentration." Since the developing red cells acquire iron only from diferric transferrin, which carries iron in plasma in about 3 μM concentrations, they have the capacity to increase this concentration 7,000-fold. Based on the value of 2.5 μg non-heme Fe per 100 mL erythrocytes [2], non-heme iron concentrations in erythrocytes are ~40,000-fold lower than those of heme iron. Additionally, the efficacy with which immature red blood cells convert transferrin-borne iron into hemoglobin iron is amazingly high [3, 4]. In the experience of these authors, reticulocytes (immediate progenitors of mature red cells) take up roughly 10 pmol Fe/10^6cells/h from diferric transferrin, corresponding to 6×10^6 atoms Fe/cell/h. Considering the above value of 12×10^8 atoms Fe per erythrocyte, it takes approximately 200 h (or 8.3 days) for iron to accumulate in total erythrocyte hemoglobin. This interval is slightly longer than the average erythroid cell maturation time (~5–6 days) but, since iron uptake by reticulocytes is probably somewhat slower than in bone marrow erythroblasts, the agreement is remarkably close. It needs to be pointed out that the rate with which iron is removed from the circulation by the developing erythroid cells is, under normal conditions, identical to the rate with which iron is released from macrophages following phagocytosis of senescent

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erythrocytes and hemoglobin catabolism by heme oxygenase 1. This very important aspect of iron metabolism is discussed in Chapter 11.

The fact that all hemoglobin iron is transported from transferrin [5] and that this delivery system operates so efficiently, leaving mature erythrocytes with comparably negligible amounts of non-heme iron, indicates that the iron transport machinery in erythroid cells is part and parcel of the heme biosynthetic pathway. It seems reasonable to propose that the evolutionary forces that led to the development of highly hemoglobinized erythrocytes also dramatically affected numerous aspects of iron metabolism in developing erythroid cells, making them unique in this regard.

The hemoglobin molecule is uniquely suited for the transport of oxygen from the lungs to peripheral tissues without oxidation of its heme[1] (a complex of protoporphyrin IX with ferrous iron) groups and to facilitate the return of carbon dioxide from the tissues back to the lungs [6, 7]. In adult humans, the two primary units of the molecule, the αβ dimers, associate to form the $\alpha_2\beta_2$ tetramer. Each chain is non-covalently bound to a single heme molecule that sits in a hydrophobic pocket. Since the ferrous iron of each heme group can bind a single oxygen molecule, the hemoglobin tetramer can reversibly bind and transport four molecules of oxygen. In addition to transporting oxygen and carbon dioxide, hemoglobin transports nitric oxide (NO) to tissues where this gaseous molecule plays an important vasodilatory role. Two mechanisms have been proposed to explain this process: (1) oxygen-linked allosteric delivery of NO from *S*-nitrosylated hemoglobin; it has been proposed that NO forms an adduct with cysteine (93) in the β-chain of oxyhemoglobin, forming *S*-nitrosohemoglobin [8], and (2) a nitrite reductase activity of deoxygenated hemoglobin that reduces nitrite to NO and vasodilates blood vessels along the physiological oxygen gradient [9]. Free hemoglobin in the bloodstream is very rapidly catabolized and can be toxic. Hence, one important function of erythrocytes is to prolong the hemoglobin's life span up to 120 days (in humans). Moreover, encasement within erythrocytes allows attainment of a remarkably high hemoglobin concentration of about 5 mM. It is likely that this is the maximal concentration of hemoglobin that, under normal conditions, can be reached in erythrocytes, since "hyperchromic" erythrocytes can be found only in patients with spherocytosis when red blood cells lose their biconcave shape [10].

Hemoglobin synthesis occurs using three independent but stringently coordinated pathways: globin synthesis, which is erythroid specific; heme synthesis that requires protoporphyrin IX synthesis; and the supply of iron from plasma transferrin to mitochondrial ferrochelatase. The two latter ubiquitous pathways are dramatically upregulated in developing red blood cells. One of the goals of this chapter is to convince its readers that in erythroid cells, and only in these cells, the path of iron from transferrin to ferrochelatase and protoporphyrin IX biosynthesis are highly integrated and are, in fact, essential components comprised by one metabolic pathway. Hemoglobin synthesis occurs in the developing red blood cells in the bone marrow in a process known as erythropoiesis that will be briefly discussed below.

2 Erythropoiesis

The average adult's blood contains about 24 trillion (2.4×10^{13}) erythrocytes with a wet weight of approximately 2.4 kg. Red blood cells are produced at a rate of 2.3×10^6 cells/s by a dynamic and exquisitely regulated process that is an integral part of the development of all blood cells, hematopoiesis. In humans, hematopoiesis occurs in the bone marrow of the adult and in the liver of the developing fetus. In addition to the bone marrow, the spleens of mice and rats are also important sites of

[1] Heme is ferroprotoporphyrin IX; hemin is ferric protoporphyrin IX. In this chapter, the term heme is used as a generic expression denoting no particular iron oxidation state.

erythropoiesis. The mature erythrocyte is the product of complex and highly regulated cellular and molecular processes that initiates at the level of the hematopoietic stem cells which have the potential to develop into all morphologically and functionally distinct blood cells. Stem cells, which are present in hematopoietic tissues in very small numbers (<0.01%), are self-renewing, slowly cycling cells that express receptors for stem cell factor (SCF) also known as c-kit receptor (tyrosine-kinase type). Although the regulation of stem cell proliferation and commitment is poorly understood, SCF and some other hematopoietic growth factors seem to be involved in the regulation. The process of commitment is characterized by restriction in the stem cell differentiation capacity, leading to the formation of progenitor cells that differentiate along one lineage [11–13].

Progenitor cells committed toward the erythroid lineage cannot be distinguished by morphologic criteria, but their existence and characteristics can be inferred from their capacity to generate colonies of hemoglobinized cells in vitro [11]. The earliest functionally detectable erythroid precursor is known as the BFU-E (burst-forming unit, erythroid), an early descendant of the hematopoietic stem cell. The BFU-E is detected by its capacity to generate multi-clustered colonies ("erythroid bursts") of hemoglobin-containing cells when marrow is incubated in semisolid medium in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), and erythropoietin. BFU-Es are relatively insensitive to erythropoietin both in vitro (requiring a high concentration of the hormone to generate the bursts of erythropoietic colonies) and in vivo.

The BFU-E in turn further develops to yield a class of more mature erythroid precursors, termed CFU-E (colony-forming units, erythroid). These cells are detected by virtue of their capability in cell culture to generate a small cluster of erythroid cells, which mature all the way to erythrocytes. The proliferative capacity of CFU-Es is limited to four or five cell divisions, generating 16–32 progeny red cells. Virtually all CFU-Es are proliferating, and they have an absolute requirement for erythropoietin to maintain viability, undergo cell division and, namely, to differentiate into proerythroblasts.

The first morphologically recognizable erythroid element is the proerythroblast, which is the immediate progeny of a CFU-E. Maturation of the proerythroblast to the circulating red cell involves four to five cell divisions; production of characteristic red cell proteins (hemoglobin, enzymes, and cytoskeletal proteins), surface antigens, and metabolic machinery; loss of replicative capacity and eventually of the nucleus itself; loss of organelles and acquisition of characteristic red cell morphologic features. The proerythroblast stage is succeeded by the basophilic erythroblast. This erythroblast is basophilic because of the high concentration of cytoplasmic ribosomes that accumulate in preparation for the onset of hemoglobin synthesis, which already occurs in these cells at a relatively low rate. The next cell, polychromatophilic erythroblast, displays increasingly deep staining for hemoglobin and a progressive decrease in the concentration of cytoplasmic ribosomes. Cell division continues until the stage of orthochromatic erythroblast in which hemoglobin synthesis continues on relatively stable ribosome-globin mRNA complexes. Extrusion of nuclei from the orthochromatic erythroblasts leads to the formation of reticulocytes. These non-nucleated cells still contain active globin-synthesizing polyribosomes as well as mitochondria that produce heme. At this stage, the reticulocytes are released into the circulation. During the first 24–36 h of circulation in the blood, the reticulocyte is transformed into the mature erythrocyte [14, 15]. The maturation of reticulocytes, which is not fully understood, is characterized by a progressive decrease in the number of polyribosomes and mitochondria, loss of hemoglobin synthetic capacity, loss of transferrin receptor 1 (TfR1), as well as by a decrease in size, and assumption of the biconcave disk shape [14, 15]. During the period of mitochondrial loss, Bcl-X(L), an anti-apoptotic protein that accumulates during erythroblast differentiation and maintains mitochondrial membrane integrity, demonstrated progressive decreases and changes consistent with deamidation [16]. Interestingly, two groups recently reported a role for a Bcl-2 family member, Nix (also called Bnip3L), in the regulation of erythroid maturation through mitochondrial autophagy. $Nix^{-/-}$ mice developed anemia with reduced mature erythrocytes and a compensatory expansion of erythroid precursors. Erythrocytes in the peripheral blood of $Nix^{-/-}$ mice exhibited mitochondrial retention and reduced life span in vivo [17]. Additionally, mitochondria

are depolarized in wild type but not Nix-deficient reticulocytes, a feature that appears to be required for the selective incorporation of mitochondria into autophagosomes [18]. Johnstone and her coworkers [19–22] and Blanc et al. [23] showed that reticulocytes lose their TfR1 by "shedding." The loss of TfRs seems to be preceded by the formation of multivesicular bodies containing encapsulated receptors. Fusion of multivesicular bodies with the plasma membrane leads to release of vesicles (exosomes), containing receptors whose extracellular domain is positioned externally, into the circulation. It is unknown how the above processes are triggered, but it is tempting to speculate that reaching a critical concentration of hemoglobin in reticulocytes represents a signal for their activation.

It is remarkable that, although three different and totally distinct pathways are involved in hemoglobin synthesis, virtually no intermediates, i.e., globin chains, intermediates of PPIX synthesis or iron, accumulate in the developing erythroblasts and reticulocytes. This is achieved, at least in part, by a series of negative and positive feedback mechanisms in which heme plays a crucial role. In general, in erythroid cells heme inhibits cellular iron acquisition (reviewed in [5, 24, 25]) and, consequently, heme synthesis, and is essential for the synthesis of globin. The effect of heme on its own synthesis and iron metabolism will be discussed later; here we shall briefly describe the role of heme in globin synthesis. Numerous reports indicate that heme stimulates globin gene transcription and is probably involved in promoting some other aspects of erythroid differentiation. Hemin treatment of erythroid precursors leads to a rapid accumulation of globin mRNA, whereas heme deficiency leads to a decrease in globin mRNA levels [26–30]. These heme-mediated effects likely involve the negative transcriptional regulator Bach1 which functions as a repressor of the Maf recognition elements (MARE, present in the β-globin locus control regions [LCR]) by forming antagonizing heterodimers with small Maf family proteins. Heme positively regulates the β-globin gene expression by blocking the interaction of Bach1 with the MARE in the LCR. This allows the binding of the transcription factor NF-E2p18/Mafk within this region, resulting in β-globin transcription [31, 32].

It has long been known that the translation of globin in intact reticulocytes and their lysates is dependent on the availability of heme [33–37]. Heme deficiency inhibits protein synthesis in part through activation of the heme-regulated inhibitor (HRI). HRI is a cyclic AMP-independent protein kinase which specifically phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2α). Phosphorylation of eIF-2α blocks initiation of protein synthesis. Heme regulates eIF-2α in the other direction by binding to and inhibiting HRI through the formation of disulfide bonds, possibly between two HRI subunits. Disulfide bond formation reverses the inhibition of globin synthesis seen during heme deficiency (reviewed in [37]). The expression of HRI seems to be confined to erythroid cells and, hence, HRI plays an important physiological role in the translation of globin and probably other proteins synthesized in erythroid cells.

In conclusion, in erythroid cells iron is not only the substrate for the synthesis of hemoglobin but also participates in its regulation. The iron protoporphyrin complex appears to enhance globin gene transcription, is essential for globin translation, and supplies the prosthetic group for hemoglobin assembly. Moreover, heme may be involved in the expression (at transcriptional as well as translational levels) of numerous other erythroid-specific proteins.

3 Iron Metabolism and Heme Synthesis in Erythroid Cells

In vertebrates, erythroid cells have by far the greatest need for iron which is used for hemoglobin synthesis with remarkable efficiency and speed [5, 24]. On a per-cell basis, the rate of heme synthesis in the erythron (According to Steadman's Medical Dictionary, Erythron = "the total mass of circulating red blood cells, and that part of the hematopoietic tissue from which they are derived") is at least an order of magnitude higher than in the liver, the second highest heme producer in the organism. Such an exceptionally high capacity of immature erythroid cells to synthesize heme is primarily a result of the

unique control of iron metabolism as well as the distinct enzyme of heme biosynthesis, 5-aminolevulinic acid synthase (ALA-S). Because of such an intimate link between iron metabolism and heme synthesis in hemoglobin-synthesizing cells, an overview of this process has to be included in this chapter.

3.1 Overview of Heme Synthesis

Current mechanistic aspects of heme biosynthesis have been thoroughly described in numerous review articles and books [5, 24, 25, 38–40]. It is well established that the synthesis of heme involves eight enzymes, four of which are cytoplasmic and four which are mitochondrial; all of which are encoded by nuclear genes. The first step occurs in the mitochondria and involves condensation of succinyl CoA and glycine to form ALA, a reaction catalyzed by ALA synthase (ALA-S) (Fig. 10.1). The next four biosynthetic steps take place in the cytosol. ALA dehydratase (ALA-D) converts two molecules of ALA to a monopyrrol porphobilinogen (PBG). Two subsequent enzymatic steps convert four molecules of PBG into the cyclic tetrapyrrole uroporphyrinogen III which is then decarboxylated to form coproporphyrinogen III. The final three steps of the biosynthetic pathway, including the insertion of Fe^{2+} into protoporphyrin IX by ferrochelatase, occur in the mitochondria (Fig. 10.1).

All enzymes of the mammalian heme pathway have been cloned and crystallized [5, 24, 38–40]. The genes encoding these enzymes reside on different chromosomes. There are two different genes for the first pathway enzyme, ALA-S. One of these is expressed ubiquitously (ALA-S1, or housekeeping ALA-S) and is encoded on chromosome 3 [42], while the expression of the other is specific to erythroid cells (ALA-S2, or erythroid ALA-S) and is encoded on the X chromosome [43]. These two genes are responsible for the occurrence of ubiquitous and erythroid-specific mRNAs for ALA-S and, consequently, two corresponding isoforms of the enzyme. No tissue-specific isozyme is known for ALA-D but there are subtle differences in 5′ untranslated regions (UTRs) in housekeeping and erythroid ALA-D mRNAs [44]. PBG deaminase (PBG-D) exists in two isoforms, one being present in all cells while the other is expressed only in erythroid cells [45]. However, these isoforms arise from differential splicing of a single gene and from two mRNAs which differ solely in their 5′ ends. There is no evidence that the ubiquitous and the erythroid enzymes would be different in the rest of the pathway. However, variations in mRNAs, caused by the alternative use of the two polyadenylation signals have been reported for ferrochelatase [46]. Interestingly, the preferential utilization of the upstream polyadenylation signal appears to be an erythroid-specific characteristic of ferrochelatase gene expression [47]. Importantly, murine [48] and human [49] ferrochelatase were shown to contain a [2Fe-2S] cluster which seems to be essential for enzyme activity and plays either a redox or regulatory role.

3.2 Overview of Iron Metabolism

All animal cells possess an absolute requirement for iron. However, the very properties of iron that make it indispensable for cells, namely, its ability to donate and accept electrons, also make it potentially hazardous. Iron is virtually insoluble in an aqueous environment under physiological conditions and its participation in one-electron redox reactions produces harmful hydroxyl radicals (also see Chap. 18). Therefore, specialized mechanisms and molecules have evolved for the handling of iron in a soluble, nontoxic form in order that cellular and organismal requirements of this metal can be met [5, 50–57]. In higher organisms, these functions are fulfilled by a number of specialized

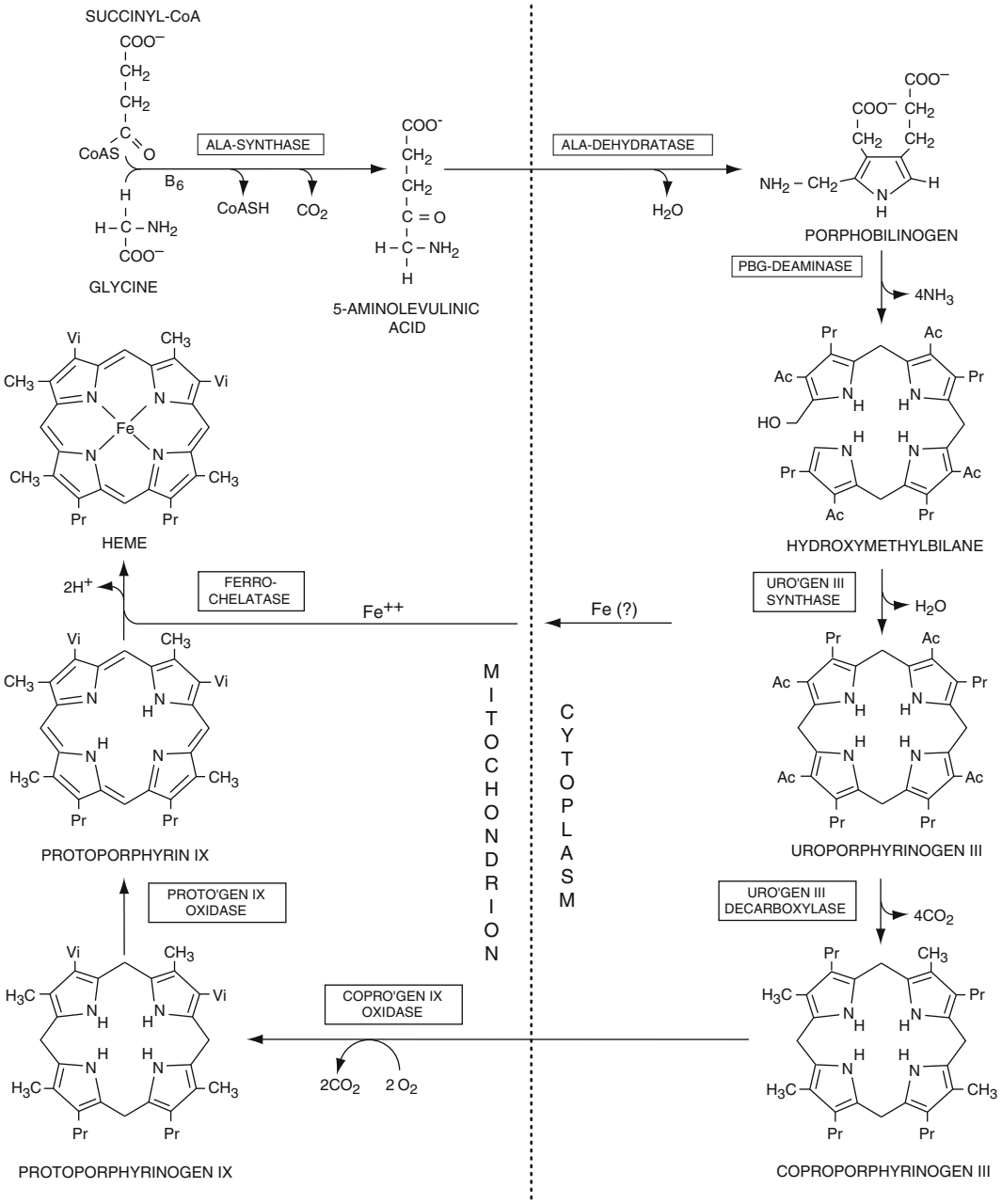

Fig. 10.1 The pathway of heme biosynthesis. *B6* pyridoxal-5′-phosphate, *URO'GEN* uroporphyrinogen, *COPRO'GEN* coproporphyrinogen, *PROTO'GEN* protoporphyrinogen, *Ac* acetate, *Pr* propionate, *Vi* vinyl (Source: Adapted from [41])

proteins, some of which are presented in Table 10.1. Moreover, an elegant regulation system exists that maintains the size of the intracellular pool of "iron-in-transit" at appropriate levels.

Iron is transported between sites of absorption, storage, and utilization by the 80 kDa plasma glycoprotein transferrin which contains two specific high-affinity iron-binding sites with an apparent stability constant for Fe(III) of approximately 10^{23} M^{-1} at neutral pH. Fe(II) does not bind specifically to these sites, and the affinity of transferrin for Fe(III) decreases progressively with decreasing

Table 10.1 Proteins involved in erythroid iron metabolism

Protein	Function	Result of deficiency	Role in erythropoiesis
ABCB7	Required for [Fe–S] on [Fe–S] proteins	XLSA with ataxia	Direct
ABCB10 (ABC-me)	Mitochondrial transport function related to heme synthesis	Unknown	Direct
ALA-S2/eALA-S	First enzyme of heme synthesis; erythroid-specific 5-aminolevulinic acid synthase	X-linked sideroblastic anemia	Direct
Ceruloplasmin (Cp)	Oxidizes exported Fe^{2+}	Neurodegeneration; hypochromic microcytic anemia	Indirect
DMT1/Nramp2	Vesicular and plasma membrane transporter for Fe^{2+}	Hypochromic microcytic anemia in mice. Hypochromic microcytic anemia with iron loading in humans	Direct
Ferritin (H and L)	Prevents Fe toxicity in all cells and during erythropoiesis	H-Ft: embryonic lethality	Indirect
Ferrochelatase	The last enzyme of heme synthesis; Fe^{2+} insertion into protoporphyrin IX	Erythropoietic protoporphyria (EPP)	Direct
Heme oxygenase-1 (HO-1)	Recycling of hemoglobin Fe	Severe anemia and inflammation	Indirect
Huntingtin (Htt) [58]	TfR1 trafficking	Hypochromic anemia in Htt-deficient zebrafish	Probably direct
IRP (1 and 2)	Fe "sensors"; bind to IREs	IRP2: brain Fe overload; microcytic anemia; EPP	Direct (IRP2)
Mitochondrial ferritin	Mitochondrial Fe storage (?)	Unknown; high expression in "ring" sideroblasts	Unknown
Mitoferrin (coded for by *SLC25A37*)	Fe transport to mitochondria	Hypochromic anemia	Direct
Sec15l1	"Vesicle docking"; required for efficient transferrin cycling	"Hemoglobin-deficit" (hbd) mice	Direct
Sideroflexin 1	Mitochondrial transport function related to Fe metabolism	Siderocytic anemia (mice)	Unknown
Steap3	Endosomal ferrireductase	Hypochromic anemia (mice)	Direct
Transferrin (Tf)	Fe(III)-carrier in plasma	Severe anemia (Fe unavailable for erythropoiesis; generalized Fe overload)	Direct
Tf receptor 1 (TfR1)	Membrane receptor for Fe_2-Tf	Embryonic lethality	Direct

pH below neutrality. Physiologically, most cells acquire iron from transferrin by a multistep process involving the specific attachment and internalization of transferrin bound to its cognate receptors (TfR1), followed by release of iron from transferrin by endosomal acidification via the v-ATPase H^+ pump (also see Chap. 7). After iron is released from transferrin, the metal is then translocated to intracellular sites of utilization or storage while the apotransferrin:TfR1 complex is recycled to the cell surface where the apotransferrin is released.

Once iron is released from transferrin within the endosome, the metal is transported across the organellar membrane by a promiscuous, divalent metal transporter, DMT1 (also known as Nramp2 or DCT1 [59, 60]). Since the form of iron bound to transferrin is in the +3 oxidation state, a reduction step, likely catalyzed by Steap3 [61], or another member of the Steap family [62], is required before transport out of the endosome. In erythroid cells, when iron reaches the outer mitochondrial membrane, it is entrapped by an as-yet-unidentified ligand and transferred across the inner membrane

to ferrochelatase. The group of Paw has recently demonstrated that mammalian homologs to yeast Mrs3/Mrs4 [63, 64], dubbed "mitoferrins," are responsible for iron import, probably in its Fe^{2+} form, through the mitochondrial inner membrane [65, 66]. Mitoferrin 1 exerts its function primarily in erythroid cells, but mitoferrin 2, which is expressed ubiquitously, cannot support hemoglobinization [66]. Of considerable interest in this context is a recent discovery [67] that mitoferrin 1 physically interacts with ferrochelatase and Abcb10, a mitochondrial inner membrane ATP-binding cassette transporter highly induced during erythroid maturation. The occurrence of a mitoferrin 1-ferrochelatase complex supports the notion that erythroid iron transport consists of a relay of the metal from protein to protein, rather than simply transport of the "free" metal across membranes.

Ferritin is a ubiquitous protein whose only well-defined function is the storage of iron ([50, 51, 68, 69]; Chap. 4). Although ferritin has been postulated to act as an intermediate for heme synthesis in erythroid cells, numerous studies failed to demonstrate that ^{59}Fe from ^{59}Fe-ferritin could be incorporated into hemoglobin [70]. Particularly strong evidence that ferritin is not involved in hemoglobinization has come from a recent study of Kühn and coworkers who demonstrated that the conditional deletion of ferritin heavy chain in adult mice did not cause any decrease in hematocrit or hemoglobin levels [71]. These findings concur with the proposal that the efficient utilization of iron for hemoglobin synthesis requires a direct contact of endosomes with mitochondria [4] and the observations that the intracellular release of iron from ferritin may require its catabolism [72]; all these studies suggest limited availability of ferritin iron for metabolic purposes. These conclusions can be expanded to non-erythroid cells, since a recent report failed to provide any evidence that, in cultivated macrophages, ferritin can make its iron available for heme synthesis [73].

Arosio's laboratory has recently identified a mitochondrial ferritin isoform that can store iron within a shell of homopolymers; however, the function and regulation of this protein is not yet understood [74–76]. Notwithstanding this, it has recently been demonstrated that the overexpression of mitochondrial ferritin causes the redistribution of iron from cytosolic ferritin to mitochondrial ferritin [77] and leads to the inhibition of cancer cells growth [78]. In this context, it should be pointed out that mitochondrial ferritin shows very low expression in all tissues except for testis [74, 76]. Additionally, although mitochondrial ferritin is not expressed in normal erythroblasts, it is expressed in ring sideroblasts of patients with sideroblastic anemia [79].

3.3 Coordinate Regulation of Ferritin and TfR1 Expression

Research conducted on non-erythroid cells cultured in vitro revealed a remarkable regulation system that coordinately regulates the expression of TfR1 and ferritin and, consequently, iron uptake and storage [50–52, 80–86]. A crucial component of this control is that which senses cellular iron levels, carried out by the iron regulatory proteins, IRP-1 (formerly known as IRE-BP [the iron-responsive element-binding protein]) and IRP-2. The coordinate control of TfR1 and ferritin occurs at the posttranscriptional level, and has been mapped to regions known as iron-responsive elements (IRE) that are recognized by IRPs. IREs are nucleotide sequences that form stem-loop structures and are present in the 5′ UTRs of ferritin H- and L-chain mRNAs and in the 3′-UTRs of TfR1 mRNA [87–90]. The IRE is also present in the 5′ UTR of mRNA for erythroid specific ALA-S (ALA-S2) [91–93] (see below). A comprehensive description of this extraordinary regulatory system is beyond the scope of this chapter; however, more intensive details can be found in Chap. 3. Briefly, IRP interaction with IRE controls iron metabolism in non-erythroid cells in the following manner: When cellular iron becomes limiting, IRP-1 is recruited into the high-affinity binding state. The binding of IRP-1 to the IRE in the 5′ UTR of the ferritin mRNA represses the translation of ferritin (and ALA-S2), while an association of IRP-1 with IREs in the

3′ UTR of TfR1 mRNA stabilizes the transcript. On the other hand, the expansion of the "labile iron pool" inactivates IRP-1 and leads to a degradation of IRP-2, resulting in an efficient translation of mRNAs for ferritin and ALA-S2 (occurring in erythroid cells only) and rapid degradation of TfR1 mRNA. However, in spite of this dramatic progress in our understanding of the regulation of proteins involved in iron metabolism, fundamental questions regarding iron transport within the cell remain unanswered.

3.4 Specific Features of TfR1 Regulation in Erythroid Cells

Although it is generally assumed that the above mechanism coordinates regulation of iron uptake and storage by all cells, there are some features of erythroid TfR1 regulation that suggest either the existence of erythroid-specific TfRs or at least distinct regulation of their expression: (1) There is evidence from the use of monoclonal antibodies that human erythroid cells may express a unique TfR1 isoform [94]. (2) Murine erythroleukemia (MEL) cells induced to erythroid differentiation respond only slightly to iron chelating agents which stimulate TfR1 expression in proliferating non-erythroid cells. This may be explained by an observation that iron chelators only modestly increase the binding activity of IRPs in differentiating MEL cells [95]. Moreover, in hemoglobin-synthesizing MEL cells, as compared to their undifferentiating counterparts, TfR1 mRNA is virtually unaffected by high concentrations of iron [95]. (3) Heme synthesis inhibitors were shown to strongly inhibit TfR1 expression at both the mRNA and protein [95, 96] levels in nucleated erythroid cells, but had virtually no effect on the expression of TfR1 in cells that did not synthesize hemoglobin [95]. (4) Whereas in non-erythroid cells the transcriptional control of TfR1 expression in response to altered growth rates or iron deprivation does not seem to play a significant role, TfR1 is transcriptionally regulated and "overexpressed" in chick embryo erythroblasts [97] as well as MEL cells induced to synthesize hemoglobin [95]. (5) The recent findings of Schranzhofer et al. suggest that, rather than TfR1 expression being unresponsive to the activity of IRPs, the ability of the IRPs to sense incoming iron decreases, possibly by a more efficient routing of the metal to its site of use [98].

The above discussion would seem to suggest that iron metabolism in hemoglobin-synthesizing cells, in particular those that are in late stages of their maturation stage, may escape the control of the IRE/IRP system. However, two recent reports [99, 100], showing that IRP2$^{-/-}$ mice develop hypochromic microcytic anemia, suggest that IRP2 probably plays at least some role in TfR1 expression in erythroid cells. IRP2$^{-/-}$ animals have increased protoporphyrin IX levels (caused by ALA-S2 hyperexpression in their erythroid precursors) associated with a decrease of TfR1 expression that is a likely cause of anemia. In this context, it is pertinent to mention that the hematopoietic-specific transcription factor Stat5 was recently shown to regulate cellular iron uptake by erythroid cells either by transcriptionally controlling TfR1 [101] or doing so via IRP2 [102].

4 Erythroid-Specific Regulation of Iron Metabolism and Heme Synthesis

Apart from the above-described unique regulation of TfR1 in hemoglobin-synthesizing cells, several other considerations emphasize the idea of erythroid-specific metabolism of iron as well as its critical influence on heme and, consequently, hemoglobin production. As already pointed out, heme synthesis in erythroid cells accounts for about 80% of total body iron turnover and the iron in hemoglobin contains almost 80% of the total iron content of a normal adult. Also, transferrin is the only

physiological source of iron for erythroid cell heme synthesis, which is best documented by observations in humans and mice with hereditary atransferrinemia. Both patients and mice with atransferrinemia have severe hypochromic microcytic anemias [5, 50] which can be explained only by the stringent dependency of hemoglobin synthesis on transferrin-bound iron. Furthermore, iron delivery to ferrochelatase, but not a step in protoporphyrin IX synthesis, is rate limiting for heme synthesis in erythroid cells [5]. In other words, either TfR1 levels or a component of the iron transport pathway from Tf-TfR1 association to delivery to the mitochondrial matrix determines the efficiency of heme production in these cells. This conclusion is based on experiments showing that transferrin-independent iron uptake (from iron-salicylaldehyde isonicotinoyl hydrazone complex) in excess of the maximum amount of iron obtained from diferric transferrin, stimulates the synthesis of heme in erythroid cells but is without effect in non-erythroid cells [103–105].

In hemoglobin-synthesizing cells, iron is specifically targeted toward mitochondria which continue to take up the metal with gluttonous appetite even when the synthesis of protoporphyrin IX is suppressed [3, 106–109]. In contrast, in non-erythroid cells, iron in excess of metabolic needs ends up in ferritin [67, 68]. Hence, some specific mechanisms and controls are involved in the transport of iron into mitochondria in erythroid cells, but the nature of these processes, besides the likely role of erythroid-specific mitoferrin 1 [65, 66], is unknown. Based on the fact that transferrin-bound iron is extremely efficiently used for hemoglobin synthesis, that iron is targeted into erythroid mitochondria, and that no cytoplasmic iron transport intermediate can be identified in erythroid cells, an alternative hypothesis of intracellular iron transport has been suggested [3–5]. This model proposes that after iron is released from transferrin in the endosome, it is passed directly from protein to protein until it reaches ferrochelatase in the mitochondrion. Such a transfer would bypass the cytosol, as the transfer of iron between proteins could be mediated by the direct interaction of the endosome with the mitochondrion (Fig. 10.2a). We have recently collected strong experimental evidence, using erythroid cells, supporting this hypothesis that is as follows: (1) iron, delivered to mitochondria via the transferrin-TfR1 pathway, is unavailable to cytoplasmic chelators [4, 119]; (2) transferrin-containing endosomes move to and contact mitochondria [4, 119]; and (3) endosomal movement is required for iron delivery to mitochondria [4, 119]. We have also established that "free" cytoplasmic iron is not efficiently used for heme biosynthesis and that the endosome–mitochondrion interaction increases chelatable mitochondrial iron [119]. Since the majority of cellular iron is processed in the mitochondria of all cells, and not just red ones, it is tempting to speculate that this direct interorganellar iron transport mechanism is ubiquitous. However, Shvartsman et al. have recently documented in a leukemia cell line that the transfer of transferrin-derived iron to mitochondria is blocked by cytosolic chelators [120]. This study quantified compartmental iron levels with the aid of iron-sensitive fluorescent chelators, which are likely to obscure intracellular distribution and may therefore overestimate iron levels.

A further distinction between red-colored cells and the others is that in erythroid cells heme synthesis is controlled by a feedback mechanism in which "uncommitted" heme inhibits iron acquisition from transferrin [121–124]. Although it is still unresolved whether heme inhibits transferrin endocytosis [122] or iron release from transferrin [124], the lack of heme as a negative feedback regulator can help in explaining the aforementioned mitochondrial iron accumulation. This may provide a clue to clinical hematologists as to the pathogenesis of mitochondrial iron accumulation in erythroblasts of patients with sideroblastic anemia. It is essential to point out that this effect of heme is specific for hemoglobin-synthesizing cells, since heme does not inhibit iron uptake in non-erythroid cells [5].

Shortly after the identification of erythroid-specific ALA-S (ALA-S2), it became obvious that most hereditary X-linked sideroblastic anemias (XLSA) cases were caused by mutations in the *ALA-S2* gene [118]. Figure 10.2b provides a schematic representation of mechanisms attributed to XLSA: decreased PPIX formation, slightly increased iron uptake, and substantial accumulation of iron in MtF.

However, there is a certain proportion of patients with hereditary sideroblastic anemia who exhibit autosomal recessive inheritance. Recently, Guernsey et al. [125] described that at least some such patients have a defect in the gene encoding the erythroid-specific mitochondrial carrier protein, SLC25A38. They demonstrated that this transporter is important for the biosynthesis of heme in eukaryotes and conjectured that this protein may be translocating glycine into mitochondria [125]. Needless to say, defects in a putative mitochondrial glycine transporter would be expected to generate a phenotype identical to that seen in patients with defects in ALA-S2 (Fig. 10.2b). It is tempting to speculate that in erythroid cells a common control mechanism, which regulates acquisition of the

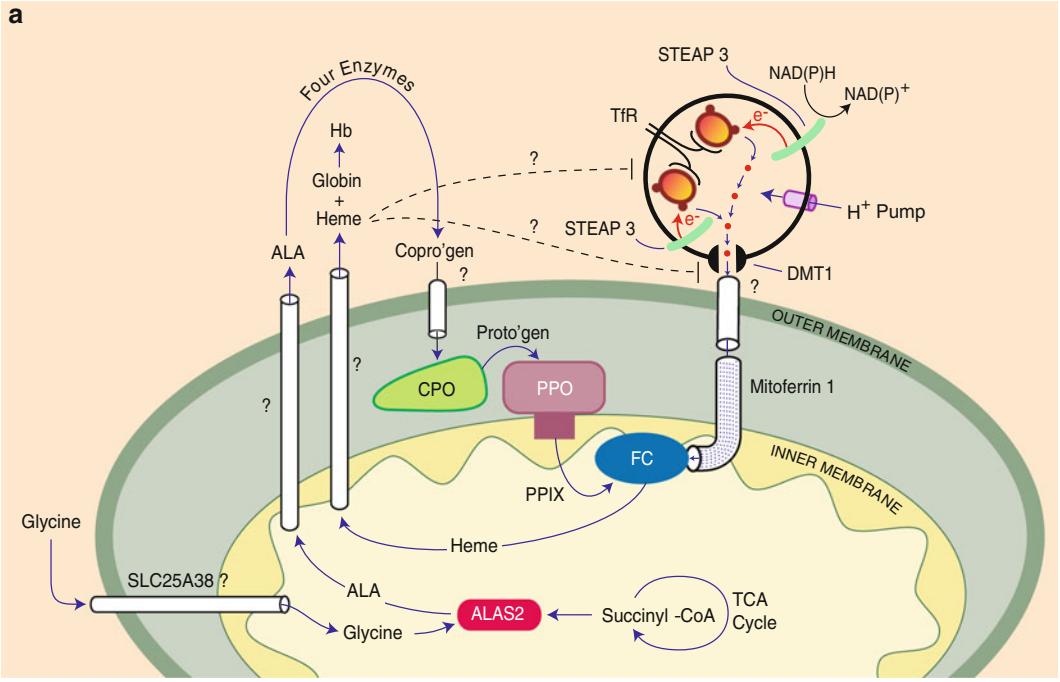

Fig. 10.2 Schematic representation of endosomal and mitochondrial steps involved in iron metabolism in normal erythroid cells (**a**) or those with inhibited heme synthesis (**b**). Iron is released from transferrin within endosomes by a combination of Fe^{3+} reduction by Steap3 (likely when it is still bound to TfR1 [110]) and a decrease in pH (~pH 5.5); following this, Fe^{2+} is transported through the endosomal membrane by DMT1. The post-endosomal path of iron in the developing red blood cells remains elusive or is, at best, controversial. It has been commonly accepted that a low-molecular-weight intermediate chaperones iron in transit from endosomes to mitochondria and other sites of utilization; however, this much sought iron-binding intermediate has never been identified. In erythroid cells, more than 90% of iron must enter mitochondria, since ferrochelatase (*FC*), the enzyme that inserts Fe^{2+} into protoporphyrin IX, resides on the inner leaflet of the inner mitochondrial membrane. Recent research supports the hypothesis that in erythroid cells, a transient mitochondrion–endosome interaction is involved in iron translocation to its final destination. It has been proposed that coproporphyrinogen (Copro'gen; please note that its generation is indicated in Fig. 10.1) is transported into mitochondria by either peripheral-type benzodiazepine receptors [111–113] or ABCB6 [114]. Neither the mechanisms nor the regulation of heme transport from mitochondria to globin polypeptides is known; however, it has been proposed that a carrier protein, heme-binding protein 1 (gene: *HEBP1*), is involved in this process [115–117]. (**b**) Pathological-ringed sideroblasts (iron loaded mitochondria surrounding the nucleus) can arise in patients with defective ALAS2 or SLC25A38 (a putative importer of glycine into erythroid mitochondria) because (**a**) heme, a negative regulator of iron uptake, is deficient; (**b**) iron is specifically targeted to erythroid mitochondria; (**c**) mitochondrial iron cannot be adequately utilized due to lack of PPIX and accumulates in MtF; (**d**) iron normally exits erythroid mitochondria only after being inserted into PPIX [5, 118]. *Hb* hemoglobin, *CPO* coprporphyrinogen oxidase, *PPO* protoporphyrinogen oxidase

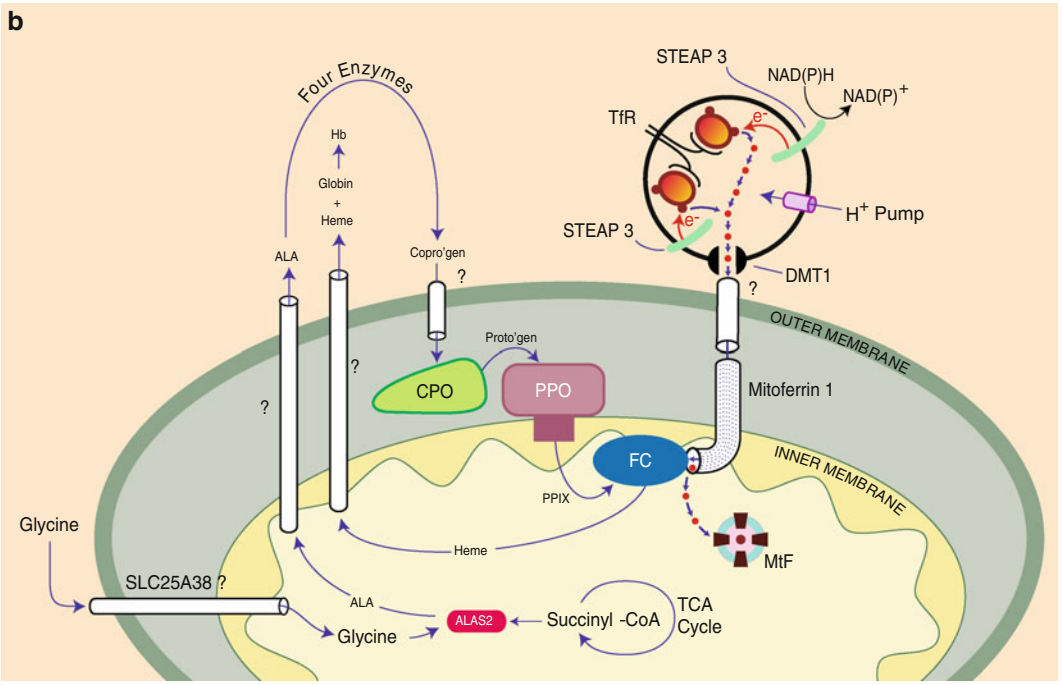

Fig. 10.2 (continued)

two substrates for heme synthesis (iron and glycine), exists. It needs to be pointed out that there are sideroblastic anemias not associated with heme synthesis defects. These conditions, which usually accompany one subtype (RARS) of myelodysplastic syndrome, are outside the scope of this chapter and are discussed elsewhere in this monograph (Chap. 16).

When discussing erythroid-specific ALA-S, there is a need to bring up an interesting and conceptually important development. Until recently, all reported mutations in ALA-S2 were shown to cause XLSA. However, Whatley et al. [126] described several families with ALA-S2 deletions resulting in frameshifts that lead to replacement or deletion of the 19–20 C-terminal residues of the enzyme. Prokaryotic expression studies showed that both mutations markedly increased ALA-S2 activity. These gain-of-function mutations caused a formerly undisclosed form of X-linked dominant protoporphyria, characterized by a high level of zinc-PPIX in erythrocytes; this symptom is reminiscent of erythropoietic protoporphyria caused by ferrochelatase defects in which there is accumulation of free PPIX. The authors explain this finding as indicating that the rate of ALA formation is increased to such an extent that the insertion of Fe^{2+} into PPIX by ferrochelatase becomes rate limiting for heme synthesis. It must be pointed out that this limitation is not ferrochelatase activity, which the authors later point out is in excess of the demands of hemoglobin synthesis, but due to the unavailability of iron for insertion into PPIX. The accumulation of zinc-PPIX is thus caused by a limitation of iron for erythroid heme synthesis, as extensively documented earlier [103–105].

Finally, as already mentioned, erythroid-specific ALA-S is uniquely regulated in erythroid cells. ALA-S2 mRNA contains an IRE in its 5′ UTR that is responsible for the translational induction of ALA-S2 protein by iron [91–93]. This means that in erythroid cells, the rate-limiting and, thus, controlling step in heme synthesis is not the production of ALA but the availability of iron. Moreover, heme has no inhibitory effect on either the activity or synthesis of ALA-S2 [5]. Furthermore, whereas

heme inhibits the import of ALA-S1 into mitochondria, it does not inhibit mitochondrial import of erythroid-specific ALA-S [127]. Finally, ALA-S2 enzyme, but not ALA-S1 protein, has been found to associate with succinyl CoA synthetase in mitochondria [128].

5 Integrated View: The Availability of Iron Controls Hemoglobinization

Erythropoiesis is a remarkably complex process that comprises both differentiation of erythroid cells from hematopoietic progenitor cells and induction of hemoglobinization in these cells. During differentiation, the three pathways that lead to the formation of hemoglobin are either transcriptionally induced de novo (globin) or transcriptionally increased (heme synthesis enzymes and TfR1, the gatekeeper for iron entry into the cell). It can be postulated that erythroid differentiation will be associated with a change ("differentiation") in mitochondria that will allow a specific targeting of iron into this organelle; however, it is currently impossible to foresee whether such an alteration is qualitative (induction of new proteins) or simply a quantitative one. Importantly, some changes in mitochondria during erythroid differentiation have already been defined. First, the induction of ALA-S2 is associated with a decrease in the housekeeping ALA-S1 [128]. Second, the erythroid transcription factor GATA-1 has been shown to induce a mitochondrial ABC transporter termed ABCB10 (ABC-me [129, 130]) that localizes to the inner mitochondrial membrane and is postulated to transport (a) yet-to-be-identified substrate(s) from the matrix into the intermembrane space. Importantly, ABCB10 overexpression enhances hemoglobin synthesis in MEL cells [129]. Hence, it seems likely that ABCB10 mediates critical mitochondrial transport functions related to heme synthesis [129]. Notwithstanding the above, recent research has revealed that ABCB10 physically interacts with mitoferrin 1 to enhance its stability and promote mitoferrin 1-dependent mitochondrial heme biosynthesis [131]. Moreover, it can also be assumed that erythroid differentiation is associated with the induction of a heme transporter involved in the export of heme from the mitochondria to the cytosol. Furthermore, a cytosolic heme-binding protein may be needed to safely carry heme from mitochondria to globin chains but, unfortunately, very little is known about this process (Fig. 10.2).

Hence, based on the experimental evidence and above discussions, it can be proposed that one aspect of erythroid differentiation involves an "iron metabolism switch" during which the erythroid-specific pathway and control mechanisms are turned on, leading to their prevalence in erythroblasts and eventually total predominance in reticulocytes. Once all the machinery required for hemoglobin synthesis is induced, its formation is controlled by a series of fine-tuning mechanisms depicted in Fig. 10.3a. In contrast to non-erythroid cells (Fig. 10.3b), heme does not inhibit either the activity or the synthesis of ALA-S, but does inhibit cellular iron acquisition from transferrin in erythroid cells. This negative feedback is likely to explain the mechanism by which the availability of transferrin-derived iron limits the heme synthesis rate, and also clarifies why the system transporting iron to ferrochelatase operates so efficiently, leaving normally mature erythrocytes with negligible amounts of non-heme iron. Since the 5′ UTR of ALA-S2 mRNA contains an IRE, the availability of iron controls ALA-S2 translation, the rate of ALA formation and, consequently, the overall rate of heme synthesis in hemoglobin-synthesizing cells. Because heme is required for globin mRNA translation [37], the overall hemoglobin synthesis rate appears to be controlled by the capacity of erythroid cells to acquire iron from transferrin. Therefore, it is tempting to speculate that erythroid cells with their high requirement for iron, whose biologic availability is so limited, have evolved regulatory mechanisms in which iron controls hemoglobinization.

In conclusion, the regulation of heme synthesis has extensively been studied only in erythroid cells that synthesize approximately 80% of organismal heme, and in the liver, which synthesizes

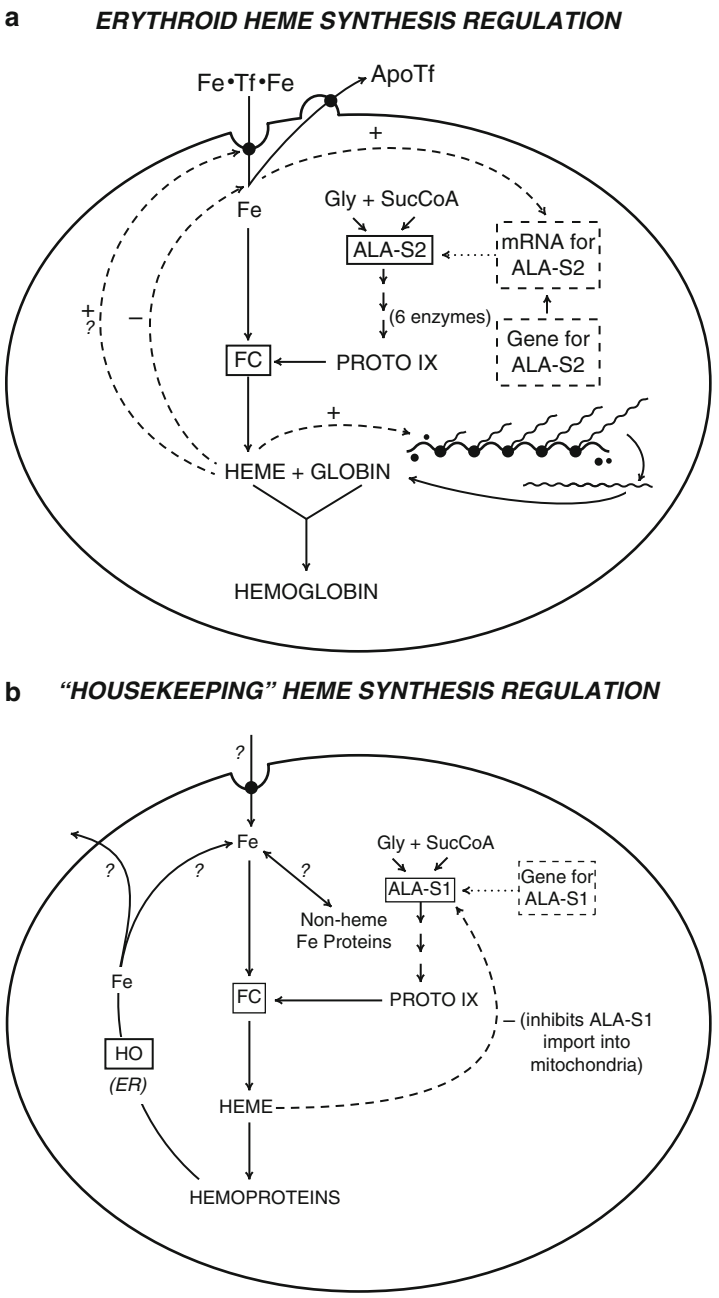

Fig. 10.3 Distinct aspects of heme synthesis regulation in erythroid (**a**) and non-erythroid (**b**) cells. Differences in iron metabolism and in genes for ALA-S likely account for the differences in the rate of heme synthesis and its regulation in erythroid cells as compared to other cells in mammals. (**a**) In erythroid cells, heme inhibits (–) cellular iron acquisition from transferrin (*Tf*) but also serves as a positive feedback regulator (+) that maintains high TfR levels [95, 96]. Heme does not inhibit either the activity or the synthesis of ALA-S2. Because the 5′-UTR of *ALA-S2* mRNA contains an IRE, the availability of iron controls ALA-S2 translation, the rate of ALA formation and, consequently, the overall rate of heme synthesis in hemoglobin-synthesizing cells. Heme is also essential for both globin transcription and translation. In contrast, (**b**) in non-erythroid cells, iron uptake is not regulated by intracellular heme and since the ubiquitous *ALA-S1* mRNA does not contain an IRE, iron availability does not control the overall heme synthesis rate. Additionally, heme represses ALA-S1 by decreasing the half-life of its mRNA [132] and by blocking enzyme's entry into mitochondria [127]. *Gly* glycine, *PROTO IX* protoporphyrin IX, *HO* heme oxygenase, *FC* ferrochelatase, *ER* endoplasmic reticulum (Source: Adapted from [5] and printed with permission)

most of the remaining heme molecules in the human body. One major difference between these two tissues is that the liver synthesizes and degrades heme continuously, whereas erythroid cells generate enormous amounts of heme that stay within the circulating erythrocytes for their life span of 120 days. Hence, the basic principles of the regulation of heme synthesis in the liver and erythroid cells are conceptually totally divergent, but are extremely well tailored for the needs of the respective tissues. Compared to the developing red blood cells, the liver produces heme with much lower rates, but in quantities that satisfy the requirements for the synthesis of hemoproteins. This is achieved by two major factors: the rate-limiting nature of ALA-S1 and the fact that the production of this enzyme is feedback inhibited by heme. In contrast, as extensively discussed above, the synthesis of heme in erythroid cells is comparable to "breaking a dam."

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