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Heme biosynthesis and its regulation: towards understanding and improvement of heme biosynthesis in filamentous fungi

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Abstract Heme biosynthesis in fungal host strains has acquired considerable interest in relation to the production of secreted heme-containing peroxidases. Class II peroxidase enzymes have been suggested as eco-friendly replacements of polluting chemical processes in industry. These peroxidases are naturally produced in small amounts by basidiomycetes. Filamentous fungi like *Aspergillus* sp. are considered as suitable hosts for protein production due to their high

capacity of protein secretion. For the purpose of peroxidase production, heme is considered a putative limiting factor. However, heme addition is not appropriate in large-scale production processes due to its high hydrophobicity and cost price. The preferred situation in order to overcome the limiting effect of heme would be to increase intracellular heme levels. This requires a thorough insight into the biosynthetic pathway and its regulation. In this review, the heme biosynthetic pathway is discussed with regards to synthesis, regulation, and transport. Although the heme biosynthetic pathway is a highly conserved and tightly regulated pathway, the mode of regulation does not appear to be conserved among eukaryotes. However, common factors like feedback inhibition and regulation by heme, iron, and oxygen appear to be involved in regulation of the heme biosynthesis pathway in most organisms. Therefore, they are the initial targets to be investigated in *Aspergillus niger*.

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Introduction

The production of enzymes by microorganisms as eco-friendly replacements for chemical and polluting industrial processes has gained increasing attention over the years. The use of enzymes produced by microorganisms as filamentous fungi like *Aspergillus* sp. are considered as preferred hosts due to their high capacity for producing secreted proteins. The production of heterologous proteins, however, is often relatively low compared with the amount

of homologous protein produced (Punt et al. 2002). Several mechanisms are responsible for this low level of protein production which, among others, includes the unfolded protein response (UPR) and ER-associated degradation (ERAD) (Guillemette et al. 2007), protease activity (Braaksma and Punt 2008), and limitations at the level of cofactor incorporation (Punt et al. 2002).

Cofactor availability and incorporation has been shown to be a limiting factor in the production of fungal peroxidases which require heme as a cofactor. Peroxidase production can be increased by the supplementation of hemoglobin or hemin to the medium (Conesa et al. 2000; Elrod et al. 1997); however, the mechanism behind heme uptake is poorly understood and the approach is too costly to be suited for industrial purposes (Elrod et al. 1997).

The preferred situation in order to overcome the limiting effect of heme would be the increase of intracellular heme levels. In animals, fungi, and in prokaryotes belonging to the α -proteobacteria (Ferreira et al. 1993; Panek and O'Brian 2002), heme is synthesized by eight enzymatic

steps (Fig. 1; Table 1), starting with condensation of glycine and succinyl CoA to form 5-aminolevulinic acid (ALA). In the eukaryotes mentioned above, the reaction is mediated by 5-aminolevulinic acid synthase (ALAS) and takes place in the mitochondria. ALA is then transported to the cytosol where it is condensed to porphobilinogen (PBG) by ALA dehydratase (ALAD). Four molecules of PBG are subsequently used to form the unstable hydroxymethylbilane (HMB) by PBG deaminase (PBGD) followed by the cyclization to uroporphyrinogen III (UroIII) by uroporphyrinogen III synthase (UROS). Uroporphyrinogen III is the final common intermediate of heme and siroheme synthesis, siroheme being a cofactor for sulfite and nitrite reductases (Raux et al. 2003). The synthesis of heme continues by decarboxylation of all four acetic side chains to methyl groups by uroporphyrinogen III decarboxylase (UROD) to form coproporphyrinogen III (Copro). With the exception of *Saccharomyces cerevisiae*, Copro is subsequently transported back to the mitochondria. The synthesis is continued by

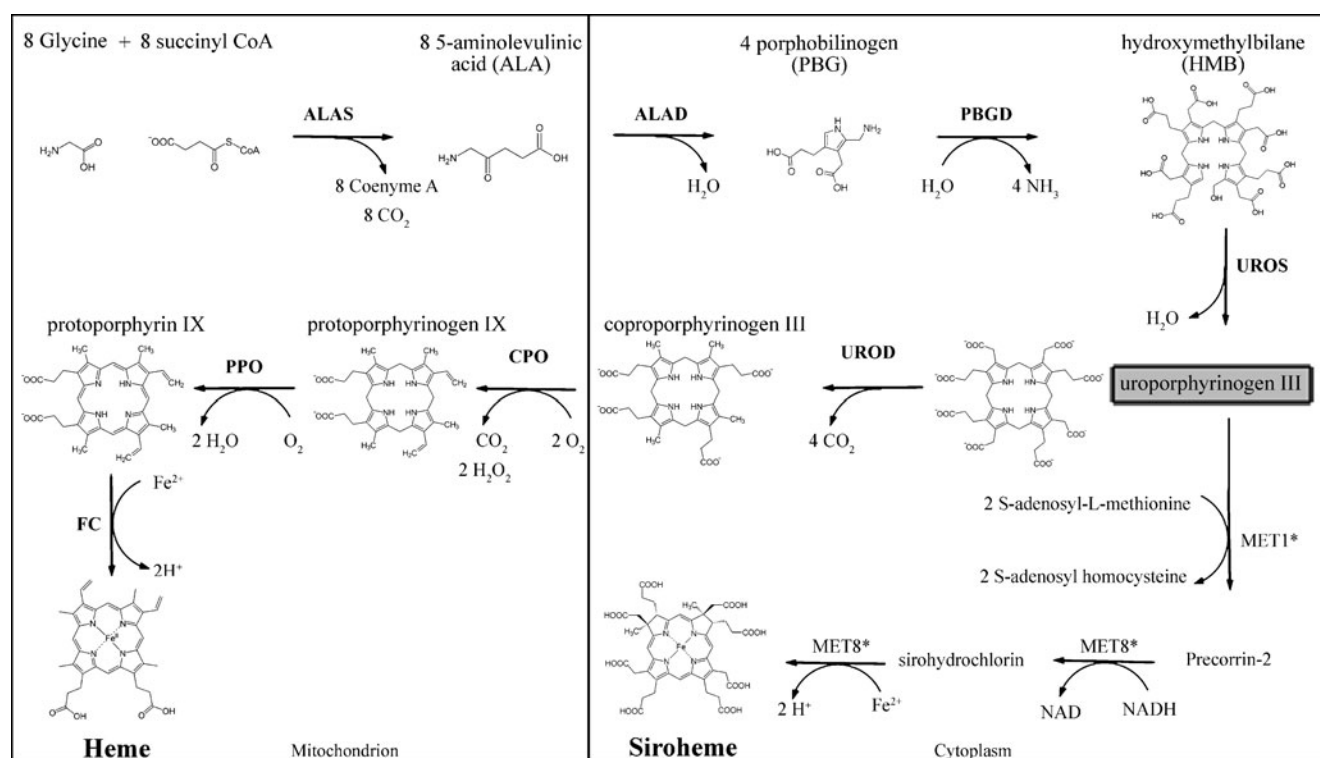


Fig. 1 Chemical heme biosynthesis pathway. Biosynthesis is initiated in mitochondria with the condensation of glycine and succinyl CoA to 5'-aminolevulinic acid (ALA) by 5'-aminolevulinic acid synthase (ALAS). ALA is subsequently exported into the cytosol to be processed to uroporphyrinogen III (UroIII), the final common intermediate between heme and siroheme synthesis. For heme biosynthesis, UroIII is decarboxylated by UroIII decarboxylase (UROD) to coproporphyrinogen III, which in turn is redirected to mitochondria. Heme biosynthesis is finalized in mitochondria in three

subsequent enzymatic reactions. Siroheme synthesis also derives from uroporphyrinogen III synthesis. Siroheme is synthesized in four subsequent reactions by one multifunctional (CysG in *E. coli*) or two enzymes (Met1p and Met8p in *S. cerevisiae*). ALAS 5'-aminolevulinic acid synthase, ALAD 5'-aminolevulinic acid dehydratase, PBGD porphobilinogen deaminase, UROS uroporphyrinogen III synthase, UROD uroporphyrinogen III decarboxylase, CPO coproporphyrinogen III oxidase, PPO protoporphyrinogen oxidase, FC ferrochelatase

Table 1 BlastP results of *S. cerevisiae* and *E. coli* heme and siroheme synthesis proteins against *Aspergillus niger* CBS 513.88 (taxid: 425011)

Enzyme (Elrod et al. 1997)	Enzyme abbreviation	Protein (version)		<i>A. niger</i> hypothetical protein	E-value		TargetP	WoLF PSORT	MitoProt II
		<i>E. coli</i>	<i>S. cerevisiae</i>		<i>E. coli</i>	<i>S. cerevisiae</i>			
Aminolevulinic acid synthase (5-aminolevulinic acid synthase)	ALAS	N.P.	hem1p (CAA88511.1)	An17g01480		7.00E-167	Mito	Mito	Mito
5-Aminolevulinic acid dehydratase (porphobilinogen synthase, 5-aminolevulinic acid dehydratase)	ALAD	hemB (CAQ30840.1)	hem2p (NP_011475.1)	An08g00010	2.00E-55	2.00E-128	O	Cyto	–
Porphobilinogen deaminase (hydroxymethylbilane synthase, Uro I synthase)	PBGD	hemC (ABD20330.1)	hem3p (NP_010076.1)	An18g02970	4.00E-49	5.00E-78	Mito/O	Mito	–
Uroporphyrinogen III synthase (UroII synthase, UroIII cosynthase)	UROS	hemD (AP_003996.1)*	hem4p (NP_014921.1)	An08g10680	4.00E-45	2.00E-67	O	Mito	–
Uroporphyrinogen III decarboxylase (Uro D, uroporphyrinogen decarboxylase)	UROD	hemE (CAQ34344.1)	hem12p (NP_010332.1)	An01g04250		1.00E-75	O	Cyto	–
Coproporphyrinogen III oxidase (coproporphyrinogenase)	CPO	hemF (CAQ32815.1)	hem13p (NP_010329.1)	An07g10040	2.00E-67	3.00E-99	Mito	Mito	Mito
Protoporphyrinogen oxidase	PPO		hem14p (NP_010930.1)	An11g01580		4.00E-32	Mito	Mito	Mito
Ferrochelatase (ferrolyase, protoheme ferrolyase, heme synthase)	FC	hemH (CAQ30948.1)	hem15p (NP_014819.1)	An15g02690	8.00E-19	4.00E-114	Mito	Mito	Mito
Siroheme synthesis	Met1	cysG (CAQ33688.1)	met1p (NP_012995.1)	An11g09700	1.00E-38	2.00E-114	O	Cyto	–
	Met8		met8p (NP_009772.1)	An07g02200	2.00E-12	4.00E-18	O	Cyto	–

Prediction tools used: WoLF PSORT, MitoProt II, and TargetP (available on <http://www.expasy.org/tools/> and <http://wolfpsort.org/>)
N.P. not present, *no significant blast and no bidirectional match, *Mito* mitochondria, *Cyto* cytoplasm, *Nucl* nucleus, *O* any other location

coproporphyrinogen III oxidase (CPO) with the formation of protoporphyrinogen IX (PP'genIX). Next, protoporphyrinogen oxidase (PPO) mediates a six-electron oxidation forming protoporphyrin IX (PPIX). The final product, heme, is formed by ferrochelatase (FC), which mediates the insertion of ferrous iron in PPIX (Elrod et al. 1997; Moretti et al. 2000). Structural features of heme biosynthesis enzymes (Layer et al. 2010) and iron utilization and regulation in fungi (Haas et al. 2008) have recently been reviewed extensively and will only be discussed briefly hereafter.

Increased peroxidase production can be achieved by overproducing heme biosynthesis genes. However, this approach does not outperform external hemin addition in experiments aimed at the improved production of secreted proteins from *Aspergillus oryzae* (Elrod et al. 1997). This is most likely caused by the strict regulation of the heme biosynthetic pathway on multiple levels. Heme is an essential molecule for the cell due to its involvement in many essential processes. However, elevated levels of free heme and accumulation of its porphyrin intermediates are toxic to the cell. Porphyrins absorb light leading to photosensitization and consequently cellular damage due to singlet oxygen release. The toxic effect of heme is mainly mediated by the iron catalyzing the Fenton reaction, generating hydroxyl radicals and subsequently damages DNA, membrane lipids, proteins, etc. (Krishnamurthy et al. 2007; Hamza 2006; Hou et al. 2006). Therefore, in order to increase intracellular levels of heme to levels appropriate for overproduction of peroxidases, insight into the complete biosynthetic pathway and its regulation is necessary. This review will outline the heme biosynthetic pathway and discuss potential rate-limiting steps. The final aim of this research would be further understanding and resolution of bottlenecks in hemoprotein synthesis in filamentous fungi

5-Aminolevulinic acid synthase (EC 2.3.1.37)

5-Aminolevulinic acid synthase

The first reaction in the heme pathway is the formation of 5-aminolevulinic acid performed by 5-aminolevulinic acid synthase (ALAS) from succinyl-CoA and glycine (Ferreira and Gong 1995; Elrod et al. 2000; Layer et al. 2010). After synthesis in the cytosol, in eukaryotes, the ALAS precursor protein is translocated to the mitochondria. This translocation involves the proteolytic removal of the leader sequence allowing the protein to fold, subsequent dimerization, and addition of its cofactor pyridoxal phosphate (Dailey et al. 2005; Layer et al. 2010).

Most ALAS proteins contain two or three so-called heme regulatory motifs (HRMs) (amino acid motif CPV/F—

Elrod et al. 2000; Ferreira and Gong 1995; González-Domínguez et al. 2001). Interestingly, one HRM is present in the mature protein, whereas the other(s) resides on the mitochondrial targeting sequence (Elrod et al. 2000; Lathrop and Timko 1993).

Most bacteria and plants also have another pathway towards the synthesis of ALA (for review see Layer et al. 2010).

Regulation of ALAS

The abovementioned HRMs are responsible for the rate-limiting character of the enzyme by feedback inhibition of heme at the posttranslational level. Excess intracellular heme is highly toxic to cells and is therefore tightly controlled at low levels (Hou et al. 2006). Due to the binding of heme, translocation of the newly synthesized peptide into the mitochondria is hindered and results in lower amounts of ALAS present in the matrix. Binding of heme to the HRM in the mature protein is not yet understood but might abolish proper folding, making the protein more susceptible for proteolytic degradation (Dailey et al. 2005). This negative feedback is abolished when the cysteine residue in the HRM is either mutated to serine or deleted (González-Domínguez et al. 2001; Lathrop and Timko 1993). Interestingly, *S. cerevisiae* ALAS does not contain HRMs (González-Domínguez et al. 2001) and the rate-limiting step in this organism is not at the level of ALAS. Instead, in *S. cerevisiae*, the second enzyme in the pathway presents a rate-limiting step (Hoffman et al. 2003).

As well as this posttranslational regulation, ALAS activity is also regulated at transcriptional level. One important complex involved in regulation of the ALAS encoding gene is the CCAAT–core binding complex (CBC). The CBC is a highly conserved complex in eukaryotes ranging from yeast to humans. The complex was first identified in *S. cerevisiae* in which it consists of three subunits, which as a complex, interact with transcriptional activator Hap4p in response to growth on non-fermentable carbon sources. Whereas a homologous CBC is present in all eukaryotes, Hap4p appears to be restricted to yeast species *S. cerevisiae*, *Kluyveromyces lactis*, and *Hansenula polymorpha* (Hortschansky et al. 2007 and references therein). In other organisms, a protein (Php4, hapX) bearing little resemblance with Hap4p has been identified that interacts with the CBC acting in combination with the CBC as a repressor under conditions of iron depletion (Hortschansky et al. 2007; Mercier et al. 2008).

Regulation of the hap complex in *S. cerevisiae* is mediated through activation by heme and repression by glucose. Expression of the ALAS encoding gene (*HEMI*) is also regulated by this Hap complex. However, steady-state regulation by oxygen (heme being the effector mediating induction by oxygen) and carbon source is masked in the

case of *HEM1* by additional layers of control, rendering its expression constitutive (Keng and Guarente 1987).

K. lactis HEM1 responds only slightly to non-fermentable carbon sources but does respond to changes in oxygen and heme. The *KIHEM1* was found to be induced under hypoxic conditions (Ferreira and Gong 1995; Lathrop and Timko 1993; González-Domínguez et al. 1997; Núñez et al. 2008). Furthermore, heme addition repressed transcription of *KIHEM1*, like it does in mammalian organisms (Ferreira and Gong 1995; Lathrop and Timko 1993; González-Domínguez et al. 1997; Núñez et al. 2008). Recently, the *KIHEM1* promoter was analyzed and multiple regulatory sites were found and characterized. The CBC complex does not appear to be involved in regulation by carbon source in this yeast. However, the *KIHEM1* promoter contains additional motifs related to regulation by carbon source (Mig1p and Gcr1p) (Núñez et al. 2008).

5-Aminolevulinic acid dehydratase (EC 4.2.1.24)

5-Aminolevulinic acid dehydratase (ALAD)

After the formation of ALA in the mitochondria, it is exported to the cytosol, where heme synthesis continues with the formation of PBG by ALAD (Elrod et al. 1997).

ALAD is present in the cytosol as a homo-octomer. Based on metal requirement, the ALAD can be divided into two classes: the zinc-dependent ALAD which is present in animals, yeast, and bacteria, and the magnesium-dependent ALAD which is present among plants (Senior et al. 1996).

In the zinc-dependent class, two zinc ions are bound at separate sites, termed α and β . In *S. cerevisiae* and *Escherichia coli*, a zinc ion can be replaced by a magnesium ion as long as there is one zinc ion bound to the β site (Senior et al. 1996). Furthermore, the enzyme is sensitive to inhibition by lead, which displaces the zinc ions (Erskine et al. 1997), and is sensitive to oxidation of the enzymes thiol groups. Oxidation of the thiol groups subsequently leads to a decrease in activity and a stoichiometric loss of bound metal ions, demonstrating that cysteines are required for zinc binding (Senior et al. 1996).

Regulation of ALAD

The *S. cerevisiae* gene encoding ALAD (*HEM2*) is partially repressed by glucose (Núñez et al. 2008). The *HEM2* promoter contains, among others, consensus sequences for binding of the CBC. Based on these sequences, it has been suggested that besides carbon sources, heme is also involved in the regulation (Schlaepfer et al. 1994). The results of

Hoffman et al. (2003) suggest that ALAD is not subject to posttranscriptional regulation in *S. cerevisiae*. For the human heme biosynthesis, it was recently suggested that ALAD might be an early control step in heme biosynthesis when avoiding ALAS regulation (Gibson et al. 2001).

Dual function of ALAD

Besides its function in the heme biosynthetic pathway (see above), ALAD was found to be identical to and functions as the CF-2 regulatory subunit of the proteasome (Grünberg-Etkovitz et al. 2006; Guo et al. 1994). In humans, loss of ALAD activity, e.g., during lead poisoning, leads directly to a strong increase in proteasomal activity, which in turn causes, among others, life-threatening neuropathy attacks (Grünberg-Etkovitz et al. 2006). This dual role of ALAD may be an example of “gene sharing” and could explain the unexpected abundance of ALAD in earlier studies in mammalian organisms (Guo et al. 1994). Furthermore, hemin is reported to be a specific inhibitor of the catalytic activity of the proteasome. Under conditions of lead poisoning, administration of hemin has been successfully used as treatment (Grünberg-Etkovitz et al. 2006). Whether ALAD in fungi also has this dual role is currently unknown.

Porphobilinogen deaminase (EC 4.3.1.8)

Porphobilinogen deaminase (PBGD)

Synthesis is continued with the condensation of PBG to the unstable HMB by PBGD (Elrod et al. 1997). The yeast and human PBGD is reported to be present in the cytosol as a monomer and contains dipyromethane as a cofactor at the active site (Fujino et al. 1999; Keng et al. 1992; McNicholas et al. 1997). In human cells, the PBGD cellular pool is controlled by proteasome activity. The protein is also imported into the nucleus, where it fulfills a separate function possibly by interacting with the nuclear Ran-binding factor, RanBPM, whose function is still unknown (Grünberg-Etkovitz et al. 2006).

Regulation of PBGD

In *S. cerevisiae*, following ALAD, PBGD might play an additional rate-limiting role in heme synthesis (Hoffman et al. 2003). Like *HEM1*, expression of the yeast PBGD encoding gene *HEM3* appears constitutive but is actually activated by the Hap4p/CBC complex and repressed on fermentable carbon sources (Keng et al. 1992). The CBC complex also appears to be involved in *HEM3* regulation in *Schizosaccharomyces pombe* in response to iron deprivation.

Despite the absence of a canonical CCAAT consensus in its promoter, an approximate 3.5-fold *Php4*-dependent de-repression was observed (Mercier et al. 2008). Recently, it was also demonstrated in hepatic cells that PBGD transcripts are reduced under hypoxia (Vargas et al. 2008).

Uroporphyrinogen III synthase (EC 4.2.1.75)

Uroporphyrinogen III synthase (UROS) and regulation

The formation of HMB is followed by subsequent flipping of the D-ring during ring closure by UROS, yielding UroIII (Elrod et al. 1997). HMB is an unstable molecule and can be non-enzymatically converted to uroporphyrinogen I (Jordan and Berry 1980; Mathews et al. 2001). However, this product is not physiologically relevant. UroIII forms the final common intermediate in the formation of all tetrapyrroles (Schubert et al. 2002b).

Currently, other than the observed *Php4*-dependent repression in *S. pombe* (Mercier et al. 2008) and hypoxia-dependent repression in human hepatic UROS (Vargas et al. 2008), no data is available upon regulation of UROS. On the other hand, UROS is not expected to become rate limiting as its substrate is unstable and its product is required for both heme synthesis as well as siroheme synthesis, which forms a branch point in the pathway.

Uroporphyrinogen III decarboxylase (EC 4.1.1.37)

Uroporphyrinogen III decarboxylase (UROD) and regulation

The fifth enzyme in the heme biosynthetic pathway is UROD (Elrod et al. 1997), which catalyzes decarboxylation of the four acetyl side groups of UroIII to yield Copro (Kurlandzka et al. 1988).

Knowledge of its regulation is limited, but the *K. lactis* *HEM12* (encoding UROD) contains a CBC consensus site and its transcriptional regulation has been studied. No regulation was found in response to increased levels of glucose or heme. A slight increase was found, however, during growth on non-fermentable carbon sources by a Hap3p-dependent mechanism (Núñez et al. 2004).

Coproporphyrinogen III oxidase (EC 1.3.3.3)

Coproporphyrinogen III oxidase (CPO)

Eukaryotic CPO (Elrod et al. 1997) forms the first oxygen requiring reaction within the heme biosynthetic pathway.

Oxygen is required in the oxidative decarboxylation at the 2- and 4-carboxyethyl side chains in Copro to yield two vinyl groups in PP'genIX (Zagorec et al. 1988).

CPO is located in the cytosol in yeast but is associated with the mitochondrial outer membrane in higher eukaryotes (Dailey 2002), the only difference being the presence or absence of the mitochondrial targeting sequence in their sequence (Phillips et al. 2004).

Although eukaryotic CPO has been reported to have an obligate requirement for molecular oxygen (Zagorec et al. 1988; Dailey 2002), *S. cerevisiae* still synthesizes 3–7% of its normal heme levels during anaerobic conditions, and oxygen is not an obligatory electron acceptor for heme synthesis during stress conditions in this organism (Hoffman et al. 2003).

Regulation of CPO by oxygen and/or heme

In *S. cerevisiae*, the gene encoding CPO (*HEM13*) is regulated on the transcriptional level by Rox1p, a repressor of hypoxic genes, in combination with the repressor Mot3p (Klinkenberg et al. 2005). *ROX1* expression is heme dependent and activated by the Heme Activator Protein 1 (Hap1) and the Hap4p/CBC complex (Zitomer and Lowry 1992) resulting in the repression of hypoxic genes. Under oxygen/heme-limiting conditions, Rox1p rapidly depletes from the cell leading to 9–20-fold de-repression (Klinkenberg et al. 2005; Keng 1992).

However, an additional mechanism in response to hypoxia has been identified in *S. cerevisiae* (Vasconcelles et al. 2001). This signal transduction pathway acts through a low-oxygen response element (LORE) and Mga2p to induce hypoxic genes (e.g., *OLE1*, *AFT1*) during hypoxia (Jiang et al. 2002). Like *S. cerevisiae*, the *K. lactis* CPO is under heme and oxygen transcriptional control (González-Domínguez et al. 2000), and recently its promoter was analyzed. A LORE site was identified next to Hap1p, CBC, Rox1p, and Mot3p sites. This search also identified an AR1 site in the promoter, a regulatory element of a specific group of anaerobic genes not under Rox1p control (the *DAN* genes), but whether induction of CPO transcription during hypoxic conditions is dependent on the AR1 and LORE site or other sequences remains to be determined (Blanco et al. 2005). CPO induction is also observed in *S. pombe* upon oxygen limitation (Todd et al. 2006).

Human CPO also appears to be regulated by heme. In vitro analysis using a full-length CPO demonstrated that hemin addition resulted in a 99% decreased availability of CPO in the mitochondria (Susa et al. 2002). However, using a CPO N-terminal–GFP fusion protein, it was shown that mitochondrial targeting was not affected (Dailey et al.

2005). These results indicate that CPO activity could also be subject to posttranslational regulation.

Protoporphyrinogen oxidase (EC 1.3.3.4)

Protoporphyrinogen oxidase (PPO) and regulation

The heme synthesis continues by a six-electron oxidation of PP^ogenIX to yield PPIX (Elrod et al. 1997). This oxygen-dependent reaction in eukaryotes is catalyzed in three independent cycles by PPO (Dailey 2002).

In eukaryotes, the protein is associated with the cytoplasmic side of the mitochondrial inner membrane as a monomer or as a homodimer, and requires flavin adenine dinucleotide (FAD) as a cofactor. The 17 N-terminal amino acids of human PPO are sufficient for proper targeting, but this sequence is not processed upon translocation. The N-terminal sequence contains an approximately 60 amino acids long dinucleotide-binding motif essential for binding of FAD (Dailey and Dailey 1998; Dailey et al. 1995).

Knowledge of PPO regulation is limited, but the CBC complex might be involved in regulation of PPO as well. A 2–3-fold increase in activity was observed when *S. cerevisiae* was grown on ethanol or galactose compared to growth on glucose (Camadro and Labbe 1996). In *S. pombe*, a 2-fold induction in mRNA was observed 1.5 h after a switch to oxygen-limiting conditions, but PPO activity was not determined (Todd et al. 2006), while in *S. cerevisiae* no increase of PPO activity was observed under anaerobic conditions (Zagorec et al. 1988). For human PPO, it has been suggested that, for its housekeeping functions, PPO activity levels are in excess (Dailey et al. 1995); therefore, PPO is not likely to become a rate-limiting factor.

Ferrochelatase (EC 4.99.1.1)

Ferrochelatase (FC)

FC forms the final step in the heme biosynthesis pathway and catalyzes the insertion of ferrous iron into PPIX (Elrod et al. 1997).

In eukaryotes, the apoprotein, synthesized in the cytosol, is translocated to its final destination, the mitochondrial matrix. Translocation of ferrochelatase is an energy-dependent process, which involves removal of the N-terminal leader sequence and assembly of the [2Fe–2S] cluster (Dailey et al. 2005). FC is found as a homodimer associated with the mitochondrial inner membrane, with its active site facing the matrix space (Dailey et al. 2005;

Ferreira 1999). It has been proposed that FC could form a complex with PPO (Dailey 2002). The localization of FC is surprising since its product is mostly used in cytoplasmic proteins like P450s and cytochrome b5 or in respiratory cytochromes located on the cytoplasmic side of the mitochondrial inner membrane (Prasad and Dailey 1995).

FCs are clearly similar in structure and gross catalytic properties among all species analyzed, although primary sequences show less than 10% identity between bacterial and higher eukaryotes (Dailey et al. 2007).

Regulation of FC

In addition to the other regulated enzymes within the heme biosynthesis pathway, FC could also be subject to regulation. Studies by Taketani et al. (2000) in human erythroleukemia cells showed that the amount of FC and activity as well as heme content decreased upon a decrease in the available iron, though mRNA levels and PPIX content were unchanged. Addition of ferric ion–nitrilotriacetate [Fe (III) NTA] restored ferrochelatase activity. The additive effect of iron was tested as well and a 1.5-fold increase in FC activity was observed upon the addition of 100 μ M Fe (III) NTA, and higher concentrations resulted in a decrease of FC activity. Furthermore, *E. coli* FC was insensitive to treatment with iron chelators. *E. coli* does not contain [2Fe–2S] clusters, and the human FC apoprotein is sensitive to proteolytic degradation. Therefore, it is suggested that FC is under the positive control of intracellular iron and that this possibly correlates with the formation of the [2Fe–2S] cluster (Taketani et al. 2000).

Transport of heme

Transporters of heme

Next to heme synthesis, transport of heme or its intermediates might also be a rate-limiting factor in heme biosynthesis and/or in production of hemoproteins. Directed transport is probably required to provide different organelles like the ER, nucleus, and peroxisomes with sufficient amounts of heme. Due to the hydrophobic and reactive nature of heme, “free” diffusion through the cytosol is not likely (Hamza 2006).

Research on transport of heme and its intermediates is only beginning to emerge in eukaryotes, and a few transporters have been identified in mammalian cells: Heme Carrier Protein 1 (HCP1), Feline Leukemia Virus C Receptor (FLVCR), and Breast Cancer Resistance Protein (BCRP) (Hamza 2006 and references therein). Next to these heme transporters, ABCB6 was found for the transport of Copro (reviewed by Krishnamurthy et al. 2007).

Mitochondrial porphyrin import

ABCB6 was first found to be localized on the mitochondrial outer membrane and has the highest affinity for Copro but can also transport other porphyrins. Due to this affinity, ABCB6 can shuttle heme back into the mitochondrial intermembrane space where it might be able to regulate ALAS import. Furthermore, expression of ABCB6 is induced by heme and its intermediates. Overexpression of ABCB6 resulted in increased expression of several rate-limiting enzymes (CPO, ALAD, and ALAS) in the heme biosynthetic pathway, resulting in higher heme levels (Krishnamurthy et al. 2006, 2007). However, ABCB6 is also reported to be localized in the Golgi apparatus (Tsuchida et al. 2008) and the plasma membrane (Paterson et al. 2007). In the latter study, two different versions of ABCB6 were detected: one is located at the mitochondrial outer membrane (ABCB6-L), and the other is modified posttranslationally and resides at the plasma membrane (ABCB6-H). ABCB6-H appears functionally similar to ABCB6-L and may be involved in the cellular efflux of porphyrins from the cell (Paterson et al. 2007).

The 2-oxoglutarate carrier (OGC) is responsible for the mitochondrial transporter of 2-oxoglutarate but exhibits similar features to ABCB6. Like ABCB6, OGC also has a general affinity for porphyrins and is able to translocate porphyrins and heme into the mitochondrial matrix. However, unlike ABCB6, OGC is a mitochondrial inner membrane transporter (Kabe et al. 2006).

Mitochondrial heme export

ABCB10 is considered a putative heme exporter and is also located on the mitochondrial inner membrane as a homodimer. However, it can assemble in a similar way in the ER when it is expressed without its N-terminal mitochondrial targeting pre-sequence (Graf et al. 2004). ABCB10 is negatively regulated by heme as well and overexpression of ABCB10 increased hemoglobin content 2–4-fold (Shirihai et al. 2000).

Transporters in fungi

A screen to identify genes involved in heme uptake in *Candida albicans* resulted in the identification of a fungal gene family involved in FAD transport into the endoplasmic reticulum (ER). Analysis of Flavin Carrier Protein 1 (Flc1) showed that Flc1p normally resides in the ER. Although heme transport is not the primary function of Flc1p, it does significantly contribute to heme uptake in

C. albicans. However, whether Flc1p is directly or indirectly involved in heme uptake remains to be determined. Two homologues are also present in *C. albicans* (*CaFLC2* and *CaFLC3*) which are strongly predicted to have similar, if not identical, biochemical activities. Overexpression of *S. cerevisiae* *FLC1* and *FLC2*, but not *FLC3*, improved heme uptake (Protchenko et al. 2006).

Recently, a transporter, Pug1p (porphyrin uptake gene), was identified in *S. cerevisiae* which is located exclusively at the plasma membrane. Overexpression of Pug1p increased utilization of PPIX but reduced uptake of exogenous heme in a heme-deficient strain. Pug1p belongs to a family of fungal proteins which may possibly function as efflux channels or pumps induced under conditions of enhanced uptake and dysregulation of intracellular small molecules. The authors suggest that Pug1p may function to facilitate excretion of excess porphyrins under conditions of porphyrin accumulation, for instance during hypoxia (Protchenko et al. 2008). Despite the transporters described above, little is known about eukaryotic heme transport, and many questions remain to be answered.

Siroheme side branch

As mentioned earlier, UroIII is a common intermediate for both heme and siroheme synthesis. Therefore, feedback regulation on the heme biosynthesis pathway originating from siroheme synthesis may be considered. We will therefore also briefly discuss the current knowledge of siroheme biosynthesis.

Siroheme is a heme-like prosthetic group for sulfite and nitrite reductases which are involved in the reduction of sulfite and nitrite to sulfide and ammonia, respectively (Schubert et al. 2002a). Via sulfite reductases, the siroheme pathway is also required for the synthesis of methionine and cysteine (Keng and Guarente 1987). It is synthesized from UroIII in four enzymatic steps: two *S*-adenosyl-L-methionine-dependent transmethylation, a dehydrogenation, and a ferrochelation (Raux et al. 1999). In *E. coli*, one protein (cysG) performs all these enzymatic reactions (Leustek et al. 1997), whereas in *S. cerevisiae* these steps are performed by Met1p and Met8p. Met1p performs the transmethylation reaction and Met8p is responsible for both the dehydrogenation and ferrochelation reactions. As the N terminus of Met1p is not required for any enzymatic transformations of siroheme synthesis, its function remains unknown. It may act in some regulatory fashion to mediate control over the branch point in *S. cerevisiae* (Raux et al. 1999).

Heme biosynthesis and regulation in filamentous fungi

Filamentous fungi in general

The current knowledge about heme biosynthesis in other filamentous fungi is extremely limited and is restricted to research in *Neurospora crassa*, *A. oryzae*, and *Aspergillus nidulans* and recent work related to iron in *A. nidulans* and *Aspergillus fumigatus* (Hortschansky et al. 2007; Schrettl et al. 2008; Elrod et al. 1997, 2000; Chandrika and Padmanaban 1980; Muthukrishnan et al. 1969, 1972; Bradshaw et al. 1993).

Whereas Hap4p plays an important role as transcriptional activator in response to non-fermentable carbon sources in *S. cerevisiae* (see above), this transcriptional activator was initially only found in yeast. A search directed to identify a Hap4p homologue in *A. nidulans* resulted in identification of HapX. However, besides a 17 amino acid N-terminal motif, HapX shares no similarity with Hap4p and was initially discarded as a functional homologue (Tanaka et al. 2002). A later study verified HapX/CBC interaction which mediates repression of iron-dependent processes like, e.g., the heme biosynthesis pathway under iron-depleted conditions (Hortschansky et al. 2007). *HemA* is one of the target genes of HapX/CBC-mediated regulation. Deletion of *hapX* or *hapC* (encoding a CBC complex subunit) resulted in transcriptional de-repression of iron-consuming pathways, including heme biosynthetic *hemA* (encoding ALAS), during iron starvation. In agreement, ALAS was found to be 19.2-fold increased at the protein level during iron starvation conditions in the *hapX*-deletion mutant compared to wild type. In support of the data mentioned above, inactivation of HapX or the CBC resulted in vast cellular accumulation of protoporphyrin IX and other iron-free heme precursors during iron starvation. A similar phenotype has been found in *hapX* lacking *A. fumigatus* (Schrettl et al. 2010). Moreover, inactivation of HapX or the CBC caused deregulation of heme biosynthetic genes also in *Cryptococcus neoformans* (Jung et al. 2010).

Iron homeostasis is regulated by *sreA* (siderophore biosynthesis repressor) in *A. nidulans* and *A. fumigatus* (Hortschansky et al. 2007; Schrettl et al. 2008). Deletion of *sreA* in *A. fumigatus* results in an increased iron uptake and microarrays showed upregulation of *hemA* and *hemH*. As a result, this Δ *sreA* strain contained increased heme content and accumulated a significant amount of later heme intermediates (Schrettl et al. 2008). This observation poses an interesting question as HRMs are the target for feedback inhibition by heme. Studies on the production of *Coprinus cinerea* peroxidase in *A. oryzae* demonstrated the rate-limiting character of ALAS in *A. oryzae*. By overexpression of genes encoding ALAS, but not ALAD, production could

be increased 2-fold. Co-overexpression however, led to a 4-fold increase in amounts, indicating that the next limitation in the pathway is located at the second enzyme, ALAD (Elrod et al. 1997). Interestingly, ALAD forms the rate-limiting step in *N. crassa* (Chandrika and Padmanaban 1980). In this organism, it was shown that PPIX, and to a lesser extent hemin, is a co-repressor of ALAD in iron-deficient cultures (Muthukrishnan et al. 1969) and coproporphyrinogen III (0.1 mM) inhibited ALAD activity completely (Chandrika and Padmanaban 1980; Muthukrishnan et al. 1969). As genome mining in *N. crassa* does not reveal large differences between *N. crassa* and *A. niger*, except the two putative porphobilinogen deaminase proteins, it is possible that this regulation on ALAD exists in *Aspergilli* as well.

Another important regulatory mechanism for heme biosynthesis is activated in response to hypoxia. Sterol synthesis is a heme-dependent pathway with several enzymes directly utilizing oxygen in their reaction. During hypoxia, sterol levels are reduced and this activates sterol regulatory element binding proteins (SREBP) required for the adaptation to hypoxia. This system appears to be conserved in the fungal kingdom, albeit with some differences and absent from *C. albicans* and *S. cerevisiae* (for detailed review see Bien and Espenshade 2010 and Espenshade and Hughes 2007). In general, the sterol regulatory element binding protein *sre1* in *S. pombe* (Hughes et al. 2005) and *C. neoformans* (Chang et al. 2007), *srbA* in filamentous fungi (Willger et al. 2008), modulates oxygen requiring pathways like, e.g., ergosterol, heme, sphingolipid, and ubiquinone synthesis (Hughes et al. 2005; Todd et al. 2006; Chang et al. 2007) but also affects pathogenicity (Chang et al. 2007, 2009; Willger et al. 2008).

Sterol synthesis requires enzymes with heme as a cofactor, e.g., *erg11* encoding sterol C-14 demethylase, and therefore it comes as no surprise that *sre1* was demonstrated to be involved in the expression of *HEM13* in *S. pombe* (Hughes et al. 2005). However, no significant change in heme biosynthetic enzymes (transcriptional level or at proteome level) was found in a *srbA*-dependent manner in *A. fumigatus* or *C. neoformans* under hypoxia (Chang et al. 2007; Willger et al. 2008), although *hem13* expression was significantly upregulated in the latter. This indicates that additional mechanisms operate to control adaptation to hypoxia. This is further demonstrated by the 4-fold increase in heme content in an *A. fumigatus* chemostat culture under severe oxygen deprivation (Vödisch et al. 2011). These results suggest that maybe even minor changes of the heme pathway genes at transcriptional and/or posttranscriptional level can result in significant changes in heme biosynthesis, further suggesting the tight regulation of this pathway.

An interesting aspect in relation to hypoxia and heme biosynthesis is the strong upregulation of a heme containing flavohemoglobin under hypoxia (Vödisch et al. 2011). As several filamentous fungi were found to be capable of denitrification and ammonia fermentation under conditions of hypoxia (Takaya 2009; Masuo et al. 2010), the upregulation of flavohemoglobin might assist in the detoxification of NO which is formed during this process. Indeed, NO dioxygenase activity of flavohemoglobin was demonstrated in *A. oryzae* (Zhou et al. 2009). Moreover, disruption of the cytosolic flavohemoglobin resulted in hypersensitivity to NO (Zhou et al. 2011). As the hemoglobin domain of the flavohemoglobin is also suggested to be involved in O₂ uptake in the cells (te Biesebeke et al. 2006), induction of flavohemoglobin may also improve O₂ uptake under hypoxic conditions. However, this suggested that the role of flavohemoglobins would require additional experimental validation in particular as in *A. oryzae* these proteins have also been shown to promote oxidative stress (Zhou et al. 2010).

Genome data of *A. niger*

Table 1 (and Supplementary Table 1) displays BlastP results for *A. niger* of the known heme and siroheme synthesis genes from *S. cerevisiae* and *E. coli*. These results indicate that a complete biosynthesis pathway is present in *A. niger* for both heme and siroheme synthesis. The proteins involved are clearly conserved and are in general more closely related to the yeast proteins, although some differences become apparent as well. First, two putative proteins appear to exist for porphobilinogen deaminase (PBGD) in *A. niger*. This phenomenon is not restricted to *A. niger*. All *Aspergilli* contain multiple putative proteins for this enzyme with *A. oryzae* and *A. flavus* containing three putative PBGD (data not shown). One hypothesis for this occurrence could have something to do with the reported dual function for human PBGD (Grünberg-Etkovitz et al. 2006), and that these functions are separated in fungi. Furthermore, localization of these two *A. niger* proteins is unclear as different results and low probabilities are obtained by different programs MitoProt II (Claros and Vincens 1996), TargetP (Emanuelsson et al. 2007), and WoLF PSORT (Horton et al. 2007) (Table 1 and Supplementary Table 1). Performing this prediction of localization on the other synthesis genes reveals another interesting aspect for *A. niger* CPO. Whereas the yeast CPO is localized in the cytosol, the *A. niger* CPO is predicted to be localized in the mitochondria. This localization would be identical to mammalian CPO (Dailey 2002; Phillips et al. 2004). The *A. niger* ferrochelatase also contains a mammalian character. The putative protein indicates that the motifs involved in [2Fe–2S] cluster formation are conserved which suggests that, like *S. pombe* (Dailey and Dailey 2002), an

[2Fe–2S] cluster in *A. niger* ferrochelatase is predicted to be present, unlike in *S. cerevisiae*.

Whereas the enzymes involved in the biosynthesis of heme are clearly conserved, much less is comprehensible when it comes to regulatory proteins (Supplementary Table 2). BlastP comparison of the major transcription factor involved in regulation of the heme biosynthetic pathway in *S. cerevisiae*, hap1p, results in identification of a hypothetical protein with only low homology. Furthermore, regulators in response to iron, Aft1p and Aft2p, are absent in the genome of *A. niger*. The absence of these transcription factors could indicate a different mechanism of regulating the heme biosynthetic pathway as is observed for the hap/CBC complex in, e.g., *K. lactis*. With respect to the other regulatory proteins, putative proteins can be identified, although caution should be taken with subscribing function to these proteins as E-values are not very low.

Continuing with transport (Supplementary Table 3), BLAST searches for almost all described transporters provide putative protein hits in *A. niger*. This would indicate that mechanisms of transport of heme and porphyrins could be conserved. Although the mammalian heme uptake protein HCP1 does not result in a clear hypothetical protein, the yeast protein Flc2p, and to a lesser extend Flc3p and Flc1p, has a clear homologue in the *A. niger* genome. These proteins could facilitate heme uptake as has been reported for *C. albicans* and *S. cerevisiae* (Protchenko et al. 2006).

Applications and concluding remarks

Production of heme containing class II peroxidases has become of particular interest due to their capability to degrade lignin. As such, these peroxidases could provide a more environmentally friendly means of processing lignin in the production of, e.g., paper and bio-ethanol. As heme is suggested to be a limiting factor during peroxidase production in *A. niger*, a thorough insight in the heme pathway is required.

Much knowledge regarding heme biosynthesis and regulation is known for *S. cerevisiae*. Although enzymes of the pathway are highly conserved, regulation of the pathway appears to be specific for *S. cerevisiae* as major differences are found already in comparison with *K. lactis* (Lamas-Maceiras et al. 2007). Major regulators in yeast appear to be absent in the genome of *A. niger*. Also, mammalian characteristics, deviating from *S. cerevisiae*, are present in several enzymes in the fungal pathway. These observations indicate that the pathway may be regulated in a different fashion in *A. niger*. Furthermore, identification of putative regulatory proteins with low homology does not directly exclude the possibility of their involvement in the

pathway. Unfortunately, information on the heme biosynthesis pathway and its regulation in other eukaryotic systems is incomplete, although common factors influencing the heme biosynthetic pathway appear to be iron, oxygen, and heme.

In order to reach elevated levels of heme, this review indicates initial targets for *A. niger*. The two most apparent options could be the removal of the HRMs in ALAS to abolish potential feedback inhibition by heme and deletion of the siderophore biosynthesis repressor *sreA*.

Under conditions of elevated heme, additional bottlenecks could be identified by making use of the autofluorescence of most intermediates in the later part of heme and siroheme biosynthesis. Accumulation of these tetrapyrroles can be detected by their autofluorescence upon exposure to near-UV light (Raux et al. 1999; Parish et al. 2005; Góra et al. 2000). This characteristic is often used to quantify tetrapyrroles for the diagnosis of porphyrias (a group of diseases that have fundamental abnormalities in the heme biosynthetic pathway in common) (Polo et al. 1988). Accumulation of porphyrins in filamentous fungi can be easily observed and quantified under iron-deprived conditions as has been demonstrated in $\Delta hapX$ strains of the filamentous fungi *A. nidulans* and *A. fumigatus* (Schrettl et al. 2010; Hortschansky et al. 2007). This characteristic could be useful as a simple assay, e.g., for mutant screening, to study regulation of this pathway in more detail.

In conclusion, heme is involved in numerous essential processes in most organisms. As a result, the heme biosynthetic pathway is a highly conserved pathway, albeit with some obvious deviations, and is tightly regulated due to the reactive nature of heme and its intermediates. This regulation, on the other hand, does not appear to be conserved among all eukaryotes. To be capable of increasing the heme content within the cell, to meet up with the requirement for peroxidase production, multiple aspects need more clarification in *A. niger* and are currently under investigation in our laboratory.

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