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Title: Heme biosynthesis and regulation in the filamentous fungus Aspergillus niger
Issue Date: 2013-12-17

Chapter 4

Analysis of the role of the A. niger aminolevulinic acid synthase (hemA) gene illustrates the difference between regulation of yeast and fungal heme and siroheme dependent pathways

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Published in *FEMS Microbiology Letters* **335**: 104-112.

Abstract

To increase knowledge on heme biosynthesis in filamentous fungi like *Aspergillus niger*, pathway-specific gene expression in response to heme and heme-intermediates was analyzed. This analysis showed that iron, 5'-aminolevulinic acid (ALA) and possibly heme control heme biosynthesis mostly via modulating expression of *hemA* (coding for 5'-aminolevulinic acid synthase (ALAS). A *hemA* deletion mutant (Δ*hemA*) was constructed which showed conditional lethality. Growth of Δ*hemA* was supported on standard nitrate-containing media with ALA, but not by hemin. Growth of Δ*hemA* could be sustained in the presence of hemin in combination with ammonium instead of nitrate as N-source. Our results suggest that a branch-off within the heme biosynthesis pathway required for siroheme synthesis, is responsible for lack of growth of Δ*hemA* in media containing nitrate as sole N-source, due to the requirement of siroheme for nitrate assimilation, as a cofactor of nitrite reductase. In contrast to the situation in *Saccharomyces cerevisiae,* cysteine, but not methionine, was found to further improve growth of Δ*hemA*. These results demonstrate that *A. niger* can use exogenous hemin for its cellular processes. They also illustrate important differences in regulation of heme biosynthesis and in the role of heme and siroheme in *A. niger* compared to *S. cerevisiae*.

Introduction

Heme is suggested to be a limiting factor in large scale production of fungal peroxidases, which require this compound as a co-factor (Andersen et al. 1992; Elrod et al. 1997). Addition of hemin, a Cl-ligand of heme, to culture medium improves this production (Andersen et al. 1992; Conesa et al. 2000; Elrod et al. 1997), but is not suited for industrial applications (Elrod et al. 1997). Also, the mechanisms by which hemin supplementation improves peroxidase production are still unknown. To achieve improved and cost-effective production of peroxidases by filamentous fungi, knowledge on heme synthesis and regulation is required as current knowledge is mainly restricted to the first two genes in the pathway (Bradshaw et al. 1993; Elrod et al. 1997; Elrod et al. 2000).

Heme is an essential molecule for almost every organism due to its requirement as a cofactor of proteins involved in many primary functions like cellular differentiation and gene regulation (Elrod et al. 1997; Ferreira et al. 1993; Hamza 2006; Panek and O'Brian 2002). Its biosynthesis in fungi has been extensively studied in *Saccharomyces cerevisiae* with mutants available for every step within the pathway (Amillet and Labbe-Bois 1995; Camadro and Labbe 1996; Gollub et al. 1977; Hoffman et al. 2003; Keng et al. 1992; Kurlandzka et al. 1988; Labbe-Bois 1990; Myers et al. 1987; Urban-Grimal and Labbe-Bois 1981; Zagorec et al. 1988). These mutants can be sustained by supplementing hemin to

their growth media or by ergosterol or Tween80 addition to supply for essential unsaturated fatty acids (Gollub et al. 1977). In aerobically grown heme mutants fatty acid synthesis is impaired due to involvement of hemoproteins at several stages in the pathway (Lorenz and Parks 1987). Furthermore, strains carrying mutations in the genes required for the formation of Uroporphyrinogen III (UroIII) contain an additional auxotrophy for methionine due to the lack of siroheme formation (Gollub et al. 1977). Siroheme is a heme-like prosthetic group required for sulfite and nitrite reductases which are essential for assimilation of sulfur and nitrogen into all life forms (Crane and Getzoff 1996; Raux et al. 2003; Schubert et al. 2002a). The highly conserved heme biosynthesis pathway is also responsible for the synthesis of Vitamin B12, chlorophyll, factor F_{430} and siroheme (Warren and Scott 1990) but only siroheme is also synthesized in fungi (Crane and Getzoff 1996; Murphy and Siegel 1973; Raux et al. 1999). As *S. cerevisiae* is unable to assimilate nitrate, lack of the siroheme pathway has no additional effects on N-metabolism.

Unlike the yeast heme-deficient mutants, growth of an 5'-aminolevulinic acid synthase (ALAS) deficient strain of *Aspergillus oryzae* (Δ*hemA*) could not be restored by hemin supplementation (Elrod et al. 2000). Also, additional supplementation with ergosterol and/or vitamin B12 was also unsuccessful and additional methionine supplementation was not examined. Therefore it remains unclear whether *Aspergillus* spp. are unable to use the external heme sources or that other metabolic processes are disturbed.

In order to generate more insight into the heme biosynthesis pathway and its regulation in filamentous fungi, pathway specific gene expression was studied and an *A. niger* Δ*hemA* mutant strain was analysed. Our results demonstrate *A. niger* is capable of heme uptake from its environment and suggest a role of the siroheme biosynthesis pathway in growth defects observed in strains deficient in the heme biosynthesis pathway. Northern analysis furthermore suggests a limiting role for *hemA* and a regulatory mechanism to direct early intermediates either to heme or siroheme synthesis.

Materials and methods

Strains and culture conditions

A. niger N402 (*cspA1* derivative of ATCC9029 (Bos et al. 1988)) and its *pyrG*$^-$ derivative, AB4.1 (van Hartingsveldt et al. 1987), were used during this study. Strains were grown on minimal medium (MM) (Bennet and Lasure 1991) or complete medium (CM) consisting of MM with yeast extract (10 g l^{-1}) and casamino-acids (5 g l^{-1}) containing sodium-nitrate (70 mM) as nitrogen source. Wherever indicated nitrate may be omitted or replaced by

ammonium chloride (10 mM). Growth medium was supplemented with 10 mM uridine when required. *Escherichia coli* DH5α was used for amplification of recombinant DNA as previously described (Inoue et al. 1990). *A. niger* transformations were performed according to Meyer *et al.* (2010). 50 μM ALA (Sigma-Aldrich) was supplemented in transformation experiments to generate *hemA* deletion mutants (Δ*hemA).*

Molecular Biological Techniques

Chromosomal DNA of *A. niger* was isolated as described by Kolar *et al.* (1988). Southern and Northern analyses were performed as previously described (Sambrook and Russell 2001). α-^{32}P-dCTP-labelled probes were synthesized using Rediprime II DNA Labelling System (Amersham Pharmacia Biotech) according to instructions of the manufacturer. Restriction enzymes were obtained from Invitrogen, New England Biolabs and Fermentas and used according to the instructions supplied by manufacturers. Ligation of DNA fragments was performed using the Rapid DNA ligation Kit (Fermentas). When required, fragments were dephosphorylated using Shrimp Alkaline Phosphatase (Fermentas). Sequencing was performed by Service XS (Leiden, the Netherlands).

Construction of deletion plasmids and complementation fragment

The pΔ*hemA* plasmid was constructed as follows: N402 genomic DNA was used as template for amplification of flanking regions. The 5'-flank of the *hemA* gene was amplified as a 1.52 kb fragment introducing a *Xba*I site at the 3'end using primers pHemA1Fw (5'-GGCGAGGGTAATTTCGATGA) and pHemA2rev (5'-tgctctagaAATGAGCGGGCAGACAATTC). The 3'flank of the *hemA* gene was amplified as a 1.56 kb fragment using pHemA3Fw (5'-GGCCAGTCGTTACCGATGA) and pHemA4rev (5'-TCCATTGTTTCACTTGGGCA). The PCR products were cloned into pBluescript SKII (Stratagene) as a *Sst*II-*Xba*I fragment *Xba*I–*Hin*dIII fragment for the 5'- and 3'-flanking region using the introduced *Xba*I restriction site and original restriction sites present in the amplified fragment. Correct clones were verified by sequencing. Next, the 3'-flank was inserted into the clone containing the 5'-flanking region as *Xba*I–*Hin*dIII. The *A. oryzae pyrG,* derived from pAO4-13 (de Ruiter-Jacobs et al. 1989), was used as selection marker and inserted between the flanking regions as a *Xba*I fragment to yield plasmid pΔ*hemA*. The plasmid was linearized prior to transformation using *Sst*II.

Complementation of Δ*hemA* was achieved by transformation of a 5 kb PCR product obtained by using pHemA1fw and pHemA4rev, using the *hemA* gene itself as selection marker. Cultures were pre-grown in CM containing 200 μM ALA. Complementation was verified by diagnostic PCR and full restoration of growth on MM.

Heme uptake and phenotypic analysis

The *hemA* deletion strain was phenotypically analyzed for growth of fresh conidia in 10-fold dilutions or point inoculation with $5 \cdot 10^3$ conidia on MM- and CM-plates containing hemin (Sigma-Aldrich). Hemin (0.5 g l^{-1}) containing media was additionally supplemented with ALA or 100 mg l^{-1} L-Methionine (Sigma-Aldrich). A methionine deficient *A. niger* strain (A897), kindly provided by Patricia VanKuyk, was used as a control strain.

Competition for ALA and hemin uptake by specific amino-acids was analysed on MM-plates using nitrate, ammonium or no specific nitrogen source, supplemented with selected amino-acids (L-methionine, glycine, glutamate, cysteine, asparagine, arginine or alanine (Sigma-Aldrich; 10 mM)). ALA growth tests were performed in CM(NO_3) supplemented by 100 μM ALA and in media that lack casamino-acids or the N-source.

Hemin growth tests were performed in CM(NH_4) media supplemented by 0.5 g l^{-1} hemin and in media that lack casamino-acids or the N-source.

Northern analysis

Northern analyses were performed on RNA samples isolated from *A. niger* N402 after 24 hours of growth on MM or MM without Iron (iron omitted from trace elements) followed by the addition of selected compounds (Table 4.1). Cultures were harvested 30 min after addition of the compound and RNA was extracted using TRIzol reagent (Invitrogen).

Expression levels of *hemA, hemB, hemF, hemH, and met1* (Table 4.2) were examined and *actin* was used as loading control.

Table 4.1: supplementation compounds in Northern analysis. Hb: Hemoglobin; ALA: 5'-aminolevulinic acid: Fe = $FeSO_4 * 7H_2O$; BSA: Bovine Serum Albumin; PPIX: Protoporphyrin IX.

condition	volume	Final concentration	Solvent
Time 0			
MQ	2,5 ml		
Hb	2,5 ml	7,75 μM (0.5 g l^{-1})	MQ
ALA	2,5 ml	250 μM	MQ
Fe	2,5 ml	1mg l^{-1} (3,6 μM)	MQ
10xFe	2,5 ml	10 mg l^{-1} (36 μM)	MQ
BSA	2,5 ml	0.5 g l^{-1}	MQ
DMSO	100 μl	0.2 %	
PPIX	100 μl	31 μM	DMSO
Hemin	100 μl	31 μM	DMSO

Table 4.2: primers used for the construction of probes. Introduced restriction sites are underlined

	primer name	sequence (5' to 3')	product size (kb)
hemA: An17g01480	hemA1fw	GGCGAGGGTAATTTCGATGA	5.0
	hemA4rev	TCCATTGTTTCACTTGGGCA	
	hemA5Fw	ATAAGAATGCGGCCGCATGGAGTCGCTTCTCCAGCA	2.1
	hemA8rev	GGAATTCGGCGCGCCTTAAGCAGCAGCCACTCCCACGG	
hemA probe: 813 bp *Xho*I fragment			
hemB: An08g00010	HemB5Fw	ATAAGAATGCGGCCGCATGTCGTTTTCAAACCTTGT	1.1
	hemB6rev	GGAATTCCATATGGGCGCGCCTTAAGAAAGCCAATCCAGAA	
hemB probe: 820 bp *Eco*RV fragment			
hemF: An07g10040	hemF1Fw	TATCCGCGGCCAGCGCCTAGCAGTCACCA	4.5
	hemF6rev	TGCTATGCGCAGACTGTACA	
	hemF7Fw	ATAAAATGCGGCCGCATGGCTGTTCCCCGACCATATATGCC	1.4
	hemF8rev	GGAATTCCATATGGCGCGCCTTACACCCATTGTCTGG	
hemF probe: 1.4 kb *Not*I-*Asc*I fragment			
hemH: An15g02690	hemH1Fw	TATCCGCGGTCCCGTCCCCGGTCGCAGTC	4.0
	hemH4rev	GGGGTACCTGGATCCTGGGTCGCTTTTC	
	hemH 6 Fw	AGAATGCGGCCGCATGGCTCTCCG	1.4
	hemH 7 rev	ATTCCATATGGGCGCGCCCTACCAAAGATGCTCCCTCTT	
hemH probe: 1.4 kb *Not*I-*Asc*I fragment			
met1: An11g09700	Met1 1Fw	ATAAGAATGCGGCCGCCTCGGGGATCAGCTTGTCAG	4.9
	Met1 4rev	GGGGTACCGATGAACATTCCAGCTGTGTAC	
Met1 probe: 759 *bgl*I fragment			
Actin	PactinP1	ATCTCCCGTGTCGACATGG	3.7
	actin_dw	GCGGTGGACGATCGAGG	

Results

Analysis of heme pathway related gene expression

Recently all potential *A. niger* heme and siroheme biosynthesis genes were identified (Franken et al. 2011). Northern analysis on several heme- and siroheme genes was carried out on mRNA samples isolated from cultures grown under different conditions, in response to supplementation with heme sources, various heme-intermediates and iron as metal-ligand of heme (Figure 4.1). Under standard iron conditions, only the expression of *hemA* was found to be responsive to addition of iron containing supplements. With the exception of ALA, all conditions appear to result in a small upregulation of *hemA* under standard iron conditions and would suggest a positive regulation by iron and possibly heme. However, the changes in expression are very limited compared to the levels obtained for solvent control conditions (MQ and DMSO). When precultured under iron limited conditions, a modest repression of *hemA, hemF* and *hemH* was observed. However *hemA* and *hemH* are directly iron-responsive upon (high)iron addition. Increased

expression of *hemA* and *hemH* was also observed upon the addition of hemin and hemoglobin, whereas the final heme-intermediate protoporphyrin IX did not alter the expression of any of the selected genes. ALA supplementation reduced the expression of all examined heme biosynthetic genes. This reduced expression was not observed for the siroheme synthesis gene *met1*. Hemoglobin addition resulted in reduced *met1* expression. The hemoglobin induced expression of the heme biosynthetic pathway under both standard and iron limited conditions, might not be specific as addition of another heme-free protein BSA had a similar effect.

Analysis of hemA deletion strain

A deletion strain of *hemA* (An17g01480) was constructed in *A. niger*. 50 μM ALA was supplemented during transformation of pΔ*hemA* to AB4.1, as the deletion was expected to be conditionally lethal. Transformants were prescreened on MM and MM containing 50 μM ALA. ALA requiring mutant strains were analyzed by Southern analysis. One of the strains showing to be a correct deletion strain was designated Δ*hemA* (results not shown). Growth of Δ*hemA* could be restored to wild-type by supplementing 100 μM ALA in MM or 500 μM ALA in CM. Decreasing ALA concentrations led to a strong, dose-dependent growth reduction. Complementation of Δ*hemA* on DNA level, by inserting a functional *hemA* fragment restored all phenotypic defects, indicating that the observed phenotype is specific for Δ*hemA* (results not shown).

To test whether Δ*hemA* is able to utilize exogenous heme sources, fresh conidia were spotted on MM or CM containing hemin as heme source (Figure 4.2), but no growth was observed. Also the addition of 1% Tween80 (v/v) had no effect on growth of Δ*hemA*. However, hemin supplementation in the presence of low ALA concentrations, by itself insufficient to sustain full development of Δ*hemA* (20 μM in MM or 100 μM in CM (limited ALA)), resulted in wild-type growth (Figure 4.2) indicating that hemin can be used as an external heme source.

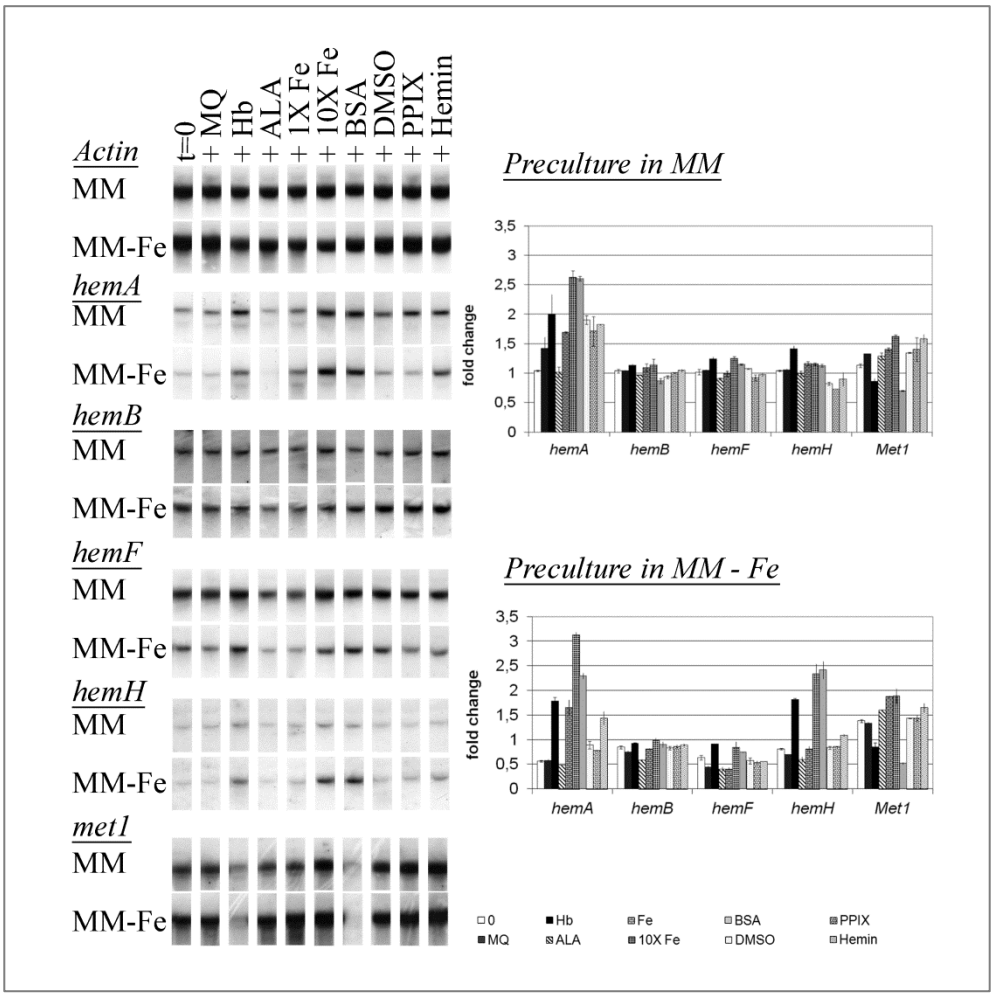

Figure 4.1: Northern analysis. *A. niger* N402 was cultured under standard or iron deprived conditions for 24h followed by the addition of a compound. RNA-Samples were frozen in liquid nitrogen 30 min. after the addition and analysed for the expression of *hemA, hemB, hemF, hemH* and *Met1*. *Actin* levels were used to correct for loading differences. Samples are quantified relative to N402 after 24h under standard conditions and normalized. Hb: Hemoglobin; ALA: 5'-aminolevulinic acid: Fe = $FeSO_4$*$7H_2O$; BSA: Bovine Serum Albumin; PPIX: Protoporphyrin IX.

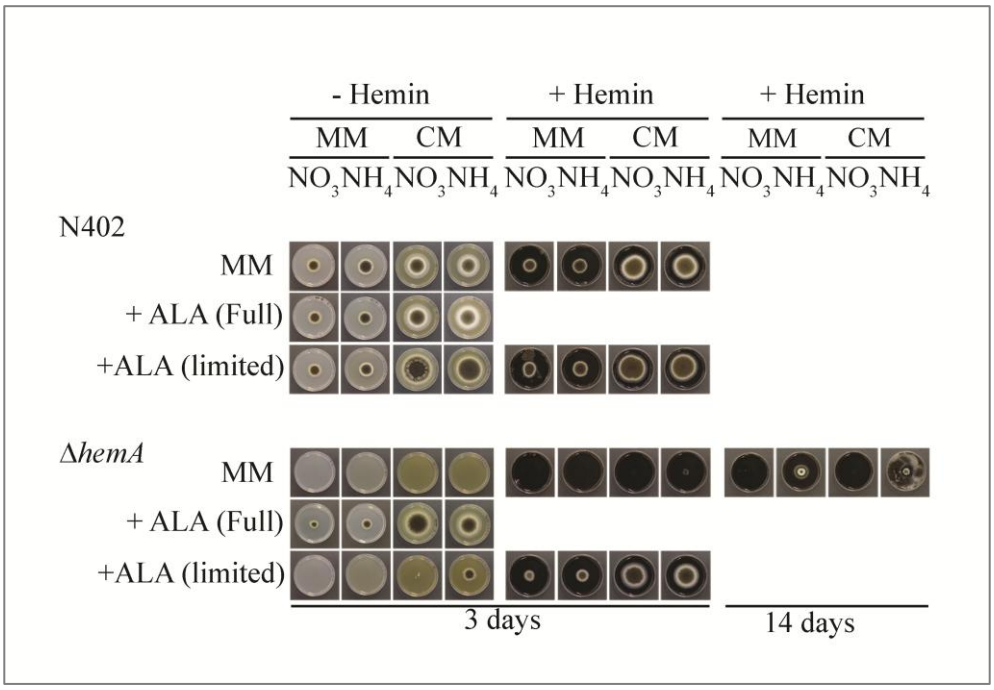

Figure 4.2: Supplementation of Δ*hemA* and siroheme involvement. $5 \cdot 10^3$ conidia are spotted and plates are incubated at 30 °C. Pictures are taken after 3 and 14 days. A) N402, B) Δ*hemA*.

Heme biosynthesis and nitrogen metabolism

Siroheme synthesis is dependent on ALA availability (Franken et al. 2011). Therefore sulfur and nitrogen metabolism could be impaired in Δ*hemA* due to inactive sulfite- and nitrite reductases. To examine whether growth of Δ*hemA* could be improved by avoiding the need for nitrite reductase activity and/or sulfite reductase activity, supplementation assays were performed using ammonium instead of nitrate as N-source and addition of L-methionine in hemin based media. Supplementation of L-methionine did not improve growth of Δ*hemA* under any of the conditions tested (results not shown). The use of ammonium, however, significantly improved the hemin supplemented growth of Δ*hemA* under limited ALA conditions and supported minimal growth when ALA supplementation was omitted, whereas no significant growth was observed on nitrate containing media (Figure 4.2). These results indicate that the inability to synthesize siroheme impaired nitrate assimilation due to lack of nitrite reductase activity in Δ*hemA*, but not sulfite reductase activity. As even in the presence of ammonium no wild-type growth is achieved without ALA supplementation, our results may indicate that some metabolic processes are still impaired, possibly due to insufficient intracellular heme levels.

Amino acid involvement in hemin or ALA uptake

Amino acids, present in CM, can serve as alternative N-source, but could also compete for uptake of components such as ALA or hemin. In the Δ*hemA* they could also supplement unexpected deficiencies. Therefore, several amino-acids (see materials and methods) were analyzed for their potential involvement in growth of the Δ*hemA* mutant. No specific altered growth was observed in combination with ALA supplementation. In combination with hemin supplementation, improved growth was observed only with cysteine addition resulting in similar growth as observed for the WT strain (results not shown). Analyses in CM media (Figure 4.3) support the finding that aminoacids do not interfere with hemin uptake or N-source utilisation as omitting all casamino-acids or ammonium does not result in an improved growth. Also competition of aminoacids with ALA-uptake is unlikely. Growth of Δ*hemA* was found to be improved when nitrate was omitted from ALA supplemented media, possibly due to inhibitory effects of impaired nitrate utilization (e.g. by forming of nitrite intermediate, nitrosative stress etc). However, no wild-type growth was achieved, as was observed in the presence of ammonium.

Discussion

Heme is thought to be a limiting factor in the heterologous production of peroxidases in *Aspergilli* but the supplementation of an external heme source during production processes is undesirable for practical and economical reasons (Elrod et al. 1997). Intracellular overproduction of heme would be preferred. However, heme biosynthesis is known to be tightly regulated (Hoffman et al. 2003; Keng and Guarente 1987) and knowledge in filamentous fungi is limited. Therefore, in order to improve the current understanding of heme biosynthesis in *A. niger,* we analyzed gene expression of several heme pathway genes in response to various heme sources and heme-intermediates. When *A. niger* N402 was cultured under standard iron containing conditions, no significant effect on gene expression was observed. However, when cultured under iron-deprived conditions repression of *hemA, hemF* and *hemH* was observed. Earlier research demonstrated control on *hemA* through iron in other *Aspergilli* by the transcriptionfactor SreA and the interaction of the CCAAT-binding core complex (CBC) with HapX (Hortschansky et al. 2007). Promoter analysis of the heme genes demonstrate the presence of CCAAT-consensus binding sites in almost all heme genes (except *hemB*). The CBC however, modulates the expression of numerous genes (Hortschansky et al. 2007), and therefore the presence of a putative binding-site alone is not indicative for regulation

by iron. As such, only *hemA*, and to a lesser extent *hemH* were found to be directly iron-responsive. The observed repression of *hemF* is more likely to be a secondary effect of the overall downregulation. This result would be consistent with a rate-limiting nature of *hemA* in most organisms (Elrod et al. 1997; González-Domínguez et al. 2001; Lathrop and Timko 1993), but not in *S. cerevisiae* (Hoffman et al. 2003). Also, increased downregulation during ALA supplementation and the presence of Heme Regulatory Motifs in ALAS (involved in feedback inhibition by heme), indicate an additional level of control on this enzyme.

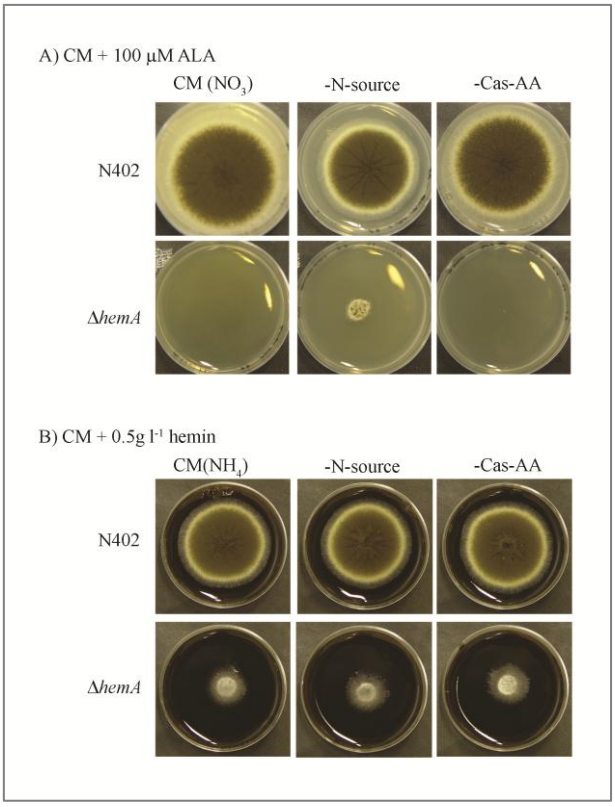

Figure 4.3: A) aminoacid involvement with ALA supplementation. Improved growth of Δ*hemA* was observed relative to nitrate-based medium when the N-source was omitted from CM, but not by omitting casamino-acids indicating aminoacids are not competing with ALA-uptake. B) aminoacid involvement with hemin supplementation. No altered growth is observed by omitting N-source or casamino-acids from CM(NH_4) media indicating that N-source regulation is not interfering with heme uptake.

Surprisingly however, supplementation of a heme source, but not protoporphyrin IX, resulted in upregulation of *hemA* and *hemH*. This would imply that heme is transported into the cell, although siderophore deficient *Aspergillus* mutants were unable to utilize heme-bound iron present in the environment (Eisendle et al. 2003; Schrettl et al. 2004). An alternative explanation for our results could be that the heme source is degraded, and not heme, but iron is causing this upregulation. Classical heme oxygenases however appear absent in the genome of *A. niger* (Franken et al. 2011). Ferrochelatase, present in *Aspergillus* (Franken et al. 2011), may play a role in iron sequestering from heme as mammalian ferrochelatase was found to involved both in iron insertion in heme and iron sequestration from heme. (Sakaino et al. 2009).

When analysing the expression profile of *met1*, encoding siroheme synthase, it becomes clear this branched pathway for siroheme synthesis is not regulated similarly to the later heme biosynthesis genes. In fact, the opposite response was observed during iron deprivation and the supplementation of ALA or a heme source. This suggests that siroheme synthesis could be regulated in response to altering concentrations of early heme-intermediates. The observation that BSA supplementation renders the same effect as hemoglobin might indicate that the response is not hemin-specific. However, interfering iron impurities in the BSA used cannot be ruled out.

Taken together, our results indicate that heme biosynthesis is regulated predominantly on *hemA* expression by iron, ALA and possibly heme, But post-translational regulation of the pathway should not be excluded.

Therefore, we analysed the role of *hemA* in more detail by means of gene-deletion. Heme is an essential molecule and deletion of *hemA* is conditionally lethal in *A. niger* as it is in most organisms. Growth could be restored by ALA supplementation in a dose dependent manner but not directly by a heme source (Figure 4.3), identical to what was observed for the *A. oryzae* Δ*hemA* (Elrod et al. 2000), indicating that *Aspergillus* spp. are not capable of using exogenous heme sources or that other compounds arising from *hemA* encoded enzymatic activity e.g. siroheme, are essential for growth as well.

Therefore, we analysed the ability for heme uptake and the role of the siroheme-branch in Δ*hemA* by using limited ALA conditions. Under these conditions there is insufficient UroIII to support both heme and siroheme synthesis and regulation of the siroheme branch-point could allow for direction of UroIII to either siroheme or heme synthesis upon requirement. Our analysis showed significantly improved growth when hemin is supplemented or ammonium is used as N-source. Growth of Δ*hemA* could even be sustained on MM using only ammonium and hemin. These results demonstrate heme uptake takes place in *A. niger* (Figure 4.2). It also indicates that siroheme synthesis is impaired in Δ*hemA* as well. Both heme and siroheme are involved in nitrate utilisation

(Figure 4.4) requiring a functional nitrate- and nitrite reductase. Nitrate utilisation is absent in *S. cerevisiae*. The nitrate reductase requires heme as cofactor (Chang et al. 1996), whereas nitrite reductase is a siroheme depending protein. As the expression of both genes is also repressed by ammonium, its use as N-source not only reliefs the requirement for siroheme but also for heme. The initial germination observed with nitrate based hemin cultures is likely the result of an active nitrate reductase but inactive nitrite reductase, leading to the accumulation of toxic nitrite which subsequently impairs growth. As such, these results would also explain the lack of growth of the *A. oryzae* Δ*hemA* strain with hemin supplementation as this strain was only analysed on nitrate containing media (Elrod et al. 2000).

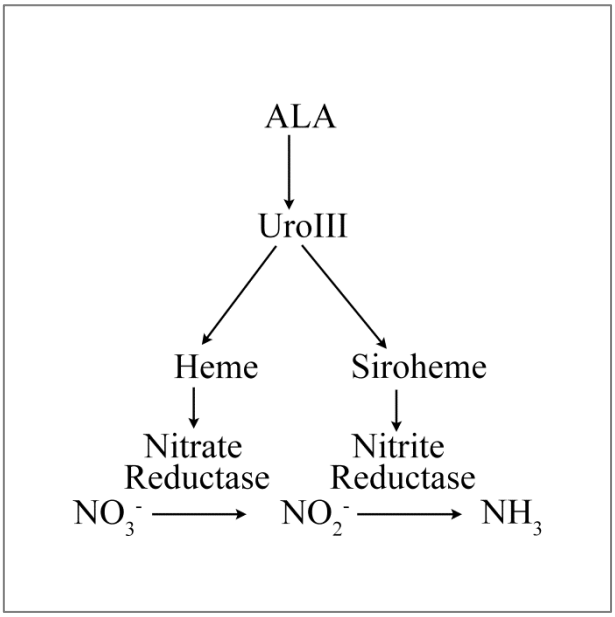

Figure 4.4: Overview of the involvement of heme biosynthesis in nitrogen metabolism. Nitrate reductase, a hemoprotein, converts nitrate into nitrite. Nitrite is subsequently converted to ammonia by nitrite reductase which requires siroheme as a cofactor. ALA: 5'-aminolevulinic acid; UroIII: uroporphyrinogen III.

Our results also suggest that the role of siroheme biosynthesis is different from *S. cerevisiae* in *A. niger* as Δ*hemA* has no methionine deficiency. The potential involvement of other aminoacids were investigated under hemin supplemented conditions, as pathways that require hemoproteins could function suboptimal in MM could be relieved by aminoacids present in CM. Surprisingly, cysteine but not methionine was found to improve growth (results not shown). Cysteine can be synthesized from methionine by

converting homocysteine to cystathionine by cystathionine-β-synthase (Banerjee and Zou 2005). Thus our results suggest that a hemoprotein is involved in the synthesis of cysteine from methionine in *A. niger*. In mammals, cystathionine-β-synthase was found to be a hemoprotein whereas the yeast cystathionine-β-synthase is not (Banerjee and Zou 2005). But, like in *S. cerevisiae,* the N-terminal heme domain is absent in the *A. niger* cystathionine-β-synthase (unpublished results). Therefore, more studies are required to indentify the origin of cysteine limitation in the *A. niger* Δ*hemA* mutant.

Aminoacids can also serve as N-source and as such compete with uptake of compounds such as ALA or hemin. For instance, the *S. cerevisiae UGA4* gene, encoding the γ-aminobutyric acid and ALA- permease, is regulated by N- and C-source (Luzzani et al. 2007). Therefore, the higher ALA requirement in CM could possibly be due to regulation of the *A. niger* ALA transporter, or possible competition on ALA uptake. However, aminoacid supplementation to ALA based MM did not result in altered growth making this hypothesis unlikely.

The results described above demonstrate *A. niger* is capable of using exogenously supplied heme for its own cellular processes and thereby strengthen the heme-limitation hypothesis during peroxidase production conditions. They further indicate the importance of the heme biosynthetic pathway in basal processes like nitrogen and cysteine metabolism. Knowledge on and regulation of those processes with regards to heme biosynthesis will make it possible to identify and resolve further bottlenecks to increase intracellular heme levels required for overproduction of heme-peroxidases by filamentous fungi (Conesa et al. 2000). From the growth analysis however, it also becomes clear that by altering media compositions, the requirement for heme for its own cellular processes can be reduced by supplementing the end-product like ammonium or cysteine. These conditions, in combination with increased iron levels, could also provide conditions for improved large-scale peroxidase production without supplementation of a heme source.

The results also show considerable differences between *S. cerevisiae* and *A. niger* regarding heme biosynthesis and regulation, making *S. cerevisiae* unsuitable as a model organism for filamentous fungi on these processes. Therefore for further understanding of heme biosynthesis, research on this pathway in filamentous fungi is currently ongoing in our laboratory.

Acknowledgements

The authors thank Elise Elliott for technical assistance. This work is funded by the Sixth Framework Program (FP6-2004-NMP-NI-4): “White Biotechnology for added value products from renewable plant polymers: Design of tailor-made biocatalysts and new Industrial bioprocesses” (Biorenew). Contract no.: 026456.