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Review

Biosynthesis of heme in mammals

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Abstract

Most iron in mammalian systems is routed to mitochondria to serve as a substrate for ferrochelatase. Ferrochelatase inserts iron into protoporphyrin IX to form heme which is incorporated into hemoglobin and cytochromes, the dominant hemoproteins in mammals. Tissue-specific regulatory features characterize the heme biosynthetic pathway. In erythroid cells, regulation is mediated by erythroid-specific transcription factors and the availability of iron as Fe/S clusters. In non-erythroid cells the pathway is regulated by heme-mediated feedback inhibition. All of the enzymes in the heme biosynthetic pathway have been crystallized and the crystal structures have permitted detailed analyses of enzyme mechanisms. All of the genes encoding the heme biosynthetic enzymes have been cloned and mutations of these genes are responsible for a group of human disorders designated the porphyrias and for X-linked sideroblastic anemia. The biochemistry, structural biology and the mechanisms of tissue-specific regulation are presented in this review along with the key features of the porphyric disorders.
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Keywords: Iron; Heme; Porphyrin; Porphyrias

1. Introduction

The total body iron content in adult humans is approximately 50 mg/kg in men and 40 mg/kg in women [1]. Almost all iron is incorporated into heme-containing proteins, particularly hemoglobin, myoglobin and cytochromes. The gender-based difference in body iron content is due to the smaller red cell, muscle and liver mass in women. The unique properties of heme, an iron molecule coordinated within a tetrapyrrole, allows heme to function both as an electron carrier and a catalyst for redox reactions. Heme is generated by the insertion of ferrous iron into the tetrapyrrole macrocycle of protoporphyrin IX, a reaction catalyzed by ferrochelatase, which resides in the mitochondrial matrix (Fig. 1). A highly conserved pathway involving both cytosolic and mitochondrial compartments is utilized to generate protoporphyrin IX but all of the heme biosynthetic genes are nuclear-encoded and translated in the cytoplasm.

Most heme synthesis takes place in developing red cells in the marrow but about 15% of the daily production takes place in the liver for the formation of heme-containing enzymes. The regulatory mechanisms controlling heme synthesis in these two organs differ. In the liver, heme biosynthetic enzymes are turned over rapidly, enabling the liver to respond to changing metabolic requirements. In erythroid progenitors, however, the pathway is regulated to permit a high steady-state level of heme synthesis and regulation is tied to the availability of iron. There are both "housekeeping" and erythroid genes for aminolevulinate synthase, the first and rate-limiting enzyme in the pathway and the next three genes have dual promoters allowing both erythroid-specific and non-erythroid regulation [2]. The remaining genes in the pathway have single promoters but nevertheless exhibit erythroid and non-erythroid expression differences. These differences will be discussed for each enzyme in the pathway.

Heme biosynthetic enzymes have been intensively studied in recent years. All of the genes involved have been cloned and the crystal structures of all of the enzymes have been determined. In this review we will dissect the complex heme biosynthetic pathway into four basic processes:

(1) Formation of the pyrrole.
(2) Assembly of the tetrapyrrole.
(3) Modification of the tetrapyrrole side chains.
(4) Oxidation of protoporphyrinogen IX to protoporphyrin IX and insertion of iron.

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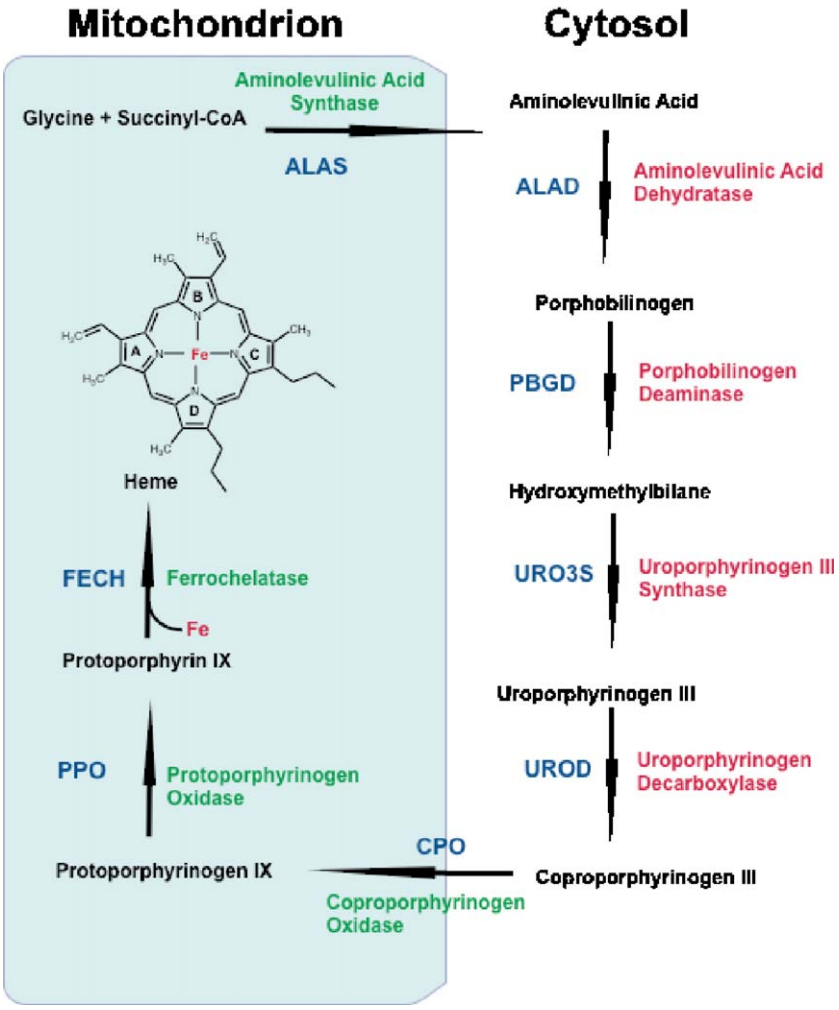


Fig. 1. The heme biosynthetic pathway. Mitochondrial enzymes are depicted in green and cytosolic enzymes in red. Abbreviations used in the text are capitalized.

Both inherited mutations and environmental factors may affect the pathway and lead to diseases including X-linked sideroblastic anemia, lead poisoning and the porphyrias. The porphyrias represent a group of disorders characterized by either acute neurovisceral attacks or photosensitivity and sometimes both. Accumulated substrates of defective pathway enzymes are responsible for disease symptoms. Compelling evidence has been generated indicating that neurovisceral symptoms are due to neurotoxic affects of porphyrin precursors. The photosensitivity is due to the fluorescent properties of porphyrins. A convenient way to classify the porphyrias is to divide them according to the dominant clinical feature (Table 1), although some authors prefer to divide the porphyrias based on whether excess substrate is generated in the liver (hepatic porphyrias) or the red cell (erythropoietic porphyrias). Here, each porphyric disorder will be described in the context of the step in the pathway which is defective.

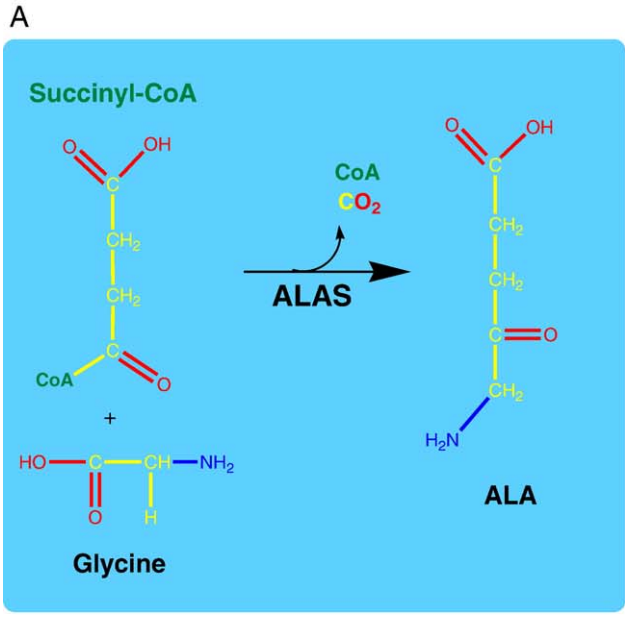
1.1. Formation of the pyrrole

The first and rate-limiting reaction in the pathway is a condensation reaction between glycine and succinyl-CoA to form 5-aminolevulinic acid (ALA) (Fig. 2A). The reaction is catalyzed by two different ALA synthases, one expressed ubiquitously (ALAS1) and the other expressed only erythroid precursors (ALAS2). The gene encoding ALAS1 maps to the short arm of chromosome 3 [3] whereas ALAS2 is encoded on the X chromosome [4]. Regulation of these two forms of ALAS is mediated by different mechanisms but both forms require pyridoxal 5-phosphate (PLP) as a cofactor (Fig. 2B) and both are expressed as homodimers. PLP binds to a specific lysine of ALAS. A PLP-glycine Schiff base complex is then formed which reacts with succinyl-CoA. The crystal structure of ALAS from *Rhodobacter capsulatus*, which is highly homologous to

Table 1
Clinical features of the porphyrias

Photosensitivity only	Neurovisceral attacks and photosensitivity	Neurovisceral attacks only
Congenital erythropoietic porphyria	Hereditary coproporphyria	Acute intermittent porphyria
Porphyria cutanea tarda	Variegate porphyria	ALAD porphyria
Erythropoietic porphyria		

both ALAS1 and ALAS2, has been determined [5]. Both forms contain an amino terminal mitochondrial targeting sequence which also contains a heme-binding motif. There are two putative heme-binding motifs (HRM) in the N-terminal mitochondrial targeting domain and a third in the N-terminus of the mature protein [6]. Binding of heme to this motif blocks mitochondrial import, representing a post-translation point of end-product feedback inhibition of the pathway. One study suggested that the heme-binding motif is functional only in ALAS1 [7]. Systematic mutations of the three HRMs suggested that HRM1 and HRM3 contributed to heme-dependent inhibition of mitochondrial transport, but HRM2 did not. Under the same experimental conditions, ALAS2 import was not inhibited by exogenous hemin. A different study found that any one of the three ALAS1 HRMs was sufficient to obtain heme-dependent inhibition of mitochondrial transport [8]. ALAS2 was not examined.



Both forms of ALAS are transcriptionally regulated but by quite different mechanisms [9–11]. In addition to heme's post-translational affect on mitochondrial import, heme down-regulates transcription of ALAS1. In humans there are two alternate splice forms of ALAS1. The major form lacks exon 1B (Fig. 4) and is subject to heme-mediated destabilization [9,12]. The minor form contains exon 1B and is resistant to heme-mediated decay and may require translation for destabilization in response to heme [13]. Transcription of ALAS1 is upregulated by the peroxisome proliferator-activated coactivator 1α (PGC-1α) [14]. PGC-1α is a coactivator of nuclear receptors and transcription factors [15]. The effects of PGC-1α are mediated by interactions with NRF-1 (nuclear regulatory factor 1) and FOX01 (a fork head family member) with the ALAS1 promoter [16]. Transcription of PCG-1α is upregulated when cellular glucose levels are low [17,18]. Upregulation of PGC-1α leads to increased levels of ALAS1, creating conditions capable of precipitating attacks of the neurovisceral porphyrias (see below). This is probably the explanation for the clinical observation that fasting may precipitate attacks whereas glucose infusions may attenuate the severity of attacks [19,20].

In contrast to ALAS1, transcriptional regulation of ALAS2 is mediated by erythroid-specific factors (including GATA1) which interact with sequences in the promoter region, sequences contained in many genes differentially regulated in red cells [10]. Translational regulation also plays an important role in determining ALAS2 expression. The ALAS2 transcript contains a 5′ iron regulatory element (IRE) which interacts with an IRE binding protein (IRP). The IRE–IRP complex prevents translation of the ALAS2 mRNA. Addition of an iron–sulfur cluster (Fe/S) abolishes the ability of IRPs to bind to the IRE and permits translation to occur. Fe/S clusters are generated and exported by mitochondria, linking regulation of heme biosynthesis in the red cell to iron availability and mitochondrial function [21–23].

Fig. 2. (A) Synthesis of 5-aminolevulinic acid (ALA). Decarboxylation of glycine followed by condensation with Succinyl-CoA is catalyzed by aminolevulinic acid synthase (ALAS). Pyridoxal Phosphate is required as a co-factor. The products are ALA, CO_2 and CoA. (B) The crystal structure of the active site of *R. capsulatus* ALAS [5]. Pyridoxal Phosphate (PLP) (fuchsia), first forms a covalent linkage with lysine (lys 391 in human ALAS). Incoming glycine induces a transient trans-aldimine (Schiff base) with PLP binding glycine rather than lysine. Succinyl-CoA (green) is condensed with the α carbon of glycine with the displacement of CoA (green) and the carboxyl group of glycine (yellow and red). ALA is released and PLP rebinds to the lysine of ALAS.

No disease-causing mutations of ALAS1 have been recognized in humans. Mutations in ALAS2, however, are causative for X-linked sideroblastic anemia (XLSA). The disorder is characterized by a microcytic hypochromic anemia and the presence of iron-laden mitochondria encircling the nucleus of developing red cells in the marrow (ringed sideroblasts). The clinical features of XLSA have been well described in recent reviews [24]. Many, but not all patients with XLSA improve with the administration of a pharmacologic dose of pyridoxine and XLSA is often referred to as pyridoxine-responsive anemia [25]. Forty-two point mutations affecting 37 residues of ALAS2 have been reported [5]. These mutations have been mapped in the crystal structure of the highly homologous ALAS of *R. capsulatus* [5]. Some directly affect the affinity of the enzyme for the PLP cofactor and are associated with pyridoxine responsiveness. Others affect domains not involved in cofactor binding and are found in cases not responsive to pyridoxine. Other rare forms of hereditary sideroblastic anemia have been associated with defects in Fe/S cluster formation, mitochondrial oxidative phosphorylation and thiamine metabolism. These disorders have been recently reviewed [24].

Following its synthesis, ALA exits the mitochondria by an unknown mechanism. Once in the cytosol, two molecules of ALA form the monopyrrole porphobilinogen by a condensation reaction catalyzed by aminolevulinate dehydratase (ALAD) (Fig. 3). The crystal structure of human ALAD has been determined [26,27]. The enzyme is a tetramer of homodimers formed from eight identical subunits with one active site per dimer [28]. The enzyme binds eight zinc atoms, one per subunit, and ALAD is inactivated by reversible replacement of zinc by lead. Four zinc molecules are essential for catalysis, whereas the other four serve to stabilize the tertiary structure of the enzyme [29–31]. Each active site binds two molecules of ALA at two distinct positions. One molecule contributes the acetate and the amino-methyl group of porphobilinogen (PBG). The other molecule of ALA contributes the propionate side chain and the pyrrole nitrogen (Fig. 3). In all tissues the activity of ALAD greatly exceeds the activity of ALAS.

In contrast to ALAS, there is only a single ALAD gene in humans, located on chromosome 9q34 [32], which consists of two alternatively spliced non-coding exons (1A and 1B) and eleven coding exons (Fig. 4) [33]. The translational start site (ATG) is located in exon 2. A “housekeeping” promoter is located upstream from exon 1A. A second erythroid-specific promoter is located between exons 1A and 1B. The erythroid-specific promoter contains several binding sites for erythroid-specific transcription factors including GATA1 [33]. The housekeeping and erythroid transcripts differ, but both encode identical proteins because they share the same translational start site located in exon 2.

There are three disorders in humans associated with deficiency of ALAD; hepatorenal tyrosinemia, ALAD porphyria and lead poisoning. Only ALAD porphyria is due to mutations of the ALAD gene. Hepatorenal tyrosinemia is an autosomal recessive disorder due to mutations of the fumarylacetoacetate hydrolase gene resulting in a defect in the last step in tyrosine catabolism, the hydrolysis of fumarylacetoacetate to fumarate and acetoacetate [34]. In the absence of enzyme activity, fumarylacetoacetate is subsequently metabolized to succinylacetoacetic acid and succinylacetone. Hypertyrosinemia is present in all patients but the presence of high levels of succinylacetone in plasma or urine is diagnostic for this disorder. Clinical manifestations include abnormalities of the liver, kidney and nerves. One of the most striking features is intermittent, painful neurological crises quite similar to the neurovisceral attacks associated with the acute porphyrias (see below). Succinylacetone is a potent inhibitor of ALAD [35]. Structurally, succinylacetone closely resembles ALA and acts as a competitive inhibitor of ALAD. ALAD activity in the liver and erythroid precursors is dramatically reduced and excess ALA is excreted in the urine. It has been proposed that ALA is neurotoxic and is the cause of the neurologic crises in patients with hepatorenal tyrosinemia [34]. Liver transplantation has proven to be curative for all of the clinical manifestations of hepatorenal tyrosinemia.

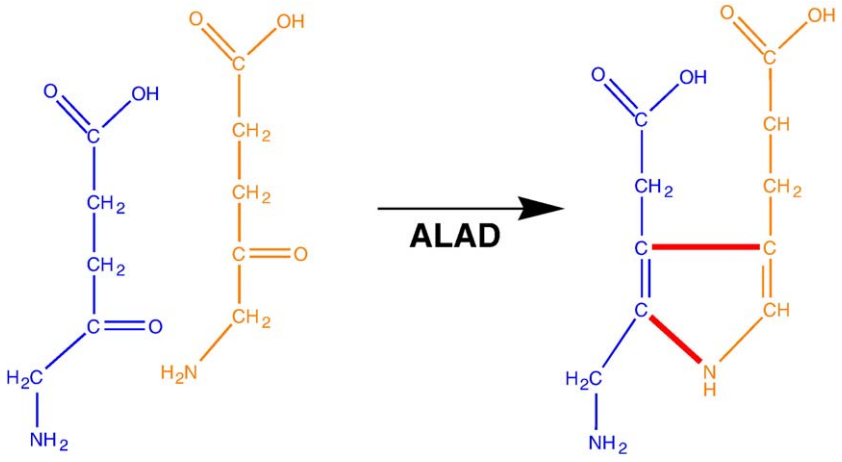


Fig. 3. Synthesis of porphobilinogen (PBG). Two molecules of ALA (blue and orange) are condensed to form PBG, a monopyrrole, by the cytosolic enzyme aminolevulinic acid dehydratase (ALAD).

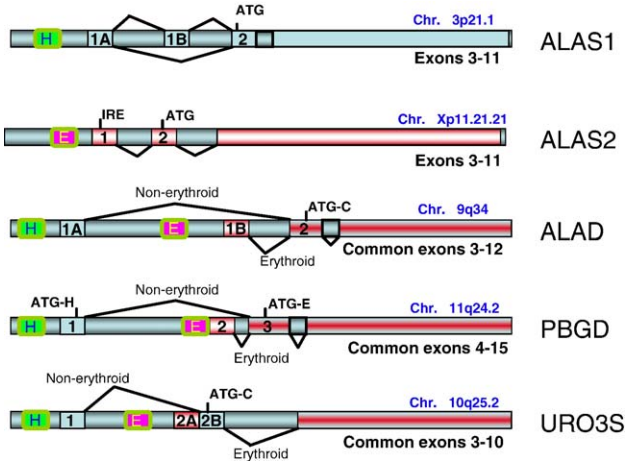


Fig. 4. Tissue-specific expression of the "early" genes in the heme biosynthetic pathway. H=Housekeeping promoter. E=Erythroid promoter. Chr.=chromosome location. ATG=translational start site. ATG-C=Common transcriptional start site. ATG-H=Housekeeping transcriptional start site. ATG-E=Erythroid transcriptional start site. The single genes for ALAD, PBGD and URO3S utilize tissue-specific promoters and alternative splicing to generate either identical proteins (ALAD, URO3S) or tissue-specific proteins (PBGD).

ALAD porphyria is a rare recessive autosomal disorder due to mutations of the ALAD gene [2]. Fewer than ten cases have been reported. ALAD activity in both the liver and erythroid precursors is markedly deficient leading to the accumulation and urinary excretion of ALA. The clinical manifestations have varied from case to case but most affected individuals have had intermittent acute neurovisceral attacks resembling those seen in patients with other acute porphyrias. In contrast to hepatorenal tyrosinemia, liver transplantation has not proven beneficial in the one patient in whom transplantation was performed. After transplantation, increased excretion of ALA in the urine persisted, suggesting that extra-hepatic generation of ALA was sufficient to sustain the clinical manifestations [36].

Lead inhibits ALAD activity by displacing zinc atoms from the enzyme, resulting in high concentrations of ALA in blood and urine [2]. Lead in plasma is taken up by red cells and is bound to ALAD. Patients with lead poisoning often develop neurologic manifestations resembling those found in the acute porphyrias. It has been suggested that the elevated concentrations of ALA are responsible for the neurologic symptoms but lead itself may have toxic effects on the nervous system. A common ALAD polymorphism (K59N) results in an enzyme that has a higher affinity for zinc and thus a lower ability to bind lead. The result is higher plasma lead levels in subjects with the K59N allele. This may facilitate delivery of lead to the nervous system and explain the association of the K59N allele with an increased risk for lead intoxication [37].

1.2. Formation of the tetrapyrrole macrocycle

A polymer of four molecules of PBG is generated in the cytosol by porphobilinogen deaminase (PBGD). The polymer is an unstable tetrapyrrole designated hydroxymethylbilane (HMB) (Fig. 5A). PBGD functions as a monomer and the crystal structure has been determined [38]. Purification of PBGD from mammals and bacteria yields an enzyme with a dipyrrole (dipyrromethane) cofactor in the catalytic site. The dipyrrole is derived by a novel mechanism. PBGD first binds HMB and then deaminates and polymerizes two additional molecules of PBG to form a hexapyrrole. The final step is cleavage of the distal tetrapyrrole and the release of HMB. The proximal dipyrrole remains covalently bound to the enzyme and is not turned over (Fig. 5B) [39].

The human PBGD gene has been cloned and mapped to chromosome 11q23-11qter [40]. The gene contains 15 exons. As with the ALAD gene, two separate promoters control transcription (Fig. 4). The housekeeping promoter lies upstream of exon 1 and the erythroid promoter lies upstream of exon 2. Transcription initiated by the housekeeping promoter results in a transcript in which exon 2 is lost by splicing of exon 1 to exon 3. Transcription initiated by the erythroid promoter results in a transcript containing exons 2–15. The erythroid promoter resembles other erythroid-specific promoters [41]. Translation of the erythroid mRNA is initiated at an AUG located in exon 3 (exon 2 lacks an AUG) [42]. The AUG translational start site encoded in exon 1 of the housekeeping transcript is spliced in-frame with the AUG in exon 3 and produces a protein 17 amino acids longer at the amino terminus compared to the erythroid protein [43].

Acute intermittent porphyria (AIP) is caused by mutations of the PBGD gene. Over 200 PBGD mutations have been detected [19]. The disorder is transmitted as an autosomal dominant trait, but the majority of heterozygotes for gene mutations remain asymptomatic. In Europe, acute attacks of porphyria occur with a frequency of approximately 1–2 per 100,000. A study of blood donors in France produced an estimate of gene frequency of at least 1:1675 suggesting that the clinical penetrance is low [44,45]. Others may have only one or a few neurovisceral attacks throughout life. Acute attacks usually occur after puberty, but in rare cases of homozygosity or compound heterozygosity for PBGD mutations, severe attacks may begin in childhood. In heterozygotes, acute attacks are often precipitated by exposure to drugs metabolized by cytochrome P450 enzymes. Induction of these enzymes depletes hepatic heme leading to the induction of ALAS. Caloric deprivation may also precipitate acute attacks and glucose infusions may attenuate or even abort attacks. The mechanism mediating the effects of caloric restriction and glucose infusions has been discussed above and is related to transcriptional regulation of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) by glucose availability. When cellular glucose levels are low, PGC-1 α production increases, leading to increased levels of ALAS1. This in turn increases levels of PBG, the substrate of PBGD. When cellular glucose is replete, PGC-1 α is suppressed and ALAS1 transcription is diminished. The preferred, and most effective treatment for acute attacks is the intravenous administration of hematin which downregulates ALAS I. During acute attacks urinary excretion of ALA and PBG is markedly increased. Hepatic overproduction of these porphyrin precursors is causative for the neurologic symptoms. In support of this

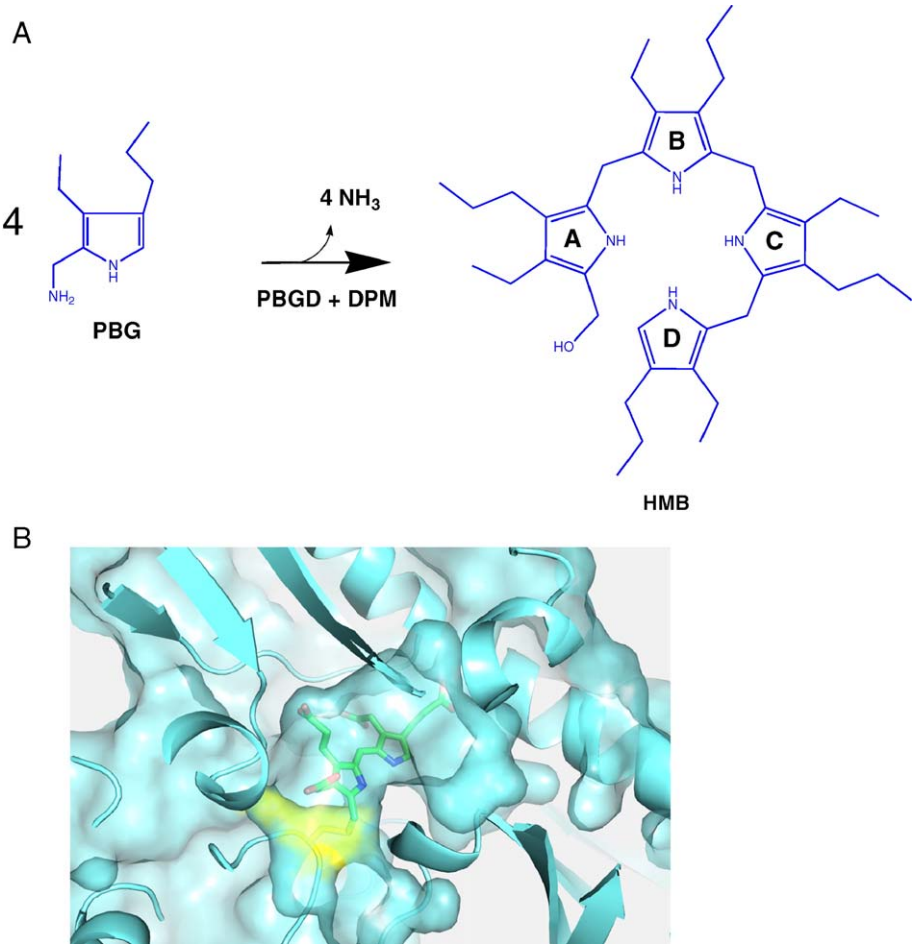


Fig. 5. (A) Synthesis of hydroxymethylbilane (HMB). The reaction is catalyzed by the cytosolic enzyme porphobilinogen deaminase (PBGD). A dipyrromethane cofactor (DPM) is covalently bound to the enzyme in the catalytic site. Four additional molecules of PBG are deaminated and polymerized to form a hexapyrrole. The distal tetrapyrrole is then cleaved and released as HMB. (B) The crystal structure of a human PBGD monomer is shown with the dipyrromethane cofactor (green) bound in the catalytic site.

conclusion was the finding that an allogeneic liver transplantation in a young woman with disabling acute attacks resulted in normalization of urinary ALA and PBG levels within a day and completely eliminated her recurrent attacks [46].

The product of the PBGD reaction, HMB, serves as the substrate for uroporphyrinogen III synthase (URO3S). URO3S catalyzes the conversion of HMB to uroporphyrinogen III (URO'GEN III) by inversion of the D-ring of HMB followed by closure of the tetrapyrrole macrocycle (Fig. 6). Spontaneous cyclization of HMB can also occur, but the product, URO'GEN I, cannot ultimately be converted to heme. The crystal structure of human URO3S has been determined [47]. The enzyme functions as a monomer. There is a single URO3S gene in humans which encodes 10 exons [48] and is localized to chromosome 10q25.2–26.3 (Fig. 4) [49]. The ATG translational start site is encoded within exon 2B. As with ALAD and PBGD, both non-erythroid and erythroid-specific transcripts are produced. An erythroid-specific promoter within intron 1 contains 8 GATA1 binding sites. The erythroid-specific mRNA contains all of exon 2. The non-erythroid promoter lies upstream of exon 1 and contains binding sites for Sp1, NF1, AP1 Oct1 and NRF2 [50]. The promoter region lacks a TATA box. The ubiquitously expressed non-erythroid transcript results from a splicing event that joins exon 1 to exon 2B which contains the ATG start site. Although the non-erythroid and erythroid-specific transcripts are expressed from alternative promoters, both produce the identical protein.

Mutations of URO3S are responsible for a rare form of porphyria in humans designated congenital erythropoietic porphyria (CEP) [2]. CEP is transmitted as an autosomal recessive trait. Markedly diminished URO3S activity results in the accumulation of uroporphyrin I (URO I) in erythrocytes. Diffusion of URO I to plasma mediates the photo-mutilation characteristic of CEP. In most cases, cutaneous photosensitivity begins in early infancy and eventually leads to cutaneous scarring and deformities. Most patients with CEP have less than 10% of normal erythrocyte URO3S activity [51]. Heterozygous carriers of URO3S mutations have no phenotype. Patients with CEP are either homozygous for a single URO3S mutation or are compound heterozygotes. Over twenty URO3S mutations have been described including missense and nonsense mutations, large and small deletions, splicing defects and intronic branch-point mutations [52]. A recent report described a patient with CEP in whom there were no URO3S mutations [53]. A mutation of GATA1, an erythroid-specific transcription factor

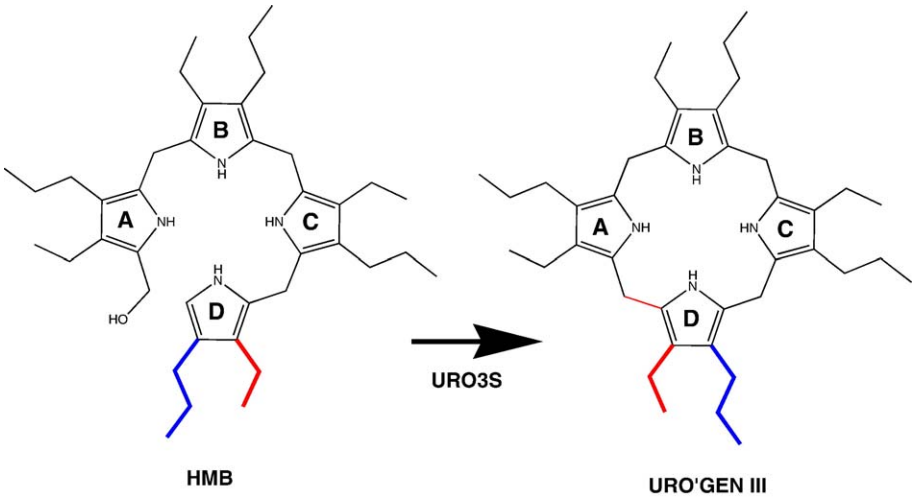


Fig. 6. Synthesis of uroporphyrinogen III (URO'GEN III). The reaction is catalyzed by the cytosolic enzyme uroporphyrinogen III synthase (URO3S). The enzyme catalyzes ring closure of hydroxymethylbilane (HMB) with concurrent "flipping" of the D ring to generate uroporphyrinogen III (URO'GEN III). The D ring acetate group is in red and the propionate is in blue.

proved to be causative. In this case, expression of PBGD was not affected and there was only a modest reduction in expression of ALAD. These findings suggest that erythroid-specific promoters are not all functionally equivalent.

1.3. Modification of the acetate and propionate side chains

URO'GEN III represents a branch point for the pathways leading to heme, chlorophyll and corrins (polyaromatic rings related to porphyrins). In the heme biosynthetic pathway, URO'GEN III is converted to coproporphyrinogen III (COPRO'GEN III) by the sequential removal of the four carboxylic groups of the acetic acid side chains, a reaction catalyzed by uroporphyrinogen decarboxylase (UROD) (Fig. 7A) [54]. URO'GEN III is the preferred substrate. URO'GEN I can also be decarboxylated but only COPRO'GEN III can be converted to protoporphyrin IX. Decarboxylation of URO'GEN III begins at the asymmetric D-ring and proceeds in a clockwise manner [55]. The crystal structure of human UROD has been determined [56]. The enzymes functions as a homodimer and each monomer contains an active site cleft. Structural studies of the active site indicate that only the flexible porphyrinogen macrocycle can be accommodated (Fig. 7B) [57]. The rigid, planar porphyrin molecule is not a substrate.

There is a single human UROD gene located on chromosome 1p34 [58,59]. In contrast to ALAD, PBGD and URO3S, there is no erythroid-specific promoter in the UROD gene [60]. The level of UROD mRNA is markedly increased in erythroid tissues but the molecular basis of erythroid-specific upregulation has not been determined [61].

Mutations in the UROD gene are causative for the familial form of porphyria cutanea tarda (PCT) [62,63]. Familial PCT is inherited as an autosomal dominant trait but, as with other dominant forms of porphyria, phenotypic expression occurs only in some heterozygotes. The disorder is clinically characterized by a photosensitive dermatosis, usually less severe than in CEP. The photosensitivity is mediated by uroporphyrin which is generated in the liver, circulates in plasma and is excreted in the urine. UROD protein and enzymatic activity is approximately half normal in erythrocytes of affected individuals and uroporphyrin does not accumulate. In the liver, UROD protein levels are half normal but enzyme activity is 25% or less. This finding strongly suggests that an inhibitor of UROD is generated in the liver, a suggestion supported by animal models of PCT [64–66]. The more common sporadic form of PCT is not associated with UROD mutations [67]. In sporadic PCT, UROD protein levels in the liver are normal but, as with familial PCT, enzyme activity is 25% or less, again suggesting the presence of a UROD inhibitor. Hepatic iron overload is found in both forms of PCT and iron depletion through phlebotomy therapy corrects the phenotype [2,68]. Approximately 20% of patients with PCT have proven to be homozygous for the C282Y mutation of the hemochromatosis gene (HFE) [69]. There is no clear explanation for the hepatic siderosis in the other 80% of cases. Several environmental factors are associated with clinical expression of PCT including alcohol abuse, hepatitis C and exposure to medicinal estrogens [69].

In rare instances homozygosity or compound heterozygosity for UROD mutations have been recognized and result in a disease called hepatoerythropoietic porphyria (HEP). The clinical manifestations are similar to PCT, but occur earlier in life and are more severe [2]. Residual UROD activity has varied widely, but is usually less than 10% of normal. Complete loss of UROD activity is incompatible with life as homozygosity for a null mutation in mice proved to be an embryonic lethal [66].

The next side chain modification is the sequential oxidative decarboxylation of the propionate groups of pyrrole rings A and B of COPRO'GEN III to form protoporphyrinogen IX (PROTO'GEN IX). This reaction is catalyzed by coproporphyrinogen oxidase (CPO), an enzyme located in the mitochondrial intermembrane space in mammals. There are both oxygen-dependent and oxygen-independent forms of this enzyme, termed *hemN* and *hemF*, respectively. Both enzymes decarboxylate the A and B pyrrole ring propionates to vinyl groups.

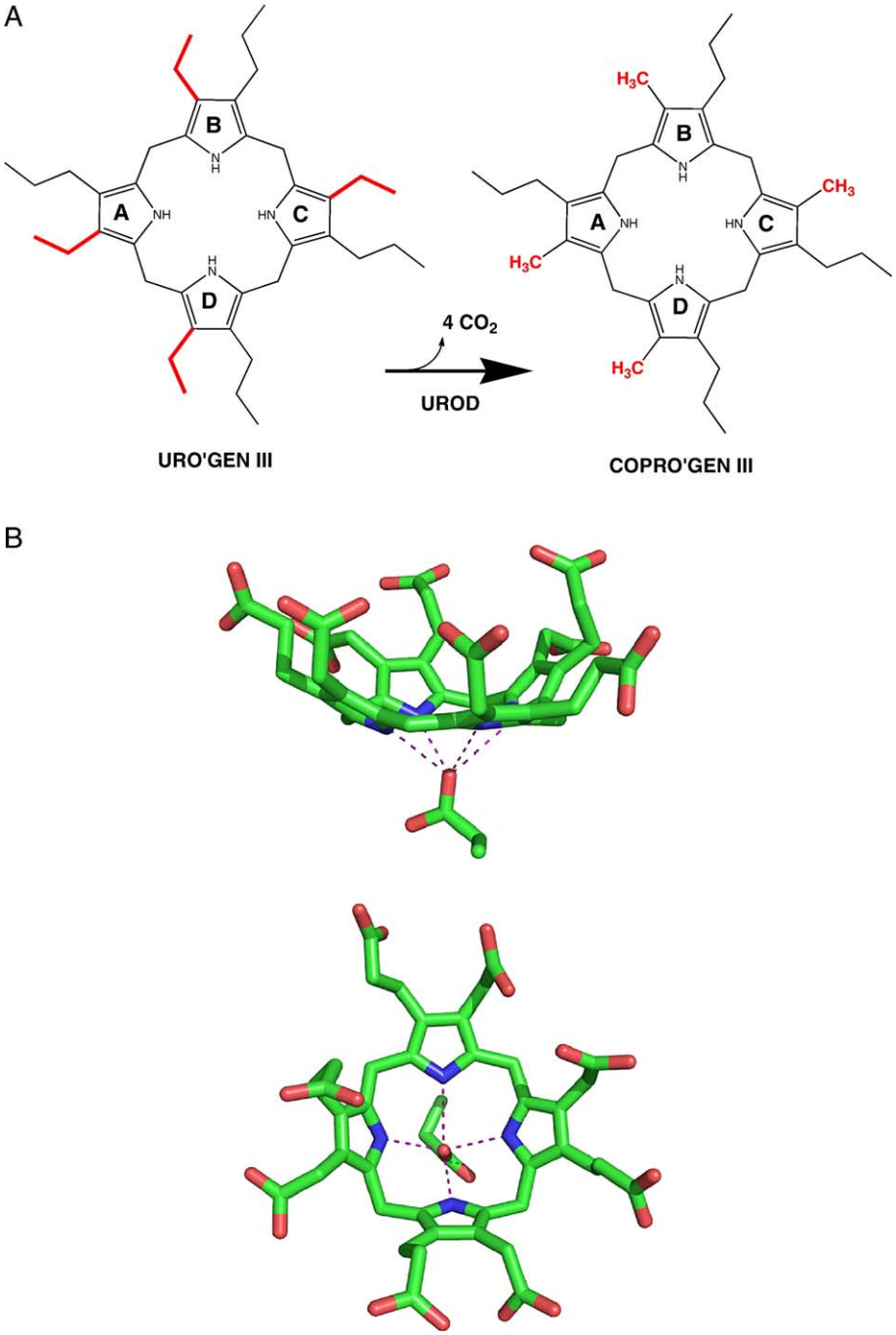


Fig. 7. (A) Synthesis of coproporphyrinogen III (COPRO'GEN III). The reaction is catalyzed by the cytosolic enzyme uroporphyrinogen decarboxylase (UROD). The enzyme sequentially decarboxylates the four acetate side chains of URO'GEN III (red) starting at the asymmetric D ring to generate the tetra-carboxylic, tetra-methyl COPRO'GEN III. (B) Flexibility of the porphyrinogen substrate. The four pyrrole nitrogens (blue) of URO'GEN III form hydrogen bonds with asparagine 86 of UROD. The porphyrinogen macrocycle assumes a domed configuration. Sufficient flexibility to form the domed configuration is a property of porphyrinogens and not of the planar, more rigid porphyrins. Upper panel: front view. Lower panel: top view. Adapted from [57].

Mammals and other higher eukaryotes utilize only the oxygen-dependent form which releases two molecules of CO_2 and H_2O_2 (Fig. 8) [70]. It has been suggested that oxygen-dependent CPO catalyzes an unusual metal- and cofactor-independent oxidative decarboxylation in which molecular oxygen acts as the immediate electron acceptor [71]. The protein possesses an unusually long leader sequence that is required for mitochondrial localization [8]. Access of COPRO'GEN III to the intermembrane space may be mediated by a peripheral-type benzodiazepine receptor [72]. CPO in eukaryotes and some prokaryotes is an oxygen-dependent enzyme [73]. The crystal structures of both human and yeast oxygen-dependent CPO have been determined [71, 74]. The enzyme functions as a homodimer [73]. The structure of CPO permits the enzyme to maintain the substrate as a porphyrinogen in the oxidative environment of the mitochondrion, yet allows access of oxygen to the active site for

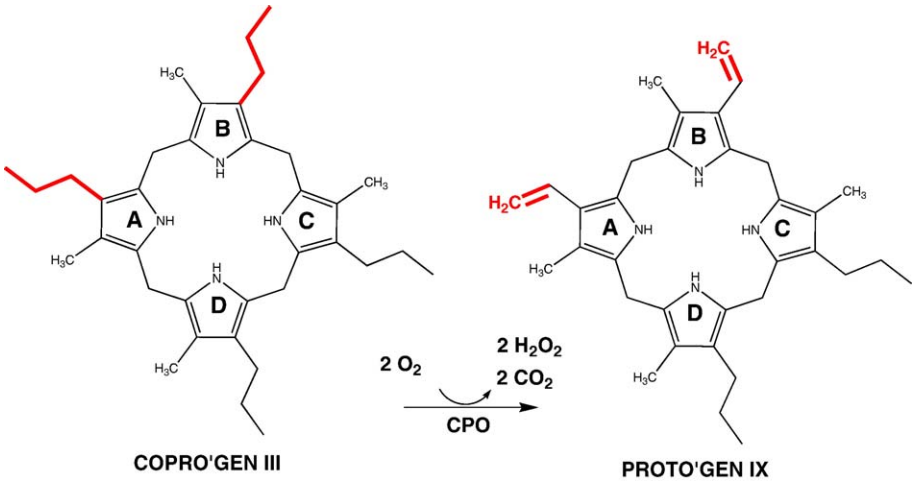


Fig. 8. Synthesis of protoporphyrinogen IX (PROTO'GEN IX). The reaction is catalyzed by the enzyme coproporphyrinogen oxidase (CPO) which resides in the mitochondrial inner membrane space. The enzyme oxidizes the propionate groups (red) of the A and B rings of COPRO'GEN III to form the two vinyl groups (red) of PROTO'GEN IX with the release of two molecules of CO_2 and two molecules of H_2O_2.

the oxidative decarboxylation reaction and the release of H_2O_2 [74]. The human CPO gene has been cloned and is located on chromosome 3q12 [75]. The promoter region encodes an Sp-1-like element, a GATA site and a novel regulatory element that interacts in a synergistic manner in erythroid cells [76]. In non-erythroid tissue the GATA site is not necessary for transcription but the novel regulatory element is required. There is differential regulation of CPO with increased expression in erythroid cells, a finding consistent with erythroid upregulation mediated by the GATA site [77].

Mutations of the CPO gene are responsible for human disorder hereditary coproporphyria (HCP). It has been suggested that most of the disease-causing mutations destabilize the enzyme [73,74]. HCP is characterized clinically by acute neurovisceral attacks similar to those seen in patients with AIP [19]. In contrast to AIP, photosensitivity may also be present. The biochemical features of HCP include a marked increase in urinary and fecal coproporphyrin content. As with AIP, urinary ALA and PGB are increased during the acute neurovisceral attacks. Infusion of hematin is the recommended treatment for acute attacks [19]. HCP is inherited as an autosomal dominant trait but only some heterozygotes develop a clinical illness. Rare, severe cases have been described in which both alleles of CPO were mutant [78, 79].

PROTO'GEN IX is oxidized to protoporphyrin IX (PROTO IX) by the enzyme protoporphyrinogen oxidase (PPO) (Fig. 9). The six electron oxidation requires oxygen as the terminal electron acceptor. The crystal structure of human PPO has been determined and the enzyme functions as a homodimer [80]. Each homodimer contains one non-covalently bound FAD. The enzyme is synthesized in the cytosol and transported to the outer surface of the inner mitochondrial membrane. The protein contains a mitochondrial targeting sequence between 151 and 175 residues from the N-terminus with a minor sequence within the first 150 amino acids [81]. The human PPO gene is located on chromosome 1q22–123 [82,83]. The 5′ flanking region of

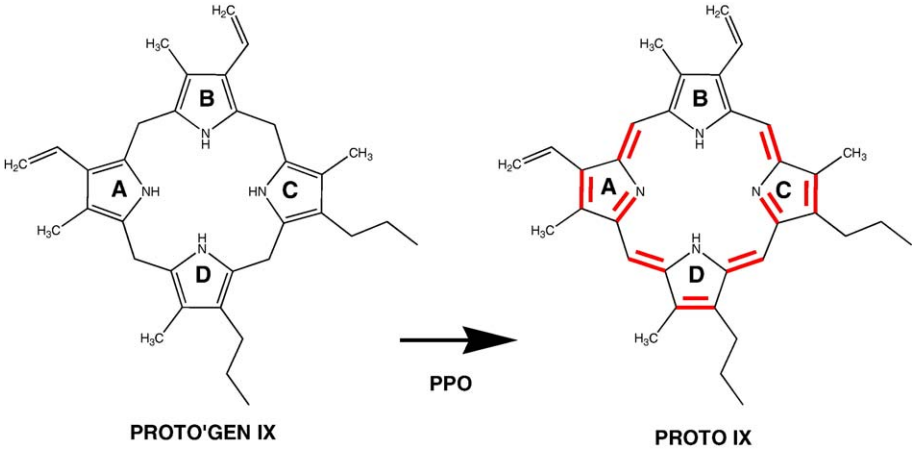


Fig. 9. Synthesis of protoporphyrin IX (PROTO IX). The reaction is catalyzed by protoporphyrinogen oxidase (PPO) which is located on the outer surface of the inner mitochondrial membrane. The six-electron oxidation to the planar macrocycle PROTO IX requires oxygen as the terminal electron acceptor. The alternating single-double bond structure of the macrocycle gives the porphyrin fluorescent properties.

the human PPO gene contains a GATA-1 binding site, suggesting the potential for erythroid-specific regulation. When mouse erythroleukemia cells were induced with dimethylsulfoxide, expression of the three terminal enzymes in the heme biosynthetic pathway increased [83,84].

Mutations of PPO are responsible for variegate porphyria (VP). More than 100 mutations of the PPO gene have been identified and include missense, nonsense and splice site mutations as well as deletions and insertions (Human Gene Mutation Database) [85]. The disorder is transmitted as an autosomal dominant trait. VP is seen in all racial groups but is particularly common in South African whites (estimated incidence 3 per thousand). A founder mutation in this population has been traced to an immigrant in the 17th century [2,86]. As with other acute porphyrias, most gene carriers do not develop a clinical illness. VP is characterized clinically by acute neurovisceral attacks, cutaneous photosensitivity or both. Symptoms rarely occur before puberty. Genotype/phenotype correlations have been unsatisfactory. The same mutation present in two unrelated individuals may result in vastly different clinical phenotypes [85]. Rare individuals with two mutant PPO alleles and markedly reduced PPO activity develop clinical manifestations in childhood [87,88]. Urinary excretion of ALA and PBG is increased during acute attacks but may be normal during periods of remission [19]. The fecal content of protoporphyrin and coproporphyrin is markedly increased. PPO activity is approximately half normal in all tissues. A patient with VP demonstrated biochemical and clinical improvement following liver transplant for alcoholic cirrhosis [89]. This result, and the previously mentioned liver transplant in a patient with AIP (see above), support the role of overproduction of ALA and PBG by the liver as causative for the neurovisceral attacks of acute porphyrias. In vitro experiments indicated inhibition of PBGD by protoporphyrinogen and coproporphyrinogen possibly explaining the accumulation of ALA and PBG in patients with VP [90]. Therapy with intravenous hematin is an effective method of treating acute attacks [19].

1.4. Insertion of iron into protoporphyrin IX

The final step in the heme biosynthetic pathway occurs on the inner surface of the inner mitochondrial membrane where iron is inserted into protoporphyrin IX (PROTO IX) by ferrochelatase (FECH) (Fig. 10). Human FECH has been crystallized and the enzyme functions as a homodimer [91]. It has been suggested that a direct interaction occurs between PPO on the outer surface of the inner mitochondrial membrane and FECH which permits direct transfer of PROTO IX from PPO to the active site of FECH [80], but experiments using isolated mitochondria suggest that a complex between enzymes is not required for heme synthesis [92]. Each monomer of FECH contains a nitric oxide-sensitive 2Fe–2S cluster [93].

The human FECH gene has been cloned and mapped to chromosome 18q21.3 [94]. The same transcriptional start site is utilized in erythroid and non-erythroid cells and contains potential binding sites for Sp1, NF-E2 and GATA1 [95]. A region (−150) containing the Sp1 sites but lacking the erythroid-specific *cis* elements was capable of inducing expression of a luciferase reporter gene in erythroid cells [96]. It was later shown that the 5′ flanking region of the FECH gene was both necessary and sufficient to obtain transgene expression in erythroid tissue [97]. FECH is synthesized in the cytosol and targeted to mitochondria by sequences in the leader peptide. The leader peptide is subsequently cleaved to produce the mature form of the enzyme [98]. Expression of FECH is regulated by intracellular iron levels, a finding presumably related to the enzyme's requirement for an iron–sulfur cluster [99]. The FECH gene is also regulated by hypoxia through interactions with hypoxia inducible factor 1 [100].

Mutations of the FECH gene result in erythropoietic protoporphyria (EPP). Early studies suggested that EPP is transmitted as an autosomal dominant trait, but a large pedigree study involving 200 patients and 91 families indicated co-inheritance of a second defective FECH gene from an unaffected parent was needed [101]. An allele resulting in low level transcription activity has been confirmed

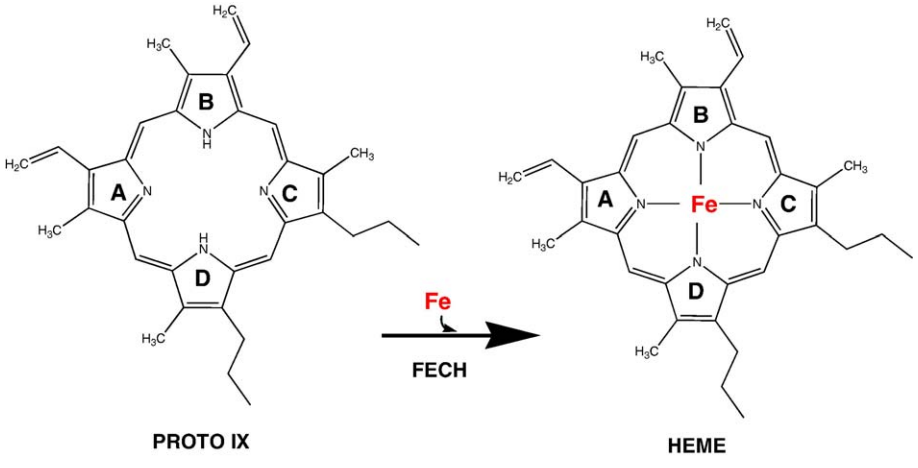


Fig. 10. Synthesis of heme. The final step in heme synthesis is catalyzed by the mitochondrial enzyme ferrochelatase (FECH). The enzyme catalyzes the insertion of one atom of ferrous iron (red) into the PROTO IX macrocycle.

and offers a reasonable explanation for a three-allelic model for morbidity [102,103]. Protoporphyrin accumulates mainly in bone marrow reticulocytes, diffuses into plasma, is taken up by the liver and excreted in the bile [2]. Protoporphyrin is present in excess in the feces but is not excreted in the urine. In a minority of patients, excess protoporphyrin in the bile leads to cholestasis and cirrhosis [104]. Patients with EPP frequently develop fluorescent gallstones containing large quantities of protoporphyrin.

The dominant clinical feature of EPP is cutaneous photosensitivity which usually begins in childhood. The cutaneous manifestations differ from other porphyrias with photosensitivity as bullous lesions are uncommon and severe scarring is unusual. Instead, the skin develops a leathery characteristic that mimics premature aging. No neurovisceral attacks are associated with EPP. Nearly ninety FECH mutations have been identified including missense, nonsense and splicing deletions and small and large deletions and insertions (human gene mutation database). Most patients with EPP have only 10–25% of normal FECH activity rather than the 50% activity expected in an autosomal dominant trait [2]. Because FECH is active as dimer, it has been suggested that a mutant allele may function in a dominant-negative fashion in which mutant homodimers and mutant-wild type heterodimers are inactive. Only wild type homodimers would be active. More recently, however, it has been demonstrated that many patients exhibiting the EPP phenotype have, in addition to a mutant FECH allele, a common wild type FECH allelic variant resulting in low expression [103].

Therapeutic measures for EPP include quenching of porphyrin fluorescence by administering β-carotene, the application of topical ointments which block long-range ultraviolet light and the administration of cholestyramine [2]. Cholestyramine, a resin capable of binding protoporphyrin, interrupts the enterohepatic recirculation of protoporphyrin and promotes fecal excretion. In selected patients with protoporphyric liver disease, liver transplantation has been beneficial, but the transplanted liver is susceptible to protoporphyrin-induced damage [105]. A patient with EPP who developed acute myeloblastic leukemia underwent bone marrow transplantation [106]. This resulted in a dramatic biochemical and clinical remission of the EPP. This case establishes that it is the bone marrow, and not other tissues, that is responsible for the clinical manifestations of EPP. It was recently demonstrated that the overproduction of protoporphyrin due to mutations in FECH can be severe enough to cause acute liver damage [107].

2. Summary

Tissue-specific regulatory features characterize the heme biosynthetic pathway. In non-erythroid cells the pathway is regulated by heme-mediated feedback inhibition. In contrast, in erythroid cells regulation is based on the availability of iron as Fe/S clusters. The first four enzymes in the pathway are regulated differently in erythroid and non-erythroid tissues, either through tissue-specific genes (ALAS1 and ALAS2) or through single genes with tissue-specific promoter regions. In eukaryotic cells the pathway is divided between mitochondrial and cytosolic compartment (Fig. 1). Cytosolic enzymes utilize unstable, reduced substrates but no data exist to support the concept that enzyme complexes exist to facilitate transfer of the product of one enzyme to the next enzyme in the pathway. Models for the spatial relationship of enzymes involved in the mitochondrial reactions have been proposed but existing data support only a potential PPO–FECH interaction. The structure of enzymes catalyzing the final four enzymatic steps are remarkably diverse even though all utilize tetrapyrrole macrocycles as substrates. This structural diversity suggests that these enzymes did not evolve through a mechanism of gene duplication.

With the exception of ALAS1, human porphyric disorders result from mutations in all of the heme biosynthetic enzymes. The phenotypes of the porphyric disorders vary widely depending upon whether porphyrin precursors, porphyrins or both are present in excess.

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