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Chapter 5

The role of coproporphyrinogen III oxidase and ferrochelatase genes in heme biosynthesis and regulation in Aspergillus niger

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Abstract

Heme is a suggested limiting factor in peroxidase production by *Aspergillus* spp*.,* which are well-known suitable hosts for heterologous protein production. In this study the role of genes coding for coproporphyrinogen III oxidase (*hemF*) and ferrochelatase (*hemH*) were analyzed by means of deletion and overexpression to obtain more insight in fungal heme biosynthesis and regulation. These enzymes represent steps in the heme biosynthetic pathway downstream of the siroheme-branch, and are suggested to play a role in regulation of the pathway. Based on genome mining both enzymes deviate in cellular localization and protein domain structure from their *S. cerevisiae* counterparts. The lethal phenotype of deletion of *hemF* or *hemH* could be remediated by heme supplementation confirming that *Aspergillus niger* is capable of hemin uptake. Nevertheless, both gene deletion mutants showed an extremely impaired growth even with hemin supplementation which could be slightly improved by media modifications and the use of hemoglobin as heme source. The hyphae of the mutant strains displayed pinkish coloration and red auto-fluorescence under UV indicative of cellular porphyrin accumulation. HPLC analysis confirmed accumulation of specific porphyrins, thereby confirming the function of the two proteins in heme biosynthesis. Overexpression of *hemH*, but not *hemF* or the aminolevulinic acid synthase encoding *hemA,* modestly increased the cellular heme content, which was apparently insufficient to increase activity of endogenous peroxidase and cytochrome P450 enzyme activities. Overexpression of all three genes increased the cellular accumulation of porphyrin intermediates suggesting regulatory mechanisms operating in the final steps of the fungal heme biosynthesis pathway.

Introduction

Aspergillus niger is a frequently used organism for the production of heterologous proteins due to its capacity secrete variety of proteins and its high production capacity (Punt et al. 2002). Due to this high production capacity, *A. niger* is considered to be a favorable host for the production of fungal peroxidases derived from white rot fungi. These peroxidases are of interest for their ability to degrade lignin thereby allowing environmental friendly utilization of cellulose (Hammel and Cullen 2008; Harris and DeBolt 2010; Martínez 2002). Expression of these genes in bacteria or yeast often results in intracellular accumulation or inactive protein due to the lack of required post-translational modifications (Pérez-Boada et al. 2002; Smith et al. 1990). While attempts for commercial production of fungal peroxidases in *Aspergillus* spp*.* resulted in active and secreted

peroxidase production, the production could be increased by supplementing culture medium with heme sources like hemin or hemoglobin or overexpression of heme biosynthesis genes early in the pathway, suggesting that heme is a limiting factor for production of peroxidases in *Aspergilli* (Andersen et al. 1992; Conesa et al. 2000; Elrod et al. 1997). Unfortunately, hemin/hemoglobin supplementation is not suitable for industrial applications due to its hydrophobic nature and costs (Elrod et al. 1997). An alternative approach for increased intracellular heme levels would be to optimize intracellular heme biosynthesis. However, the knowledge on heme biosynthesis and its regulation in filamentous fungi required to accomplish this goal is lacking.

The heme biosynthesis pathway is a highly conserved and regulated pathway among pro- and eukaryotes, as heme has an essential function in multiple cellular processes (Hamza 2006). In eukaryotes, heme is synthesized by eight subsequent enzymatic reactions (Figure 5.1; for reviews see Franken et al. 2011; Layer et al. 2010). However, knowledge on heme biosynthesis and regulation in filamentous fungi is only marginally available.

The previously obtained results with the *A. niger hemA* deletion strain demonstrated that not only heme is synthesized from 5'-aminolevulinicacid (ALA). The positive effect on growth of using ammonium as N-source together with hemin supplementation confirmed that also siroheme synthesis derives from the heme biosynthesis pathway in *A. niger* as well (Franken et al. 2012). Siroheme, a heme-like tetrapyrrole (Figure 5.1), is an essential cofactor for functional nitrite reductase (Raux et al. 2003; Schubert et al. 2002a; Tripathy et al. 2010). In this study we focused on studying the role of two heme pathway genes, coproporphyrinogen III oxidase (CPO; EC 1.3.3.3) and ferrochelatase (FC; EC 4.99.1.1) which are independent of the siroheme pathway, and had previously been shown to follow a similar transcriptional regulation as the upstream gene *hemA* (Franken et al. 2012).

Although heme biosynthesis enzymes are highly conserved amongst different organisms, two enzymes in the final steps of the pathway, CPO and FC show deviations between different organisms. CPO, the sixth enzyme within the pathway is responsible for the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX (Elrod et al. 1997). Whereas this enzyme has been reported to be associated with the inner side of the outer mitochondrial membrane in mammals, the enzyme is located in the cytosol in yeasts (Dailey 2002). The *A. niger* hypothetical protein is predicted to be localized to the mitochondria like its mammalian counterpart (Franken et al. 2011). Irrespective of its localization, the enzyme production is strongly regulated in both yeasts and mammals probably because, like heme, also porphyrins are highly reactive molecules resulting in, amongst others, radical formation (Hamza 2006). Regulation of CPO was observed in

response to heme on transcriptional level in yeast (Amillet et al. 1995, 1996; González-Domínguez et al. 2000; González-Domínguez et al. 1997; Keng 1992; Vasconcelles et al. 2001) as well as on protein level for human CPO (Dailey et al. 2005; Susa et al. 2002). Also in filamentous fungi CPO could function as a regulatory step preventing hazardous levels of intracellular heme as accumulation of coproporphyrinogen III was observed in an *Aspergillus fumigatus* Δ*sreA* strain that showed increased heme synthesis due to elevated siderophore biosynthesis and iron uptake (Schrettl et al. 2008).

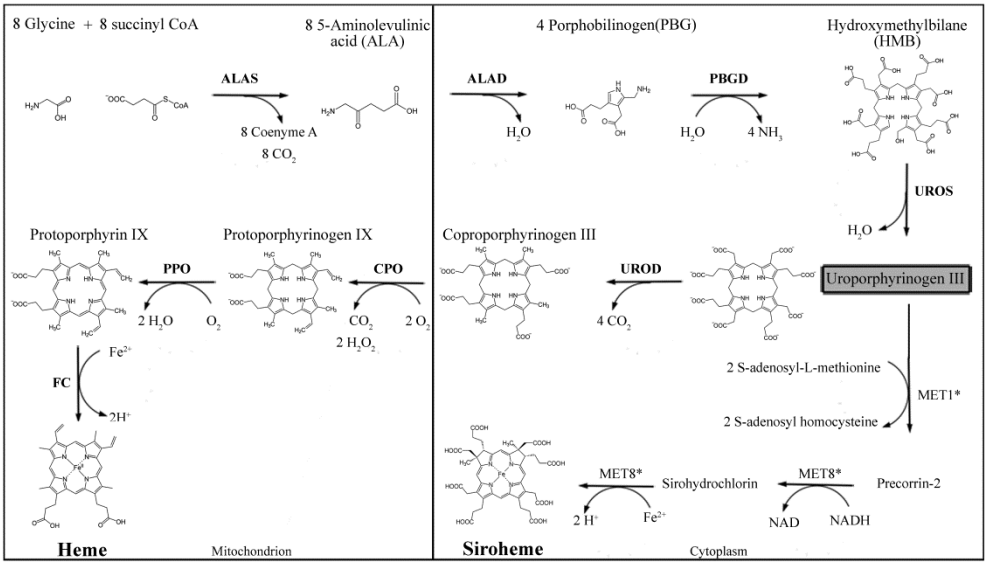

Figure 5.1 Chemical heme biosynthesis pathway. Biosynthesis is initiated in mitochondria with the condensation of glycine and succinyl CoA to 5'-aminolevulininc acid (ALA) by 5'- aminolevulinic acid synthase (ALAS). ALA is subsequently exported into the cytosol to be processed to uroporphyrinogen III (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. Reprint with permission from Franken et al. (2011).

The second enzyme, FC is also highly conserved and functions in all organisms by insertion of ferrous iron into protoporphyrin IX (PPIX) to finalize heme biosynthesis (Elrod et al. 1997). Like mammalian and *Schizosaccharomyces pombe,* the *A. niger* FC is predicted to

contain an [2Fe-2S] cluster, in contrast to *S. cerevisiae* where this domain is not present (Franken et al. 2011).

FC has been found to be a target in the regulation of heme biosynthesis in fungi. Iron was found to affect expression levels in *A. fumigatus* (Schrettl et al. 2008) and the transcription of the *Cryptococcus neoformans* FC was found to be responsive to light induction (Idnurm and Heitman 2010). These authors suggested that up-regulation of FC in light increases heme and reduces porphyrin content (Idnurm and Heitman 2010). However, it remains to be determined whether this enzyme presents a significant limitation for elevated heme biosynthesis, as the observed effects are relatively small.

Therefore, to address potential limitations of CPO and FC for elevated heme biosynthesis, we characterized mutant strains lacking and overexpressing the genes for CPO (*hemF*) and FC (*hemH*) respectively.

Materials and methods

Strains and culture conditions

A. niger N402 (*cspA1* derivative of ATCC9029 (Bos et al. 1988)) and the *pyrG*$^{-}$ derivative of this strain, AB4.1 (van Hartingsveldt et al. 1987) were used during this study. *Aspergillus* strains were grown on minimal medium (MM) (Bennet and Lasure 1991) or on complete medium (CM) consisting of minimal medium with the addition of 10 g l^{-1} yeast extract and 5 g l^{-1} casamino acids containing $NaNO_3$ (70 mM) as nitrogen source. Wherever indicated $NaNO_3$ was replaced by NH_4Cl (10 mM). Growth medium was supplemented with 10 mM uridine when required. Conidiospores were obtained by harvesting conidia from a CM plate after 4 – 6 days growth at 30 °C, using 0.9 g l^{-1} (w/v) NaCl solution and stored at 4 °C. *Escherichia coli* DH5α (Sambrook and Russell 2001) was used for the amplification of recombinant DNA. Transformation of DH5α was performed according to the heat shock protocol as previously described (Inoue et al. 1990).

Molecular Biological Techniques

Chromosomal DNA of *A. niger* was isolated as described by Kolar et al. (1988). Southern blot analysis was performed according to Sambrook and Russell (2001). α-^{32}P-dCTP-labelled probes were synthesized using Rediprime II DNA Labelling System (Amersham Pharmacia Biotech, Amersham, UK) according to the instructions of the manufacturer. Restriction enzymes were obtained from Invitrogen (Bleiswijk, The Netherlands), New England Biolabs (Ipswich, MA) and Fermentas Life Science (Leon-Rot, Germany) and used

according to instructions of the manufacturer. Ligation of DNA fragments was performed using the Rapid DNA ligation Kit (Fermentas Life Science, Leon-Rot, Germany). When required, fragments were dephosphorylated using Shrimp Alkaline Phosphatase (Fermentas Life Science, Leon-Rot, Germany). Sequencing was performed by Service XS (Leiden, The Netherlands).

Construction of deletion plasmids and complementation fragments

For the construction of the *hemF* and *hemH* deletion plasmids, N402 genomic DNA was used as a template. The *Aspergillus oryzae pyrG,* derived from pAO4-13 (de Ruiter-Jacobs et al. 1989), was used as a selection marker. For the construction of p∆*hemF* the 5' flanking region was amplified as a 1.74 kb fragment introducing a *Sst*II restriction site at the 5'end and a *Eco*RI site at the 3'end using primers pHemF1Fw and pHemF2rev (Table 5.1). The 3'flanking region was amplified as a 1.42 kb fragment introducing a *Hin*dIII restriction site at the 5'end using pHemF3Fw and pHemF6rev (Table 5.1). The 5'flanking region was cloned into pBluescript SKII (Stratagene, San Diego, CA) as a *Sst*II- *Eco*RI fragment. The 3'flanking region was cloned into pBluescript SKII (Stratagene, San Diego, CA) as a *Hin*dIII-*Xho*I fragment, using the original *Xho*I fragment present in the amplified DNA. For each flanking region two correct clones were analysed by sequencing. Next, the 3'flanking region was cloned into pBluescript SKII-5'flanking region as *Hin*dIII-*Xho*I followed by the insertion of *AopyrG* (de Ruiter-Jacobs et al. 1989) in between the flanking regions as a *Eco*RI-*Hin*dIII fragment to yield the plasmid p∆*hemF*. The plasmid was linearized prior to transformation using *Xho*I.

The construction of p∆*hemH* was performed similar to the cloning of the p∆*hemF* with the following modifications: the 5' flanking region was amplified as a 1.41 kb fragment using primers pHemH1Fw and pHemH2rev (Table 5.1). A *Kpn*I restriction site was introduced at the 3' *hemH* flanking region instead of an *Xho*I and a 1.24 kb fragment was amplified using pHemH3Fw and pHemH4rev (Table 5.1). The plasmid was linearized prior to transformation using *Bsp*HI.

Transformation of A. niger

Transformations were performed according to Meyer et al. (2010) with the following modifications. 100 ml cultures inoculated with $1{\cdot}10^8$ conidia were grown at 30 °C at 250 rpm for 12-16 h. 200 mg lysing enzymes (Sigma-Aldrich, Zwijndrecht, The Netherlands) were dissolved in 10 ml SMC, (consisting of 1.33M sorbitol, 20 mM MES-buffer and 50 mM $CaCl_2$) and set to pH 5.6. Protoplastating was performed at 37 °C and 120 rpm and was verified every 30 minutes by microscopy. 100 µl of protoplasts were mixed with 10 µl of

DNA solution (5 to 10 µg), and 25 µl of freshly made polyethylene glycol 6000 (PEG) buffer. Top-agarose was added to a volume of 40 ml, mixed and poured onto two 15 cm selective transformation plates.

Table 5.1 Primers used in this study. Introduced restriction sites are underlined.

primer	sequence (5' to 3')
hemF1Fw	TATCCGCGGCCAGCGCCTAGCAGTCACCA
hemF2rev	CACAGAATTCTGTTGGAGTAAATTGAGTTG
hemF3Fw	CCCAAGCTTGAATTAGAGAGATCATCAGC
hemF6rev	TGCTATGCGCAGACTGTACA
hemF7Fw	ATAAAATGCGGCCGCATGGCTGTTCCCCGACCATATATGCC
hemF8rev	GGAATTCCATATGGGCGCGCCTTACACCCATTGTCTGG
hemH1Fw	TATCCGCGGTCCCGTCCCCGGTCGCAGTC
hemH2rev	GACAGGTAATTGTACTGAAT
hemH3Fw	CCCAAGCTTTGTCAGGGGGTTTGTCGTTT
hemH4rev	GGGGTACCTGGATCCTGGGTCGCTTTTC
hemH 6 Fw	AGAATGCGGCCGCATGGCTCTCCG
hemH 7 rev	ATTCCATATGGGCGCGCCCTACCAAAGATGCTCCCTCTT
BN041	CGGGGTACCGTGCAATCCAGGACCTGGACACAAA
pAO9	AATGTCAATTCCAGCAGCG
pAO10	TTCGCGAGACTGAATGCGG
NC39	AAAGCGGTCGACGGTTGATCTTGTCCAGCAGC
NC49	AAATGAGTGCGACGCGGA
pGlaA FW 1	TGCTCTAGAACAGGAGCCTCGCAATCGT
pGlaA Rev 2	ATAAGAATGCGGCCGCTGCTGAGGTGTAATGATGCT
tTrpC FW 1	GGAATTCCATATGGGCGCGCCTCGTTGGTGTCGATGTCAGC
tTrpC Rev 2	CACAGAATTCCCTGTGCATTCTGGGTAAACG

Isolation of A. niger coproporphyrinogen III oxidase and ferrochelatase mutants

Deletion strains of *hemF* and *hemH* were constructed in *A. niger* by transforming the linearized disruption vectors p∆*hemF* and p∆*hemH* into strain AB4.1 and plated on selective media supplemented with 100 mg l^{-1} hemin. Obtained colonies were analyzed for porphyrin accumulation by detection of auto-fluorescence after mycelia transfer to MM. Colonies (heterokaryons) displaying red auto-fluorescence under uv-light were selected for further studies and maintained on hemoglobin supplemented media by mycelium transfer. Selection of homokaryons was eventually achieved by increasing hemin concentration up to 1 g l^{-1} and addition of 1% (w/v) Tween 80 (Merck, Darmstadt, Germany) improving visible colony formation from 31 to 7 days of incubation. Due to the absence of spore formation on hemin supplemented media, mycelia of pinkish colored colonies was transferred to hemoglobin supplemented media. The obtained

transformants were analyzed by Southern blotting to confirm correct integration of the disruption cassette in the genome (data not shown).

Complementation of hemF and *hemH deletion strains*

For complementation of the deletion strains 4 - 4.5 kb PCR products were obtained using the outer primers (Table 5.1) of the deletion constructs. Complementation of the deletion strain was performed by transforming respective PCR fragment, using either the *hemF* or *hemH* gene as the selection marker. Cultures were pre-grown in CM containing 10 mM NH_4Cl and 0.5 g l^{-1} hemin at 30 °C and 80 rpm for 3 days. Complementation was verified by diagnostic PCR (Table 5.2 and Figure 5.S1 in the supplementary material) and full restoration of growth on MM.

Table 5.2 primer combinations and expected product size used for the verification of complementation of the deletion strains.

complementation Δ*hemF*				
Primer Fw	Primer rev	N402	Δ*hemF*	Complementation Δ*hemF*
hemF7Fw	hemF8rev	1.4 kb		1.4 kb
BN041	hemH8rev	4.1 kb		
hemF1Fw	hemF6rev	4.5 kb	6.0 kb	4.5 + 6.0 kb
hemF1fw	pAO9		1.9 kb	1.9 kb
BN041	pAO9		2.9 kb	2.9 kb
pAO10	hemF6rev		1.4 kb	1.4 kb
complementation Δ*hemH*				
Primer Fw	Primer rev	N402	Δ*hemH*	complementation Δ*hemH*
hemH 6 Fw	hemH 7 rev	1.4 kb		1.4 kb
NC49	pAO9		1.6 kb	1.6 kb
hemH1fw	hemH4 rev	4.0 kb	5.5 kb	4.0 + 5.5 kb
NC49	NC39	1.9 kb		
hemH1 fw	pAO9		1.5 kb	1.5 kb
pAO10	hemH4 rev		1.3 kb	1.3 kb

Construction of overexpression strains

The expression vector pFMM2.2 was constructed as follows: The glucoamylase promoter (P*glaA*) was amplified from N402 genomic DNA as a 2.1 kb fragment, introducing an *Xba*I restriction site at the 5'end and an *Not*I restriction site at de 3'end of the promoter prior to the start codon using the primers pGlaA Fw 1 and pGlaA rev 2 (Table 5.1). The *trpC*

terminator (T*trpC*) was amplified as a 0.66 kb fragment, introducing *Nde*I-*Asc*I restriction sites at the 5'end and an *Eco*RI site at the 3'end using the primers pTtrpC fw 1 and pTtrpC rev 2 (Table 5.1). T*trpC* was cloned into pUC21 (Vieira and Messing 1991) as a *Nde*I-*Eco*RI fragment and P*glaA* was cloned into pUC21 as a *Xba*I-*Not*I fragment. After verification by restriction analysis, the inserts of two clones for P*glaA* and four clones for T*trpC* were verified by sequence analysis (Service XS; Leiden, The Netherlands). Next, P*glaA* was cloned into pUC21-T*trpC* as *Xba*I- *Not*I, resulting in the plasmid pFMM2.1. The selection marker *amdS* from p3SR2 (Corrick et al. 1987) was inserted into pFMM2.1 as an *Xba*I fragment, resulting in the plasmid pFMM2.2.

The heme biosynthesis genes selected for over expression (*hemA, hemF* and *hemH*) were isolated by PCR amplification of 4 - 5 kb around the identified gene locus (Table 5.1). The coding region subsequently was amplified with the insertion of *Not*I restriction site prior to the ATG and *Asc*I-*Nde*I directly after the stop codon (Table 5.1). The respective PCR fragments were cloned into pBluescript SKII (Stratagene, San Diego, CA) and verified by restriction analysis. Clones that displayed a correct restriction pattern were sequenced (Service XS, Leiden, The Netherlands) and subsequently cloned into pFMM2.2 as *Not*I-*Asc*I fragments, followed by a second round of restriction analysis verification.

Heme uptake and phenotypic analysis

The *hemF* and *hemH* deletion strains were phenotypically analyzed by a plate assay. Fresh conidia ($5 \cdot 10^3$) were point inoculated on MM and CM containing hemin or hemoglobin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Hemin containing media was additionally supplemented with 1% (w/v) Tween 80. All media compositions were also tested using 10 mM NH_4Cl as nitrogen source. The inability of the *hemF* and *hemH* deletion strains to germinate on hemoglobin containing plates was tested by adding the culture supernatant of N402 cultured for 12 h or 48 h in MM(NH_4) in a 1:1 ratio to 2x MM plus hemoglobin.

Strains overexpressing *hemA, hemF* or *hemH* were phenotypically analyzed for altered sensitivity towards hydrogen peroxide by inoculating 10-fold dilutions of conidia (starting with ~$5 \cdot 10^3$ conidia) on MM containing hydrogen peroxide (0.2 μM - 0.6 μM). Sensitivity towards voriconazole was analyzed by E-test strips (containing a gradient in voriconazole, AB Biodisk, Solna, Sweden.) on RPMI-1640 (Sigma-Aldrich, Zwijndrecht, The Netherlands) plate containing 165 mM MOPS inoculated with a lawn of conidia and incubated at 30 °C . Plates were examined at 24 and 45 hours of growth.

Porphyrin analysis

Accumulation of porphyrins was determined by microscopy of four day old cultures of the Δ*hemF* and Δ*hemH* grown in CM containing NH_4Cl as the nitrogen source and 0.5 mg l^{-1} hemin at 30 °C and 80 rpm. Zeiss Axioplan 2 imaging (Zeiss, Jena, Germany) was used for microscopy using the Zeiss Axiocam MRC5 camera (Zeiss, Jena, Germany) to capture images. Images were processed using ImageJ (NIH image Bethesda) and Adobe Photoshop (Adobe, San Jose, CA). Following microscopic analysis, the accumulating porphyrins in the deletion strains were verified from three day old cultures by HPLC with UV and fluorescence detection and normalized to the sample protein content. Porphyrin quantifications in heme overexpression strains were performed on biological duplicates of 24 h and 48 h old cultures grown in MM containing 5% maltose. Briefly, strains were cultivated in darkness and freeze dried before analysis. Proteins were extracted from 40 mg ground mycelium in 0.8 ml phosphate buffered saline (Sambrook et al. 1989). All non-covalently bound heme and its intermediates were extracted from freeze dried cultures by using an equal volume of aceton/HCl (97.5:2.5 (v/v)). The mixture was vortexed and subsequently centrifuged at 12000g at 4 °C for 5 minutes. Supernatant was subsequently loaded on a RP-18 250mm Li-ChroCART® 250-4 column (Merck, Darmstadt, Germany) at 35 °C and eluted using 25 mM $(NH_4)_2HPO_4$ (pH 3.5), H_2O:MeOH (1:1) and MeOH gradient according to the method of Bonkovsky et al. (1986). PPIX (Frontier scientific, Logan, Utah) and porphyrin acid chromatographic marker kit (Frontier scientific, Logan, Utah) were used as standards.

Northern analysis

Northern analysis was performed on the additionally harvested samples from heme quantification cultures. Strains overexpressing *hemA, hemF* and *hemH* were cultured as biological duplicates in MM containing 5% (W/V) maltose for 24 h or 48 h following RNA extraction. Isolation of RNA from was performed using TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands). 10 μg of total RNA was denatured by incubating at 50 °C with 2.3 μl 6 M glyoxal, 10 μl dimethyl sulfoxide (DMSO) and 2 μl 0.1 M sodium phosphate buffer (pH 7.0) for 1 hour. RNA electrophoreses was performed at 10 °C using a SEA-2000 (Elchrom Scientific, Cham, Switzerland). Northern blotting was performed according to Sambrook and Russell (2001). α-^{32}P-dCTP-labelled probes were synthesized using Rediprime II DNA Labelling System (Amersham Pharmacia Biotech, Amersham, UK) according to the instructions of the manufacturer. Expression levels of *hemA, hemB, hemF, hemH* and *met1* were analyzed using probes described in Franken et al. (2012).

Equal amounts of RNA (10 μg) were loaded and expression levels of *actin* were used to correct for loading differences by density using Quantity one (Bio-Rad, Hemel Hempstead, United Kingdom).

Results

A. niger coproporphyrinogen III oxidase and ferrochelatase mutants

In earlier in silico studies (Franken et al. 2011) we have identified all putative heme biosynthesis genes in the genome of *A. niger*. Coproporphyrinogen III oxidase (CPO) and ferrochelatase (FC) represent the sixth and eighth (final) enzyme within the heme biosynthetic pathway and are encoded by *hemF* (An07g10040) and *hemH* (An15g02690) respectively. Deletion strains of *hemF* (CPO) and *hemH* (FC) were constructed in *A. niger* and isolated as described in 'Materials and Methods'. The resulting transformants were expected to be disrupted at different stages within the heme biosynthetic pathway and it was therefore anticipated that these strains would accumulate porphyrins. Porphyrins are brightly colored (purple/violet) and display red-fluorescence when exposed to ultraviolet light (Hamza 2006; James and Hift 2000; Moore 2009; Polo et al. 1988). The auto-fluorescent feature is a highly sensitive property. Therefore the obtained transformant colonies were analyzed for this feature. Colonies displaying red auto-fluorescence under uv-light were selected for further isolation and Southern blotting to confirm correct integration of the disruption cassette in the genome (data not shown). BR10#7 (Δ*hemF*) and BR9#25 (Δ*hemH*) were selected for further analyses. Complementation with the respective PCR fragment restored growth and all phenotypic defects of both deletion strains (data not shown), and was verified by diagnostic PCR (see Figure 5.S1 in the Supplementary Material and Table 5.2).

Deletion of *hemF* (CPO) or *hemH* (FC) is conditionally lethal (Figure 5.2a) and despite supplementation of hemin, Δ*hemF* and Δ*hemH* strains were still extremely impaired in growth (Figure 5.2b-d). However *hemF* and *hemH* deletion strains were able to grow, albeit slow, on nitrate based media, contrary to the previously analyzed *hemA* (ALAS) deletion strain (Franken et al. 2012). The *hemA* deletion strain was impaired in synthesis of uroporphyrinogen III, the common precursor for heme and siroheme synthesis. Both heme and siroheme are required to be able to utilize nitrate as N-source. Deletion strains of *hemF* (CPO) and *hemH* (FC) would be defective after uroporphyrinogen III synthesis and therefore only heme biosynthesis was affected. Similar to Δ*hemA*, growth Δ*hemF* and

Δ*hemH* was improved by the use of NH_4Cl as N-source (Figure 5.2d), CM, and/or additional cysteine supplementation (data not shown). However, also subtle differences were present between the *hemF* and *hemH* deletion strains. Although growth on CM instead of MM was improved for both strains (Figure 5.2c), growth of the Δ*hemF* was better compared to the Δ*hemH* strain. Also the use of Tween 80 led to a increased growth for Δ*hemF* on hemin containing MM but not on CM (Figure 5.2b).

We further examined the ability to utilize hemoglobin for the deletion mutants to clarify the results obtained during the initial isolation of the deletion strains (see materials and methods). No growth was observed when spores were inoculated on hemoglobin supplemented media over a period of 30 days. However, supplementing 12 h or 48 h old *A. niger* N402 spent culture medium (materials and methods) resulted in germination and continued growth for all three heme deficient mutants (Figure 5.3). These results indicate that spores are not capable of hemoglobin uptake and may not contain sufficient proteolytic activity to degrade the hemoglobin molecule in time to be utilized for germination.

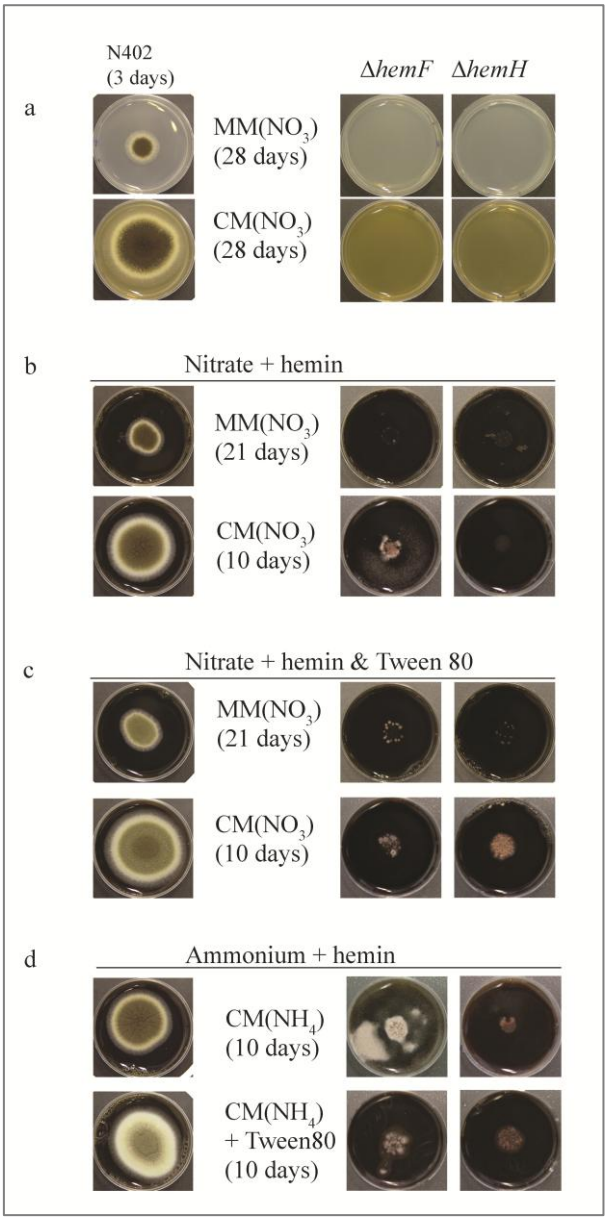

Figure 5.2 Phenotypic comparison of wild-type N402 strain with growth analysis of Δ*hemF* and Δ*hemH* strains. $5 \cdot 10^3$ conidia are spotted and plates are incubated at 30 °C for the indicated times; N402 was incubated for three days. **a** nitrate based MM and CM without additional supplementation, **b** supplementation of 0.5 g l^{-1} hemin to nitrate based MM and CM, **c** additional supplementation of 1 % (w/v) Tween 80 to nitrate based MM and CM containing 0.5 g l^{-1} hemin, **d** replacement of nitrate by ammonium (10 mM NH_4Cl) in CM media containing 0.5 g l^{-1} hemin.

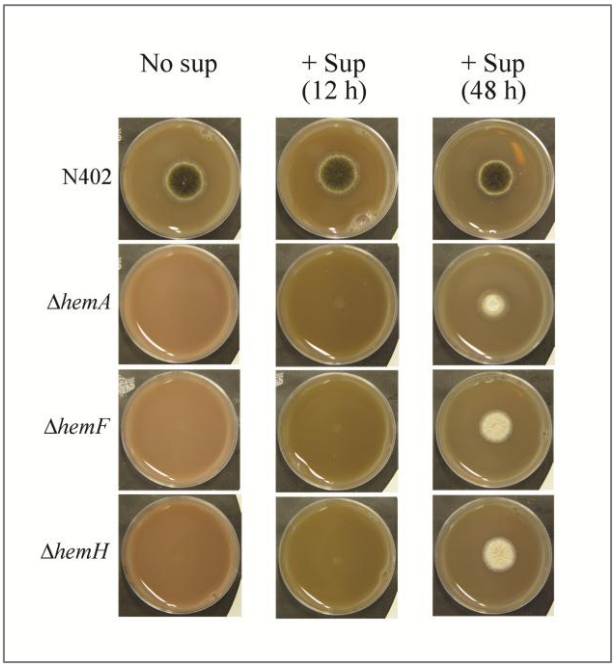

Figure 5.3 Hemoglobin utilization was impaired in Δ*hemA,* Δ*hemF* and Δ*hemH* mutant strains. Strains were inoculated onto MM(NH_4) plates containing 5 g l^{-1} hemoglobin in the presence or absence of N402 culture supernatant (sup) incorporated into the agar. Plates were incubated at 30 °C for three days.

Porphyrin analysis

When grown on hemin or hemoglobin supplemented solid media, both the Δ*hemF* and Δ*hemH* strains displayed hyphal coloration which is likely caused by porphyrin accumulation but with marked differences; Δ*hemF* was pink, while Δ*hemH* was more pink/orange (Figure 5.4). The deletion strains should accumulate different porphyrins and hence the different coloration was likely to be specific for the accumulating porphyrins. Whereas a slight auto-fluorescence was already observed in the obtained heterokaryons (see materials and methods), the purified homokaryon strains cultured in CM(NH_4) containing 0.5 g l^{-1} hemin for four days showed red auto-fluorescence further indicating porphyrin accumulation (Figure 5.5). HPLC analysis of the deletion strains confirmed the accumulation of predominantly coproporphyrinogen III and PPIX in Δ*hemF* (CPO) and Δ*hemH* (FC) strains, respectively (data not shown).

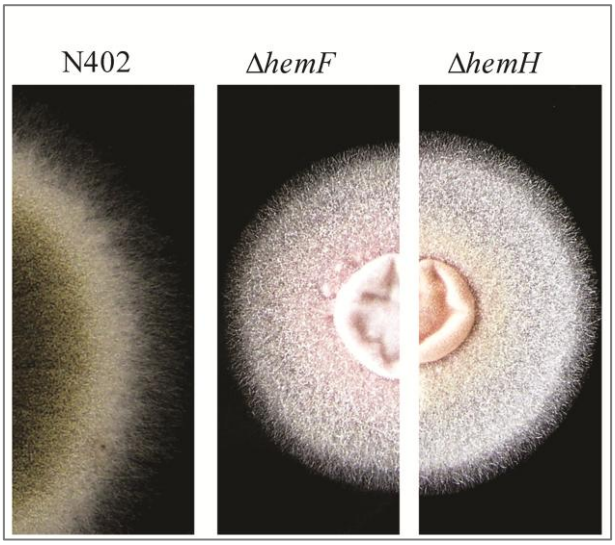

Figure 5.4 *hemF* and *hemH* deletion strains display specific pigmented phenotypes due to coproporphyrinogen III and protoporphyrin IX accumulation respectively**.** Strains were inoculated onto CM plates containing 0.5 g l^{-1} hemin. Plates were incubated at 30 °C for three days.

Overexpression of heme pathway genes *hemA, hemF, hemH*

Overexpression of *hemF* (CPO)*, hemH* (FC)*,* and the previously analyzed *hemA* (ALAS) were studied to analyze their effects on heme biosynthesis and on expression of heme biosynthetic genes. Overexpression was achieved by cloning of the respective genes in the expression vector pFMM2.2 which contained the strong and inducible glucoamylase promoter, followed by transformation into N402. Transformants were purified twice on MM containing acetamide and multicopy integration was confirmed by Southern blot analysis (data not shown). Three *hemA* multicopy strains (BR15#1, BR15#4 and BR15#5), varying in copy-number integration (containing 3, 2 and 1 additional copies respectively), were selected for further studies. One multicopy strain was selected for *hemF* (BR19#3; 3 additional copies), as well as for *hemH* (BR21#1; 1 additional copy).

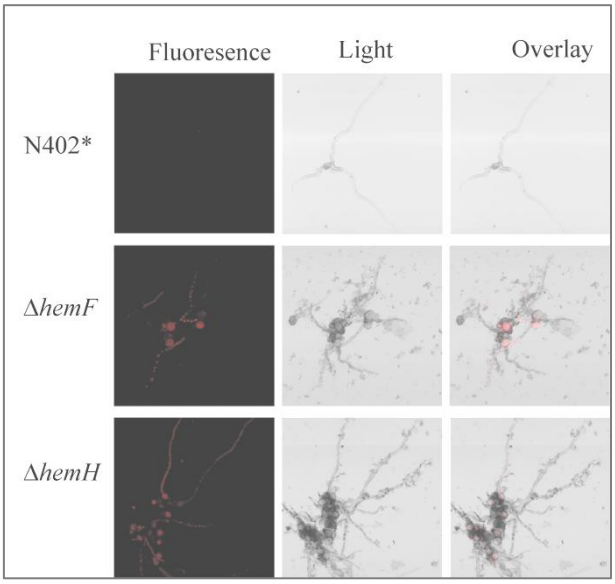

Figure 5.5 Auto-fluorescence of *hemF* and *hemH* deletion strains. Deletion strains in the final part of the heme biosynthetic pathway accumulate porphyrins. Characteristic for porphyrin accumulation is the presence of red auto-fluorescence. Both the *hemF* and *hemH* deletion strains display localized red auto-fluorescence after 4 days of incubation at 30 °C in CM(NH_4) containing 0.5 g l^{-1} hemin. N402 does not accumulate porphyrins and therefore does not display auto-fluorescence (O/N incubation at 30 °C).

The overexpression strains were subsequently cultured on MM containing 5% maltose, to induce expression, for 24 h or 48 h. Northern analysis was used to analyze mRNA levels of the over-expressed genes and verified overexpression of the respective gene in the selected transformants (Figure 5.6).

Heme and heme intermediates were identified and quantified by HPLC. After 48 h strains overexpressing *hemA* (ALAS) displayed elevated levels of uroporphyrinogen III and coproporphyrinogen III but no increase was observed for PPIX and heme (Figure 5.7). Overexpressing *hemF* (CPO) generated a depletion in uroporphyrinogen III and coproporphyrinogen III, but no difference was observed in PPIX and heme concentrations. Strikingly, only overexpression of *hemH* resulted in a modest increase in heme content, together with a slight increase in PPIX. However, also in this strain, uroporphyrinogen III and coproporphyrinogen III levels were reduced at this time point.

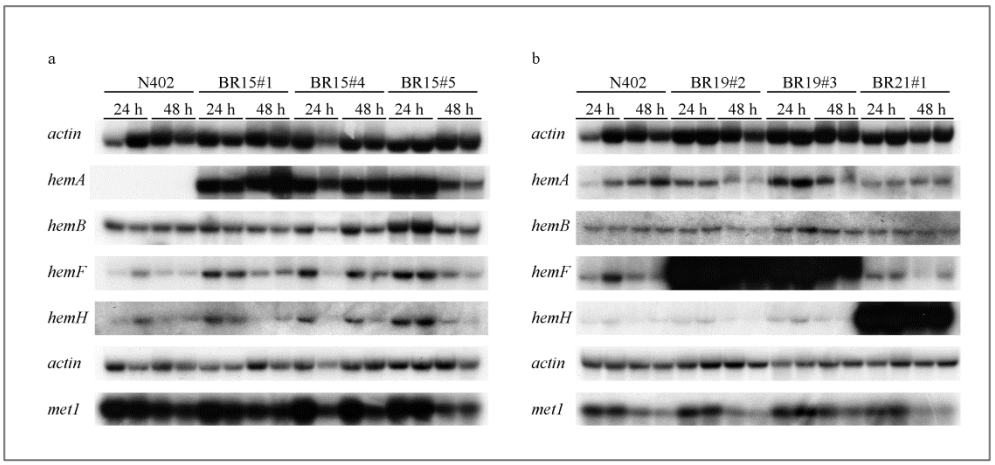

Figure 5.6 Northern analysis of *hemA, hemF* and *hemH* overexpressing strains verified overexpression of the respective genes. **a** BR15 strains; overexpressing *hemA*, **b** BR19 and BR21 strains overexpressing *hemF and hemH* respectively. Strains were cultured in duplo in MM containing 5% (w/v) Maltose for 24 h or 48 h following RNA extraction. Expression levels of *hemA, hemB, hemF, hemH* and *met1* were analyzed. Equal amounts of RNA (10 μg) were loaded and expression levels of *actin* were used to correct for loading differences.

Northern analyses (Figure 5.6) demonstrated that the accumulation or depletion of porphyrins in the overexpressing strains was not the result of a change in gene expression of native heme biosynthesis genes as no significant change in expression levels were observed after quantification. This would indicate that an increase in heme biosynthesis does not require upregulation of other genes in the pathway and/or that heme biosynthesis is not directly downregulated by transcriptional regulation upon a forced change in heme or heme intermediates.

To analyze whether the observed effects on heme (intermediate) levels resulted in increased availability of heme for heme dependent proteins, we analyzed sensitivity towards hydrogen peroxide, as a measure for increased endogenous heme-peroxidase activity. In addition voriconazole resistance was analyzed, as a measurement for heme dependent cytochrome P450 enzyme activity. In both cases no significant change was observed for the overexpression strains (data not shown). These results indicated no significantly increase in heme content was present in the strains overexpressing one of the heme biosynthesis genes to provide increased resistance, and thereby support the HPLC results.

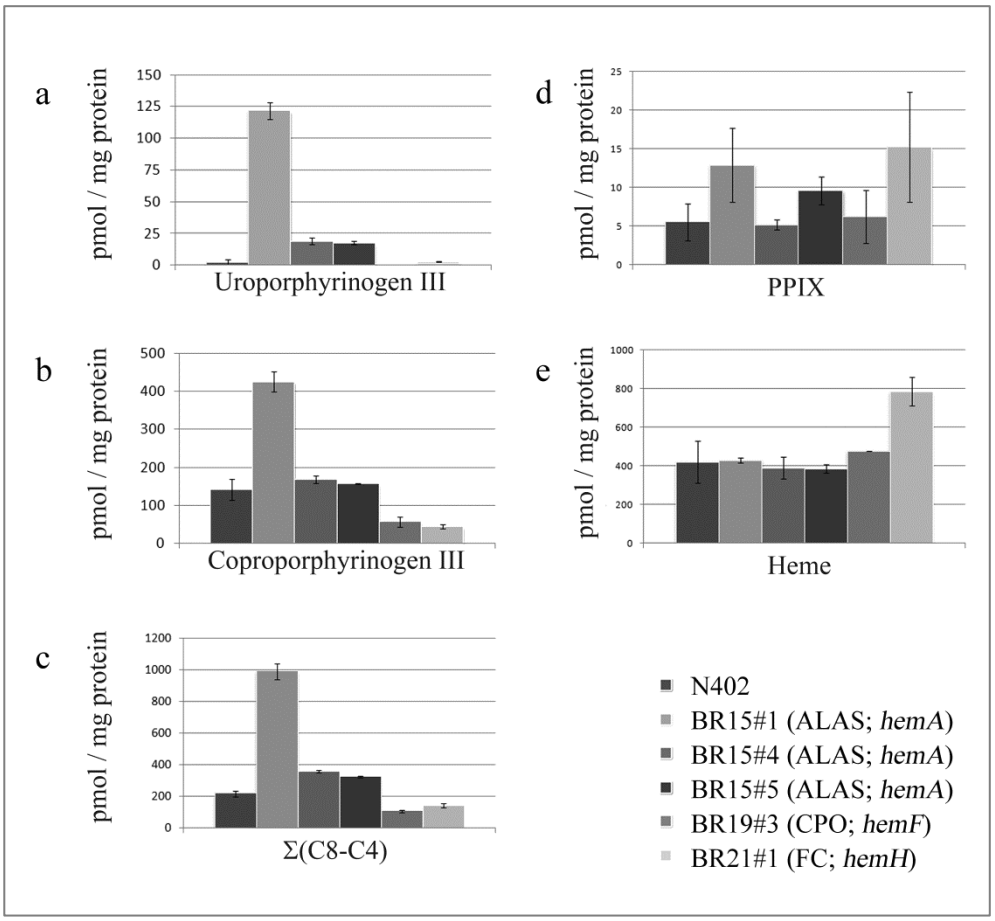

Figure 5.7 Measurement of heme and heme- intermediates in *hemA, hemF* and *hemH* over-expressing strains. Strains were cultured at 30 °C for 48 h in MM containing 5% maltose. Intermediates were extracted from freeze-dried mycelium and quantified by C-18 reverse phase chromatography according to the method of Bonkovsky et al. (1986). Values are given in pmol / mg protein. **a** concentration of uroporphyrinogen III, **b** concentration of coproporphyrinogen III, **c** sum of uroporphyrinogen III, coproporphyrinogen III and decarboxylation intermediates in the formation of coproporphyrinogen III (Σ (C8-C4), **d** concentration of PPIX (protoporphyrin IX) and **e** determined concentration of heme.

Discussion

We studied heme biosynthesis in *A. niger* as heme is a potential limiting factor in heterologous peroxidase production. Increasing its biosynthesis would provide an economically attractive and more sustainable solution compared to supplementation of a heme source during production. To achieve this goal, more knowledge on this tightly

regulated pathway is required. We analyzed the role of two heme biosynthetic steps downstream of the uroporphyrinogen III branchpoint. These steps have been suggested to be relevant for regulation of the pathway (Franken et al. 2012; Idnurm and Heitman 2010).

CPO and FC, were inactivated by deletion of the respective gene. Despite hemin supplementation during transformation, only heterokaryotic transformants were obtained. which displayed a faint auto-fluorescence under UV indicative for porphyrin accumulation. Increasing the hemin concentration and supplementation of Tween 80 improved growth (Figure 5.2b) resulted in colony formation of both *hemF* and *hemH* disruption strains after approximately seven days of growth. Tween 80 supplementation is described for *S. cerevisiae* heme mutants as a source of unsaturated fatty acids (Camadro and Labbe 1996). Moreover, Tween 80 also provides a more hydrophobic environment, improving the solubility of hemin in the media, and could alter the permeability of the cell wall. All of these possibilities improved hemin utilization on its own or in combinations.

Putative Δ*hemF* and Δ*hemH* colonies showed a pinkish coloration (Figure 5.4) . Similar to the auto-fluorescence (Polo et al. 1988) observed in the heterokaryons, this coloration is another indicator for porphyrin accumulation (Hamza 2006; Moore 2009). Correct deletion strains should be defective in heme biosynthesis at late steps within the pathway and therefore accumulate coproporphyrinogen III or PPIX, respectively, for Δ*hemF* and Δ*hemH* strains. Therefore, the different colors observed for both strains likely represents accumulation of the different porphyrins as was also confirmed by detailed HPLC-analysis of porphyrin content (Figure 5.4).

Although adjustments in medium composition improved growth of the deletion strains, Δ*hemF* and Δ*hemH* remained extremely impaired in growth (Figure 5.2) similar to what was observed for a *C. neoformans hem15* deletion strain (which corresponds to Δ*hemH*) (Idnurm and Heitman 2010). Furthermore, the colonies did not reach a sporulating state. Mycelium transfer to hemoglobin media significantly improved growth and sporulation, but no growth was observed when the media was inoculated with conidia. Previously, hemoglobin was found to alter the expression of heme and siroheme biosynthesis genes (Franken et al. 2012) and addition of hemoproteins contributed positively to peroxidase production (Conesa et al. 2000), thus suggesting uptake of heme via this approach. Our findings led to the hypothesis that the hemoglobin molecule needs to be degraded to liberate heme molecules before uptake can take place and that conidia do not possess sufficient proteolytic activity to release these heme molecules in time for its need during germination in these heme deficient mutants. Indeed, the addition of spent N402 culture

medium supports this hypothesis, as medium from a 12 h and older cultures facilitate germination and subsequent growth of all heme deficient strains (Figure 5.3).

A more detailed analysis of the two deletion mutants showed similar results as the previously described Δ*hemA* strain (Franken et al. 2012). The replacement of nitrate for ammonium in CM (Figure 5.2d) positively contributed to the growth of the two deletion stains as previously determined for *hemA* deletion strain (Franken et al. 2012). However, whereas the Δ*hemA* strain had an absolute requirement for ammonium based media, the *hemF* and *hemH* deletion strains were able to grow on nitrate based media when supplemented with a heme source (Figure 5.2b and 2c). These two deletion strains thereby confirm the ability of (intact) heme uptake, although uptake appears to be inefficient. However, given the essential nature of heme in multiple processes, the impaired growth could alternatively and/or additionally be caused by secondary effects. The *A. niger* genome contains putative heme transporters (Franken et al. 2011) which could help to clarify the role of heme uptake in healthy growing *Aspergillus* strains. However, in our study, improvement in heme uptake is less relevant as heme supplementation is not economically relevant for biotechnological processes. The *hemF* and *hemH* deletion strains, together with the earlier analyzed Δ*hemA* strain (Franken et al. 2012), also confirms that siroheme synthesis derives from the heme biosynthetic pathway and is involved in nitrite reductase in *A. niger*. The growth improvement by NH_4Cl observed in the Δ*hemF* and Δ*hemH* strains is caused by the relief of a requirement for nitrate reductase, which is a hemoprotein involved in nitrogen metabolism (Chang et al. 1996). Finally, like in the Δ*hemA* strain, cysteine addition also improved growth, possibly caused by the relief of a requirement for cystathionine-β-synthase which could be a hemoprotein in fungi as well as suggested earlier (Banerjee and Zou 2005; Franken et al. 2012). However, this would require further analysis beyond the topic of our research.

We subsequently analyzed strains in which overexpression of *hemA, hemF or hemH* was achieved under control of the inducible glucoamylase promoter and multicopy integration of the expression cassette. Only overexpression of the final gene in the pathway, *hemH,* actually resulted in a modest increase in heme content (Figure 5.7e), indicating a limiting role for FC for overproduction of heme. The slight increase PPIX in the same overexpression strain indicates that FC still poses a limiting factor for overproduction of heme, a role for FC that was mentioned earlier for *C. neoformans hem15* (Idnurm and Heitman 2010). Overexpression of *hemH* with the glucoamylase promoter is expected to give significantly increased FC protein levels, its expression being independent of heme and/or porphyrins. Therefore enzymatic downregulation of FC should be considered to

explaining the accumulation of PPIX in the *hemH* overexpressing strain. Another significant limitation was observed at the level of CPO. All *hemA* overexpressing strains displayed increased accumulation of uroporphyrinogen III and coproporphyrinogen III (including the intermediates in the formation of coproporphyrinogen III (Figure 5.7c) but not heme after 48 h of growth. This indicates a crucial role of CPO in controlling heme biosynthesis. As *hemF* (CPO) overexpressing strains do not synthesize additional ALA like the *hemA* overexpressing strains, it is not surprising that overexpression of *hemF* did not result in increased PPIX and heme levels after 48 h of growth. In fact, after 48 h, concentrations of uroporphyrinogen III and coproporphyrinogen III appeared to be decreased suggesting an increase in the flux of the pathway could be achieved. However, as no increased heme was measured, also regulatory mechanisms should be considered during future research.

In agreement with these results we were also unable to obtain evidence for increased availability of heme for endogenous heme proteins, such as peroxidase and cytochrome P450 enzymes (Guan et al. 2010; Wilks 2002) in the overexpression strains. Peroxidase mediated H_2O_2-sensitivity and cytochrome P450 mediated voriconazole sensitivity were unaltered in these strains.

These results described above demonstrate that elevating heme production is not a simple task. The complex regulation of several steps within the pathway on, likely, (post) translational levels prohibit major changes in heme content with crucial roles for FC and CPO. This potentially limiting effect could be (partially) overcome when working in a peroxidase overproducing strain as was done in *A. oryzae* (Elrod et al. 1997). In this situation the peroxidase would act as a "sink" for heme and thereby would relief the potential limitation of FC, allowing overproduction of the peroxidase by *hemA* overexpression. However, also in this study, accumulation of porphyrins (likely uroporphyrinogen III) was observed after overexpression of *hemA* and *hemB* (Elrod et al. 1997), indicating that also in this fungus UROD and/or CPO could act as a limitation for increased peroxidase production. A combined approach, using overexpression of multiple heme biosynthesis genes in a peroxidase producing strain, together with a more detailed understanding of regulatory mechanisms of the pathway was therefore likely required to achieve higher levels of heterologous peroxidase production in *A. niger.*

Acknowledgements

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