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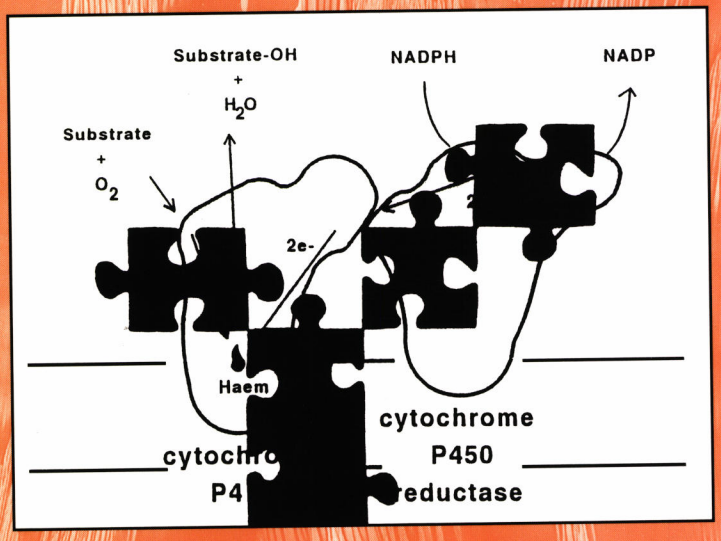
Cytochrome P450 reductase of *Aspergillus niger;* a molecular biological study



Hans van den Brink

Cytochrome P450 reductase of *Aspergillus niger:*

a molecular biological study

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Cover design by Paula Ragas.

Photograph on back-cover: *A. niger* strains carrying multiple copies of the *cyp51* (AB-D1), the *cprA* (AB2.2), or of both genes (AB-D1.15) as well as the wildtype strain N402 were plated on plates to which increasing amounts of a 14α-demethylase inhibitor were added.
Details of the experiment are described in Chapter 3 of this thesis

Cytochrome P450 reductase of *Aspergillus niger:*
a molecular biological study

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. W. A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College van Dekanen
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door

Johannes Maarten van den Brink

geboren te Barneveld in 1967

Promotiecommissie

Promotores:	Prof. dr. C.A.M.J.J. van den Hondel
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Referent:	Prof. dr. ir. P.J.G.M. de Wit

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Contents

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Ook buiten MGG zijn er een aantal mensen die extra belangrijk zijn geweest voor mijn onderzoek. De 14dm-ers in Wageningen die de gouden regel in de wetenschap (één plus één is meer dan twee) hebben helpen bevestigen. Maarten en Hans, ik denk dat ik mag zeggen dat onze samenwerking naast plezierig ook een zeer vruchtbare is geweest. One of the best memories I'll have from my TNO-time certainly will be the period I wasn't there. During my stay at Maryland University I did have a great time at the lab. Of course my very special thanks are for Dave. Not only for his enthousiastic scientific support (which continued afterwards by e-mail and art donations) but even more for his increadible hospitality. Being a huge living toy was a very special experience. Dave, Sue, Donny and Sarah; Thanks again.

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De laatste alinea is gereserveerd voor "Overigen". Maar dan wel een heel speciaal stel.
Nu dit boekje af is weet ik één ding heel zeker. Ik doe het nooit meer! Ik heb het geluk één uitzondering op die regel te kennen. Patricia, je dwong me bijna om, na het afronden van je eigen proefschrift, ook de lay-out van mijn boekje aan je over te laten. Wow!

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Chapter 1

General Introduction

1. Introduction

Filamentous fungi are found in many different environments like soil, living plants and on organic waste material. For their survival filamentous fungi possess the ability to rapidly adapt their metabolism to use different compounds as carbon or nitrogen source. To achieve this adaptation fungi are capable of producing a large set of different intra- and extracelluar enzymes to degrade complex biopolymers. Besides the production and secretion of a number of enzymes filamentous fungi can secrete a great diversity of primary and secondary metabolites (e.g. antibiotics).

The great diversity of fungal bioconversions include the hydroxylation of complex polyaromatic hydrocarbons and steroid compounds. Biochemical evidence indicates that this type of bioconversions are monooxygenase reactions, probably carried out by enzyme systems belonging to the cytochrome P450 superfamily. Other fungal bioconversion reactions mediated by cytochrome P450s include steps in the biosynthesis pathway of mycotoxins like aflatoxin and the detoxificaton of phytoalexins and thus in plant pathogenicity.

Eukaryotic cytochrome P450 enzyme systems are predominantly found to be anchored in the membrane of the endoplasmic reticulum and consist of at least two proteins, the reaction specific cytochrome P450 enzyme and the general, electron donating enzyme NADPH cytochrome P450 reductase (CPR). Whereas many different, reaction specific, cytochrome P450s can be present in the endoplasmic membrane, only one species of CPR is present. All microsomal cytochrome P450 enzyme systems catalyze the same basic monooxygenation reaction:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

R being the substrate involved. The electrons needed in the reaction equitation are generated by the conversion of NADPH to $NADP^+$ by the NADPH-cytochrome P450 reductase.

Cytochrome P450 enzyme systems have been well studied in mammalian systems and

have been shown to be involved in the conversion of different substrates like steroids and other pharmaceutically active compounds, fatty acids and many other endogenous and exogenous compounds (Lu et al. 1973). Studies on cytochrome P450 enzyme systems in filamentous fungi are scarce, mainly because low expression levels and instability of the proteins severely hampered protein isolation and subsequent characterization of fungal cytochrome P450s. The increasing availability of molecular genetical tools for many filamentous fungi may overcome these technical drawbacks and will probably lead to a rapid increase in knowledge about fungal cytochrome P450 systems.

2. Cytochrome P450 Enzyme Systems

Klingenberg (1958) and Garfinkel (1958) were the first to identify a pigment with a specific absorption at 450 nm in rat and pig liver microsomes respectively. Later, this pigment was characterized by Omura and Sato (1961) as a cytochrome like hemoprotein. The typical absorption peak at 450 nm, found after reduction of the microsomal fraction with dithionite and subsequent incubation with carbon monoxide, gave this class of enzymes their name. This characteristic absorption is caused by the binding of both a thiolate anion, donated by a conserved cysteine residue in the apoprotein, and of CO to respectively the fifth and sixth ligand of the reduced haemgroup (White and Coon 1980). Often also a peak at 420 nm is found which seems to be caused by an inactive form of cytochrome P450, cytochrome P420 (Omura and Sato 1964).

In the following decades many different cytochrome P450 enzymes were identified by protein purification or gene cloning from organisms throughout nature. To date over 400 cytochrome P450 enzymes have been reported to the International Committee on the Nomenclature of Cytochrome P450 Enzymes (available on the internet http://drnelson.utmem.edu/homepage.html) and this number is expected to reach 500 within a year. Both the absolute number of cytochrome P450 enzymes classified and the number of families and subfamilies are rapidly increasing, especially due to the identification of many cytochrome P450s from microorganisms and plants (Nelson et al. 1996; Nelson et al. 1993; Nebert et al. 1991).

Description of a cytochrome c reductase, later known as cytochrome P450 reductase, came from an even earlier timepoint. In 1950 Horecker was the first who described extraction of a flavoprotein from liver which could reduce cytochrome c. More than 10 years later a NADPH dependent cytochrome c reducing protein was isolated from protease-treated pig liver

microsomes (Williams and Kamin 1962). Since this protein was found in microsomal membranes it was unlikely that the soluble, cytosolic cytochrome c would be the natural electron acceptor of this flavoprotein. However, it was only in 1974 that Vermillion and Coon were able to show that the isolated cytochrome c reducing protein was also able to reduce cytochrome P450 enzymes. Since both the isolated cytochrome c reductase and cytochrome P450s were localized in the same compartment this observation was a strong indication that reduction of cytochrome P450s was the main physiological function of the cytochrome c reducing protein. Consequently this protein was named NADPH-cytochrome P450 oxidoreductase (CPR; Vermillion and Coon 1974). Further improvement of purification protocols resulted in more detailed kinetic analysis of the CPR (Yasukochi and Masters 1976; Dignam and Strobel 1977). To date (partial) CPR sequences from 20 organisms have been reported. Remarkably, the plant species *Arabidopsis thaliana* (Mignote-Vieux, Kazmaier, Lacroute and Pompon, unpublished) and *Helianthus tuberosis* (Hasenfratz, Jeltsch, Benveniste, Lesot and Durst, unpublished) were shown to contain two related and functional genomic copies of the *cpr* gene while in all other organisms described thus far only one copy was identified. Regularly updated information on CPR sequences and sequences of related flavoproteins is available from "the directory of P450-containing systems" (http://www.icgeb.trieste.it/p450).

CPR as well as the majority of the eukaryotic cytochrome P450s is localized in the endoplasmic membrane. Kärgel et al. (1996) showed that fusion of the N-terminal 22 amino acids of the *C. maltosa* CPR to invertase was sufficient to obtain partial ER retention of the reporter protein while full retention was achieved with the N-terminal 33 amino acids. This result indicates that insertion of the CPR in the ER membrane is probably mediated via this small N-terminal sequence which functions as a membrane anchor sequence. The remainder of the protein protrudes from the cytosolic face of the membrane. Growing evidence accumulates that ER-type cytochrome P450 enzymes are inserted in the membrane via a similar mechanism, using a small N-terminal membrane spanning domain consisting of a single helical structure. In a series of elegant experiments, using glycosylation tags attached to the N-terminal part or to the cytosolic part of the protein, Szczesna-Skopura et al. (1993; 1995) were able to show that the N-terminus is on the luminal site of the membrane, indicating the presence of only one membrane spanning domain. Integration of P450s and CPR in the ER membrane is supposed to be mediated via the signal recognition particle (Sakaguchi et al. 1992; Bar-Nun et al. 1980).

A subclass of mammalian P450s is found in the mitochondria. These differ from the ER-type P450s both by the electron donation system used, and by the presence of a mitochondrial targeting signal at their NH_2-terminus. A few prokaryotic cytochrome P450s have been described thus far. Since these organisms lack internal membranes their P450s are soluble.

The rapid increase in number of identified cytochrome P450 enzymes, from many different sources, created the need for a consensus classification system. This system was designed based on amino acid sequence identity. Cytochrome P450s exhibiting more than 40% amino acid sequence identity are defined to belong to the same gene family, whereas sequence identity between members of a subfamily should be more than 59%. In this denomination the prefix "CYP" is followed by an arabic numeral to indicate the family, a capital letter to indicate subfamilies and an arabic numeral to indicate the individual enzymes (Nelson et al. 1993 and 1996).

Although cytochrome P450 enzymes may differ considerably in amino acid composition they share some major characteristics, the most important being the interaction between a thiolate anion derived from a conserved cysteine residue with the reduced haem-bound iron. The amino acid sequence surrounding this cysteine residue is also highly conserved between different cytochrome P450 enzymes, resulting in a typical "fingerprint" for cytochrome P450 enzymes (FxxGxxxCxG; Table 1.1). Major exceptions to this fingerprint have been described, especially in cytochrome P450 enzymes isolated from lower eukaryotes (e.g. substitution of the second conserved glycine for alanine and of the conserved phenylalanine for tryptophan in the soluble CYP55A2). The largest deviation of the cytochrome P450 fingerprint sequence is found in CYP74 of flax seed and guayole. Since this enzyme uses a substrate which supplies its own oxygen in the form of hydroperoxide (Song et al. 1993), the need for an oxygen binding pocket is no longer present. This has resulted in substitutions at two positions of the fingerprint, phenylalanine was replaced by proline and the first glycine by alanine (flax) or aspartate (guayole) (Nelson et al. 1996). In most ER-type cytochrome P450 molecules another conserved region, called the PR region, is found. This PR region contains three to five proline residues and is located close to the NH_2-terminus. Mutations in the PR region resulted in incorrect folding and localization of the protein, showing the importance of this region for the structural stability of cytochrome P450s (Ishihara et al. 1995; Yamazaki et al. 1993).

The large difference in amino acid composition of cytochrome P450 molecules is not reflected in its three dimensional shape. A complete X-ray diffraction analysis was carried out on a soluble, bacterial cytochrome P450, $P450_{cam}$ (CYP101; Figure 1.1), providing insights in the general structure of cytochrome P450 enzymes (Poulos, 1986; Poulos and Raag 1992). Later crystal structures were determined for the P450 domain of $P450_{BM-3}$ from *B. megaterium* (CYP102; Ravichandran et al. 1993) and for the bacterial $P450_{terp}$ (CYP108; Haseman et al. 1994) and $P450_{cryF}$ (CYP107; Cupp-Vickery and Poulos 1995). Comparison of the structures of these bacterial proteins showed that all proteins were comparable α/β proteins, shaped like a triangular prism. Although the overall topology of these molecules is very comparable, the orientation of the structural helices differs (Degtyarenko 1995). Computer analysis, comparing the amino acid sequences of many different mammalian cytochrome P450 enzymes with the amino acid sequences of the crystallized P450s, showed no major differences in the overall structure of the molecules, even though helical formation may be very different (Black and Coon 1987; Degtyarenko 1995). However, since all crystallized cytochrome P450s are soluble, prokaryotic, proteins care should be taken if their structures are being compared with those of membrane bound eukaryotic cytochrome P450s.

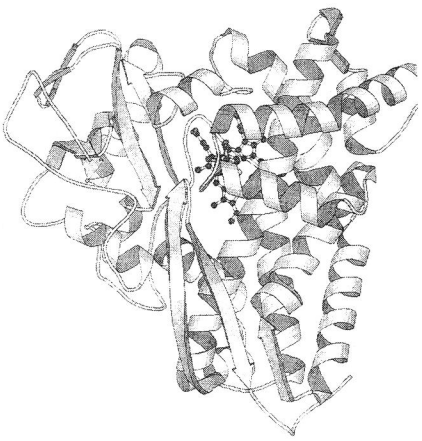

Figure 1.1 Three dimensional structure of *P.putida* cytochrome $P450_{cam}$ (CYP101). Taken from Degtyarenko (http://www.icgeb.trieste.it/p450).

3. Cytochrome P450 in Fungi

Cytochrome P450 mediated conversion steps are part of many specific fungal biotransformation processes. The involvement of cytochrome P450 enzymes in these reactions is implied either based on the NADPH-dependency of the reaction, the ability of some well known cytochrome P450 inhibitors like metyrapone and CO to inhibit the enzymatic conversion or is based on the microsomal localization of the enzyme complex. Formal proof, by purification and characterization of the enzymes involved, has only been provided in a few cases.

The best studied cytochrome P450 mediated reactions in fungi (which will be described

in more detail in the following sections of this chapter), concern the degradation of poly-aromatic hydrocarbons like benz<a>pyrene and biotransformation of important steroids like progesterone (pharmaceutical industry) or smaller aromatic hydrocarbon compounds used for the production of many different biochemicals (a.o. a strong mosquito repellent; Asakawa et al. 1991). Other well studied fungal cytochrome P450 systems are the pisatin demethylase from *Nectria haematococca* (Weltring et al. 1988) and the benzoate para hydroxylase system in *Aspergillus niger* (Van Gorcom et al. 1990; Boschloo et al. 1990).

3.1 Bioconversion of poly-aromatic hydrocarbons

Poly-aromatic hydrocarbons (PAHs) are composed of three or more fused aromatic rings and are resistant to microbial degradation. PAHs are known to be present in coal and petroleum but most of the PAHs present in the environment are the result of pyrolytic processes such as forrest fires or incomplete combustion of fossil fuels (Gibson and Subramanian 1984). PAHs are major environmental pollutants, some of them being (potentially) strong carcinogenics (Pelkonen and Nebert 1982; Autrup 1990). Since mammalian and fungal metabolism of complex PAHs follow identical pathways (Wackett and Gibson 1982; Gibson 1982) fungi are, in contrast to bacteria, excellent model organisms to study mammalian breakdown and activation of PAHs.

Many different filamentous fungi possess the ability to hydroxylate one or more PAHs (Cerniglia et al. 1984 and 1992; Griffiths et al. 1991). By screening a number of fungi isolated from a refinery dump site, Launen et al. (1995) identified several fungi capable of degrading model PAHs like benz<a>pyrene and pyrene. The highest conversion rates observed were achieved by some *Penicillium* species. By screening 180 saprophytic and plant pathogenic basidiomycetes Anke and coworkers identified *Crinipellis stiparia* as a potent species for pyrene hydroxylation (Lambert et al. 1994; Lange et al. 1994). The metabolic pattern of fungal degradation of the model PAHs used in these studies resembles the metabolic pattern observed with mammalian cytochrome P450 enzyme systems, suggesting the involvement of cytochrome P450 enzymes in the fungal bioconversions as well. However, no experimental evidence for the involvement of cytochrome P450 in these cases has been provided yet. The ability of *Aspergillus ochraceus* to hydroxylate benz<a>pyrene has been described by Dutta et al. (1983) and Ghosh et al. (1983). By inhibition studies with a number of specific inhibitors, including cytochrome c, CO and SKF-525A this hydroxylation was shown to be cytochrome P450 dependent (Dutta et al. 1983; Ghosh et al. 1983).

A thorough study of fungal P450 mediated PAH hydroxylation has been carried out by Cerniglia and coworkers (Reviewed in Cerniglia et al. 1992, 1984 and 1982). In a number of experiments they have established the capability of some fungi, most notably *Cunninghamella elegans* and *C. bainieri*, to carry out specific hydroxylations of a number of model PAH-compounds like pyrene, benz<a>pyrene, 3-methylcholanthrene and naphthalene. Indications for the involvement of a cytochrome P450 enzyme system in PAH hydroxylation carried out by *Cunninghamella* species came from the close homology between the mammalian PAH hydroxylation metabolism and the fungal metabolic pattern. Evidence for the involvement of cytochrome P450 enzyme systems was provided by partial enzyme purification followed by *in vitro* reconstitution experiments, showing the dependence of the isolated enzyme fraction on NADPH and CPR, and by studying the incorporation of molecular O_{18} in the substrate. Using a different fungal species, *Mortierella isabellina*, Holland and coworkers (e.g. Holland et al. 1994) performed comparable experiments with similar results. Both groups found a number of different fungi to be able to hydroxylate a set of different PAHs.

Besides for detoxification of PAHs fungal bioconversion reactions of PAH molecules can also be used for specific modification of complex molecules. The site of hydroxylation for one specific substrate can differ considerably between different fungal species while hydroxylation of PAH molecules by fungi is in general stereospecific (Holland et al. 1994, 1992 and 1987). This wide range of different, stereospecific bioconversions makes fungal bioconversion an attractive alternative for difficult production steps in the synthesis of aromatic compounds. For this reason many industrial PAH hydroxylations are carried out by filamentous fungi. However, application of fungi for other industrial bioconversions is severely hampered by the lack of knowledge about bioconversion capabilities of most fungi.

A number of fungi are able to employ alternative enzyme systems, besides cytochrome P450 systems, for detoxification of PAHs. Such enzymes like laccases, ligninases and peroxidases are mainly found in wood-degrading fungi like *Phanerochaete chrysosporium* (Keyser et al. 1978) and can attack their substrate by generating oxygen radicals. Many different substrates can be oxidized by these enzymes, including complex PAHs like benz<a>pyrene (Paszcynski et al. 1986; Sariaslani 1989; Haemmerli et al. 1986). As suggested by Bezalel et al. (1996) it is most likely that *in vivo* both lignolytic and cytochrome P450 enzymes are involved in the degradation of PAHs like benz<a>pyrene. According to their hypothesis the cytochrome P450 enzymes perform the initial attack followed by degradation of the PAH-derivative by the lignolytic system. This hypothesis is supported by the recent

identification of a (partly soluble) cytochrome P450 system mediating benz<a>pyrene hydroxylation in the white rot fungi *Pleurotus pulmonarius* and *Phanerochaete chrysosporium* (Masaphy et al. 1995 and 1996 respectively).

3.2 Bioconversion of steroid molecules

Another well studied class of bioconversions carried out by fungal cytochrome P450 enzyme systems is the (stereo)specific hydroxylation of pharmaceutically interesting steroids, especially progesterone (Figure 1.2). This microbiological transformation, first described by Peterson and Murray in 1952, replaced a dozen separate chemical reactions and thus was of great importance for cost-effective production of steroids. Microsomal fractions, capable of *in vitro* steroid hydroxylation, were prepared from various filamentous fungi including *Aspergillus ochraceus* (11α-hydroxylation), *Botryospaeria obtusa* (7ß), *Cochliobolus lunata* (11ß), *Mucor piriformis* (14α) and *Phycomyces blakesleeanus* (7α) (reviewed in Smith et al. 1993).

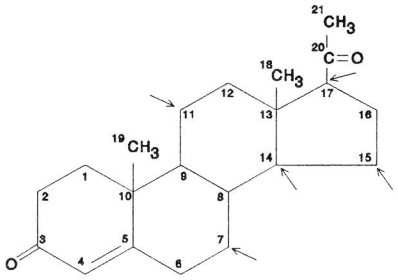

Figure 1.2, Structure of progesterone. Arrows indicate the positions hydroxylated by different fungi.

Komel and coworkers have studied the steroid hydroxylating activity of the fungus *Cochliobolus lunatus*, which has a high specificity for 11α hydroxylation of progesterone (Rozman and Komel 1992). The involvement of cytochrome P450 enzymes in this bioconversion reaction was studied by Vitas et al. (1995). Interaction of microsomal cytochrome P450s, obtained from *C. lunatus*, with progesterone was indicated by the presence of a clear type I substrate binding spectrum. In comparable experiments, using microsomal fractions, Smith et al. (1994) showed the involvement of P450 enzymes in the hydroxylation of progesterone by *A. fumigatus*. Other well studied progesterone hydroxylating fungi are *Rhizopus nigricans* (Breskvar et al. 1991; Breskvar and Hudnik-Plevnik 1977), which predominantly hydroxylates the 11α position and *P. blakesleeanus* which is more specific for the 7α and 15ß positions of the progesterone molecule. By protein purification of the progesterone hydroxylation enzyme system from *P. blakesleeanus* and subsequent reconstitution studies Ahmed et al. (1995) clearly proved the involvement of a P450 system in these bioconversions.

3.3 Cloned cytochrome P450 encoding genes in fungi

Many important fungal biotransformation reactions are carried out by cytochrome P450 enzymes but cloning of only a few of the corresponding genes has been reported yet (Table 1.1). These are categorized in the *cyp* gene families 51-62 (Nelson et al. 1996 and 1993).

One of the most wide spread cytochrome P450 genes in nature is the gene encoding eburicol- or lanosterol 14α-demethylase (EDM or $P450_{14DM}$). $P450_{14DM}$ is involved in the conversion of mevalonate to ergosterol which is an important membrane compound with a function comparable to cholesterol in higher mammalians. $P450_{14DM}$ carries out the rate limiting step in the ergosterol biosynthesis pathway. Since many azole-based fungicides are directed against $P450_{14DM}$, this enzyme is a favourable subject of agricultural research (Vanden Bossche 1988).

A large cytochrome P450 gene family (*cyp52*) is found in *Candida* species and its members are involved in *n*-alkane assimilation. Different members of this family seem to have partially different substrate specificity (Seghezzi et al. 1992). The CYP52 enzyme system is the rate limiting step for *n*-alkane assimilation as it catalyzes the terminal hydroxylation of the different substrates (Sanglard et al. 1984). Other reactions in which members of the *cyp52* gene-family are involved are the production of fatty acid alcohols and dicarboxylic acids (Bühler and Schindler 1984).

Another family of cytochrome P450 enzymes is found in aflatoxin producing fungi like *A. parasiticus*. However, for biosafety reasons this pathway has been studied more detailed in the non-aflatoxigenic fungus *A. nidulans* which contains all the enzymes needed for production of the aflatoxin precursor sterigmatocystin but misses the enzymes necessary for the final bioconversion of sterigmatocystin in aflatoxin. The genes encoding the *A. nidulans* sterigmatocystin biosynthesis enzymes are located in a gene cluster. DNA

Table 1.1 Identified fungal cytochrome P450 encoding genes.
Fungal cytochrome P450 enzymes of which cloning of the corresponding gene has been reported are described. Sequence data of some genes have not yet been made publically available. Amino acid sequences in the heme binding pocket, forming the cytochrome P450 fingerprint, are given. From these fingerprint sequences a consensus sequence was deducted. **BOLD CAPITALS** in this consensus sequence indicate completely conserved residues in mammalian cytochrome P450s according to Gonzalez (1989). *Underlined ITALICS* indicate residues which are almost completely conserved in the fungal cytochrome P450 haem binding pocket. Small writing indicates residues which are present in a significant part of the fungal cytochrome P450s. Residues written below indicate major alternatives to the consensus sequence.

cyp	Organism	Function, functional name	P450 fingerprint	Reference
cyp51	*P. italicum*	Demethylation of eburicol/lanosterol at 14α position. 14DM	YLPFGAGRHRCIGEKFAY	van Nistelrooy et al. 1996
	S. cerevisiae		YLPFGGGRHRCIGEHFAY	Kalb et al. 1986 and 1987
	C. albicans		YLPFGGGRHRCIGEQFAY	
	C. tropicalis		YLPFGGGRHRCIGEQFAY	
	U. maydis		YLPFGAGRHRCIGEQFAY	Unpublished
	S. pombe		YLPFGAGRHRCIGEQFAY	Unpublished
cyp52A3,	*C. maltosa*	*n*-alkane assimilation, Alk1a	YVPFNGGPRICLGQQFAL	Seghezzi et al. 1992
cyp52A4,		*n*-alkane assimilation, Alk3	YVPFNGGPRICLGQQFAL	Sanglard and Loper 1989
cyp52A5,		*n*-alkane assimilation, Alk2a	FVPFNGGPRICLGQQFAL	
cyp52A9,		*n*-alkane assimilation, Alk5a	FLPFNGGPRICLGQQFAL	
cyp52A10,		*n*-alkane assimilation, Alk7a	FLPFNGGPRICLGQQFAL	
cyp52A11,		*n*-alkane assimilation, Alk8a	FMPFSTGPFMCLGQQFAL	
cyp52C2,		*n*-alkane assimilation, Alk6a	YLPFNGGPRICLGQQFAL	
cyp52D1		*n*-alkane assimilation, Alk4	YVPFNGGPRICLGQQFAL	
cyp52A1,	*C. tropicalis*	*n*-alkane assimilation, Alk1	FLPFNGGPRICLGQQFAL	Sanglard and Fiechter 1989
cyp52A2,		*n*-alkane assimilation, Alk2	YVPFNGGPRICLGQQFAL	Schunck et al. 1991
cyp52A6,		*n*-alkane assimilation, Alk3	YLPFNGGPRICLGQQFAL	
cyp52A7,		*n*-alkane assimilation, Alk4	YLPFNGGPRICLGQQFAL	
cyp52A8,		*n*-alkane assimilation, Alk5	YLPFNAGPRTCLGQQYTL	
cyp52B1,		*n*-alkane assimilation, Alk6	FIPFSAGPRACLGQQLPR	
cyp52C1		*n*-alkane assimilation, Alk7	YIPFNGGPRICLGQQFAL	
cyp52E1	*C. apicola*	*n*-alkane assimilation		Unpublished
cyp53	*A. niger*	Benzoate parahydroxylase, benzoate inducible, BPH	FIPFSTGPRACVGRNVAE	Van Gorcom et al. 1990
	Rhodotorula minuta	Benzoate parahydroxylase, conversion of isovalerate to isobutene, L-phenylalanine inducible		Fukuda et al. 1994

cyp54	*N. crassa*	Cycloheximide inducible, unknown function		Attar et al. 1990
cyp55	*Fusarium oxysporum*	Reduction of nitric oxide. Self sufficient and soluble.	PLGFGFGDHRCIAEHLAK	Kizawa et al. 1991 Shoun et al. 1991
	Cylindrocarpon tonkinense		GLGFGFGPHRCIAELLAK PLGYGFGPHRCIAEHLAK	Unpublished
cyp56	*S. cerevisiae*	Formation of dityrosine (DIT2)	VTGFHGGRFACLGEKLAL	Briza et al. 1990
cyp57a1v1	*N. haematococca*	Demethylation of pisatin. *pda-T9* isolated from highly pathogenic strain	F-AFGAGSRSCIGKNISI	Weltring et al. 1988 Maloney and VanEtten 1994
cyp57a2	*N. haematococca*	*pda6-1* isolated from low virulence strain	F-AFGAGSRSCIGKNISI	Reimman and VanEtten 1994
cyp58	*F. sporotrichiodes*	Trichothecene biosynthesis, *Tri4*	ITNFSQGSRQCIGYTMAF	Hohn et al. 1995
cyp59	*A. nidulans*	Sterigmatocystin biosynthesis, *stcS*	WRAFEFGPRSCIGQTLAM	Brown et al. 1996
cyp60A1	*A. parasiticus*	Sterigmatocystin biosynthesis, *stcF*	SQPFSIGPRNCIGRQLAY	Yu et al. unpublished
cyp60A2	*A. nidulans*	Sterigmatocystin biosynthesis, *stcF*	SQPFSIGPRNCIGRQLAL	Brown et al. 1996
cyp60B	*A. nidulans*	Sterigmatocystin biosynthesis, *stcL*	YQPWSVGVRNCIGRNLAY	
cyp61	*S. cerevisiae*	Unknown function	WLVFGCGPHVCIGQTYVM	Unpublished
cyp62	*A. nidulans*	Sterigmatocystin biosynthesis, *stcB*		Brown et al. 1996
cyp63	*P. chrysosporium*	Unknown function		Unpublished
		Consensus mammalian	FLPFG-G-R-C-G-----	
		Consensus fungal	yLPF--GrR-CIGeqfAl fv pH Laq l y	

sequencing of the gene cluster revealed twenty-five open reading frames (*stcA-stcW*). Four of these genes, *stcB* (*cyp62*), *stcF* (*cyp60A2*), *stcL* (*cyp60B*) and *stcS* (*cyp59*) were shown to be members of the cytochrome P450 superfamily (Brown et al. 1996; Keller et al. 1995). A homologue of *stcF* (*cyp60A1*) has been cloned from the aflatoxigenic fungus *A. parasiticus* by Yu et al. (unpublished).

A cytochrome P450 gene from *Aspergillus niger* (*bphA*) was reported by Van Gorcom and co-workers in 1990 and encodes a benzoate para-hydroxylase. Recently protein purification from the yeast *Rhodotorula minuta* of another member of the CYP53 family was reported by Fukuda et al. (1994). This L-phenylalanine inducible[1] cytochrome P450 enzyme was shown to be able to convert benzoate into para-hydroxybenzoate as well as to catalyze the formation of isobutene from isovalerate.

Cytochrome P450 enzymes seem to play important roles in plant pathogenicity of some fungi. The fungus *Nectria haematococca* is a pathogen to garden pea (*Pisum sativum L.*). The gene product of the *pda* gene (*cyp57*) of *Nectria haematococca* is involved in the demethylation of pisatin, a phytoalexin secreted by garden pea. Genes encoding pisatin demethylases have been isolated both from the highly pathogenic PDA-T9 and from the non-pathogenic PDA6-1 *N. haematococca* strains (Weltring et al. 1988; Maloney and VanEtten 1994; Reimmann and VanEtten 1994). Expression of the *pda* gene in the high-virulence strain was shown to be inducible by pisatin. Pisatin induction of the PDA-T9 *pda* gene was shown to be mediated by a 35 bp DNA element (pisatin responsive element), present in the gene control region of the PDA-T9 *pda* gene (Straney and VanEtten 1994). Interestingly, it was shown by Miao et al. (1991) that the *pda* gene is located on a small, meiotically unstable, chromosome that is dispensable for normal growth.

The plant pathogenic fungus *Fusarium sporotrichiodes* produces the sequiterpenoid trichothecene which is needed for enhancement of virulence of this fungus (Desjardins et al. 1992). A number of genes involved in the trichothecene biosynthesis pathway is present as a gene cluster (Hohn et al. 1993). One of them, *Tri4*, has been shown to be a cytochrome P450 gene (Hohn et al. 1995), most closely related to human *cyp3A4*.

[1] Induction is defined as an increase in enzymatic activity or transcription rate in response to a specific environmental stimulus.

4. Electron donation systems

Many different biocatalytic conversions are carried out by cytochrome P450 enzymes. The general reaction equation for all these reactions is identical as was shown in the first paragraph of this thesis ($RH + NAD(P)H + H^+ + O_2 \rightarrow ROH + NAD(P)^+ + H_2O$).

In most cytochrome P450-dependent monooxygenase reactions described thus far, the electrons needed for this equation are transferred from NAD(P)H to the substrate by a second enzyme system.

In prokaryotic and mitochondrial systems two enzymes are involved in the transfer of electrons from NAD(P)H to the cytochrome P450. First, electrons are transferred from NAD(P)H via a flavin containing enzyme, ferredoxin reductase (adrenodoxin reductase in mitochondria), to ferredoxin (adrenodoxin in the mitochondrial system) and subsequently to the cytochrome P450. The soluble cytochrome P450 camphor hydroxylase ($P450_{cam}$) from *Pseudomonas putida* (Poulos and Raag, 1992) is considered a model enzyme for prokaryotic and mitochondrial cytochrome P450s and has been extensively studied. Electrons for microsomal cytochrome P450 enzymes are donated by a single enzyme, NADPH-cytochrome P450 oxidoreductase (CPR) (Figure 1.3).

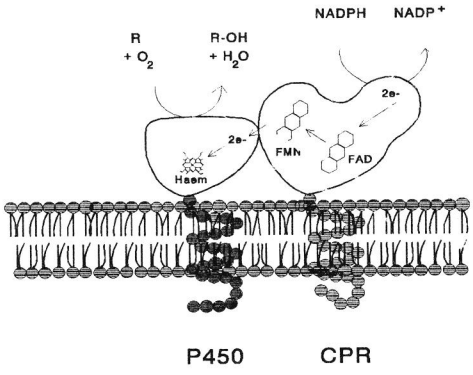

Figure 1.3, General reaction mechanism for the ER located P450 enzyme system.

Two eukaryotic cytochrome P450 enzymes have been described which are able to reduce substrates independently of an electron transfer system and of molecular oxygen; $P450_{nor}$ (CYP55) from the fungus *Fusarium oxysporum* (Kizawa et al. 1991) and flaxseed allene oxide synthase (Song et al. 1991 and 1993). The exact reaction mechanisms for these enzymes still has to be elucidated. An exceptional cytochrome P450, the soluble $P450_{BM-3}$, was found in *Bacillus megaterium*. This protein proved to be an ancestral fusion of two domains, a cytochrome P450 like and a CPR-like domain, making the reaction mechanism independent of external enzymes (Ruettinger et al. 1989). Until recently it was the only natural cytochrome P450-CPR fusion protein known. Crystallization of the complete $P450_{BM-3}$ protein (the crystal structure of the

P450 domain has already been established; Ravichandran et al. 1993) might provide insight in the interaction of eukaryotic cytochrome P450s and their major electron donor, CPR. Since $P450_{BM-3}$ has an extremely high turn-over rate for its substrate (long chain fatty acids; Narhi and Fulco 1986), probably due to its efficient electron transfer system, different research groups have tried to mimic this situation by construction of fusions between CPR and cytochrome P450 encoding genes and overexpressing them in yeast (Shibata et al. 1990; Sakaki et al. 1990). Recently a membrane bound protein was isolated from the fungus *F. oxysporum* ($P450_{foxy}$) that proved to be a fusion between a fatty acid hydroxylating cytochrome P450 domain and CPR (Nakayama et al. 1996). Except for its membrane bound nature this protein very much resembles $P450_{BM-3}$. It has a similar high substrate turn-over rate, has a comparable substrate specificity and is recognized by $P450_{BM-3}$ specific antibodies (Nakayama et al. 1996).

4.1 Cytochrome P450 reductase

CPR is a flavoprotein of ± 78 kDa, containing 1 mol each of the prosthetic factors FAD and FMN per mol protein (Figure 1.4). The pathway of electron transfer proceeds from NADPH to FAD to FMN to P450 (Vermillion et al. 1981; Kurzban and Strobel 1986). In stopped flow spectrophotometric studies the kinetics of electron transfer was unravelled (Oprian and Coon 1982; Sevrioukova and Peterson 1995). After the initial transfer of two electrons from NADPH to the reductase a rapid equilibrium occurs between the three possible reduction states (Figure 1.5). One electron can be donated to the cytochrome P450 from the FMN-reduced state after which event a second pair of electrons is accepted from NADPH to fully reduce the cytochrome P450. Alternatively, the reduced CPR can accept a second pair of electrons from NADPH followed by complete reduction of the cytochrome P450 (reviewed in Sevrioukova and Peterson 1995).

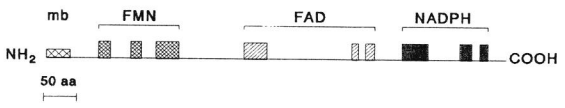

Figure 1.4, Schematical representation of the functional domains involved in electron flow in CPR as well as the membrane spanning domain (mb).

By trypsin treatment of rat liver microsomes,

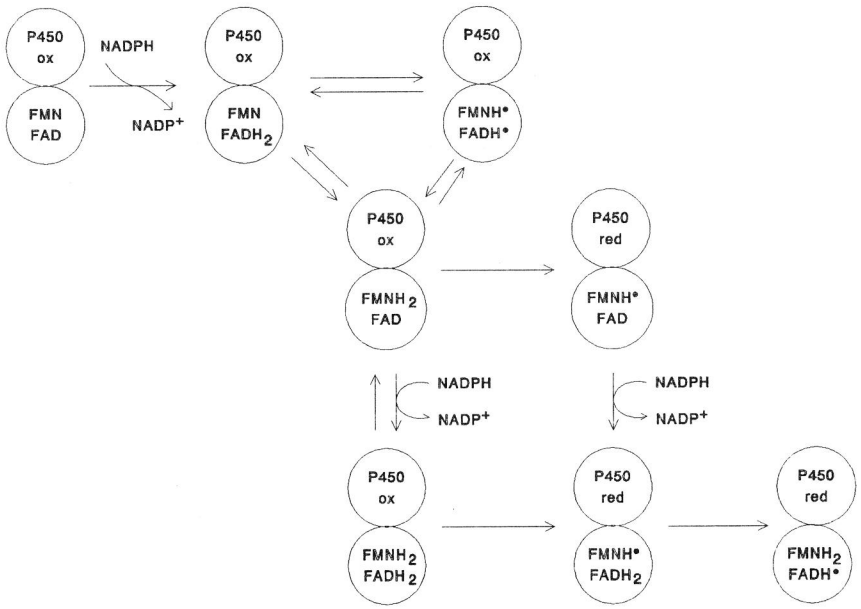

Figure 1.5, Schematical representation of the electron flow from NADPH, via the prosthetic factors FAD and FMN to cytochrome P450. Free after Sevrioukova and Peterson (1995).

CPR could be divided in two domains, a hydrophobic NH_2-terminus of ± 6 kDa which serves to anchor the molecule in the microsomal membrane and a hydrophillic domain of about 72 kDa (Black et al. 1979). In all cytochrome P450 reductase sequences determined thusfar this NH_2-terminal fragment contains a variable region followed by a segment of approximately 20-25 amino acids which is highly hydrophobic and probably involved in membrane binding (Kärgel et al. 1996).

The hydrophillic domain can be divided into four structural domains interacting with the cytochrome P450, NADPH and the cofactors FAD and FMN (Shen et al. 1989; Porter and Kasper 1986; Kasper 1971). The cofactors are important for the electron flow from NADPH to FAD to FMN and finally to the electron acceptor cytochrome P450 as was shown by selective removal of FMN from CPR (Porter 1991; Kurzban and Strobel 1986; Vermillion et al. 1981). Since the different functional domains of the protein, involved in binding of NADPH, FAD and FMN (Figure 1.4; Porter and Kasper 1986 and 1985), are in distinct parts of the

protein, Porter (1991) suggested that CPR arose by fusion of two ancestral genes, one related to flavodoxin and one related to ferredoxin-$NADP^+$ reductase. Expression of the individual domains of human CPR in *E. coli* showed their capability to incorporate the appropriate cofactor (Smith et al. 1994), supporting the hypothesis of Porter.

Although CPR proteins have been crystallized (Djordjevic 1995; Masters et al. 1994) no complete X-ray diffraction data of these CPR-crystals have been reported yet. Some predictions have been made for the structure of the functional domains, based upon comparison of CPR amino acid sequences with crystallized FMN and FAD containing proteins like flavodoxins and ferredoxin $NADP^+$ reductase (van den Brink et al. 1995; Porter and Kasper 1985 and 1986). Comparison of CPR structures with the CPR-like domain of $P450_{BM-3}$ and site directed mutagenesis experiments have been important tools for identification of critical amino acids involved in interactions with different prosthetic factors, NADPH or cytochrome P450. The importance of two invariant tyrosine residues (140 and 178) in binding of the FMN prosthetic factor of rat CPR was determined by site directed mutagenesis (Shen et al. 1989). Replacement of Tyr-178 by phenylalanine had no significant effect on cytochrome c reductase activity or FMN content, while replacement by aspartic acid resulted in an almost complete loss of FMN content and thus of CPR activity. Replacement of Tyr-140 with aspartic acid reduced CPR activity but did not significantly affect FMN content of the protein. Comparable experiments were performed with the $P450_{BM-3}$ protein of *B. megaterium* (Klein and Fulco 1993). Replacement of the conserved glycine (Gly-570) from $P450_{BM-3}$ resulted in complete loss of FMN binding. Depending on the substitution made, replacement in $P450_{BM-3}$ of the two tyrosine residues, which are present in similar positions in rat CPR resulted in complete loss of FMN or had no effect. The interaction of CPR with cytochrome P450 was studied by replacing acidic residues in rat CPR by their non-acidic counterparts in an acidic domain of CPR which was expected to be involved in the interaction with cytochrome P450s. Depending on the residues altered either a reduction of the cytochrome P450 activity or a reduction of cytochrome c reductase activity was found (Shen and Kasper 1990).

4.2 Regulation of cpr gene expression

Regulation of gene expression of some cytochrome P450 genes has been studied in great detail, but no detailed study was carried out on the regulation of the second component of the cytochrome P450 enzyme system, the electron donating system.

Some reports describe a considerable increase of *cpr* mRNA levels (upto 50 fold) in

mammalian liver microsomes in response to some well known inducers of specific cytochrome P450s, such as phenobarbital and *trans*-stilbene oxide (O'Leary et al. 1994; Gonzalez and Kasper 1982), dexamethasone and β-naphtaflavone (Gonzalez and Kasper 1982; Gonzalez et al. 1982; Sheppard et al. 1982). Some increase of CPR activity was observed in the mammalian liver as well but never reached more than 1.5 fold the basal level. One exception has been reported in a study in which an extremely high dosis of DTT was used, resulting in a 10 fold increase of CPR activity (Balakrishnan et al. 1985).

Only few studies have been described in which the relative induction of *cpr* mRNA and of CPR protein levels were compared. From these studies it becomes clear that only in some cases a direct correlation is found between the increase in mRNA levels and the increase in activity levels (Table 1.2).

Table 1.2 Comparison of increase of *cpr* mRNA levels and CPR activities in response to different inducers. Data are for rat liver microsomes after treatment with the compounds and are indicated relative to the data obtained from livers from non-treated animals which was set at 1.

Inducer	Relative increase of *cpr* mRNA	Relative increase of CPR protein/activity	Reference
Phenobarbital	10	1.0 - 1.8	Gonzalez and Kasper 1982
Dexamethasone	7	1.5	Simmons et al. 1987
Pregnenolone 16α-carbonitrile	3	1.2	Simmons et al. 1987
2-acetyl-aminofluorene	1.5	1.2	Gonzalez et al. 1982

Different mechanisms can result in an increase of CPR activity. Some compounds, like phenobarbital, have been reported to increase *cpr* mRNA levels by transcriptional activation (Hardwick et al. 1983), as was shown by *in vitro* nuclear transcription studies. In contrast to this Simmons et al. (1987) describe a sevenfold increase of *cpr* mRNA level in response to dexamethasone due to posttranscriptional mRNA stabilization (in vitro transcription studies indicated that the transcription rates remained unaltered). The role of posttranslational regulation has not been well studied. However, studying the effect of development on *cpr* mRNA levels in rat, Simmons and Kasper (1989) observed a decline in *cpr* mRNA levels 35 days after birth while CPR protein levels remained unaltered, suggesting the presence of a CPR stabilizing factor.

Besides its major activity, electron donation to cytochrome P450s, CPR is able to provide electrons to a number of different microsomal proteins like cytochrome b_5 (Ilan et al. 1981; Enoch and Strittmatter 1979), heme oxygenase (Schacter et al. 1972) and to non-physiological electron acceptors like cytochrome c, ferricyanide and menadione (Williams and Kamin 1962). Using antibodies directed against CPR, Ilan et al. (1981) were able to reduce microsomal mediated fatty acid elongation with 50%. CPR was shown to be important for the activation of anti-tumour compounds like mitomycin C (Keyes et al. 1984) and anthracycline (Bachur et al. 1978). In COS cells overexpression of the human *cpr* gene resulted in a clear increase in sensitivity to mitomycin caused by the reductive activation of mitomycin by CPR resulting in DNA cross-linking (Belcourt et al. 1996). Although CPR is involved in many important physiological processes, deletion of the *cpr* gene in *S. cerevisiae* did not result in a change in phenotype besides an increase in sensitivity to the fungicide ketoconazole (Sutter and Loper 1989).

Another important side effect of CPR activity is the production of oxygen radicals. CPR can perform its NADPH oxidase reaction without the presence of a redox partner (Mukhopadhyay and Chatterjee 1994; Winston and Cederbaum 1983). In reconstitution experiments using cytochrome P450 reductase and cytochrome P450s isolated from phenobarbital treated rats, Winston and Cederbaum (1983) showed the formation of OH-radicals with different scavengers. The formation of OH-radicals was shown to be completely dependent on the concentration of CPR in the reconstituted system while changing the cytochrome P450 concentration or adding heat denatured CPR did not have any effect on the scavenger conversion rate. Since the reactions were strongly inhibited by catalase and superoxide dismutase it seems likely that the hydroxyl radicals are formed from H_2O_2. However, a role of cytochrome P450s, and not CPR, in the formation of oxygen radicals can not be excluded. Incubation of microsomes obtained from the liver of ethanol fed rats resulted in strong production of H_2O_2 which was inhibited by the addition of a number of inhibitors and ligands of cytochrome P450 (Kukielka and Cederbaum 1995). However, formation of active oxygen radicals can also be of physiological importance since it may result in initiation of lipid peroxidation (Sevanian et al. 1990).

Some data are available about the regulation of *cpr* gene expression in lower eukaryotes. In the yeast *S. cerevisiae,* Stansfield et al. (1991) showed a clear decrease in *cpr* transcript level when *S. cerevisiae* was grown in (semi) anaerobic conditions while the levels of

cytochrome b_5 and *cyp51* were increased under low oxygen conditions. In the *n*-alkane-assimilating yeasts *Candida maltosa* and *Candida tropicalis*, each containing a family of closely related *cyp* genes involved in the *n*-alkane assimilating pathway, the expression of the *cpr* genes was shown to be increased 6-8 fold after the addition of *n*-alkanes to the medium (Ohkuma et al. 1995; Sutter et al. 1990). Induction of CPR activity in filamentous fungi seems to be highly strain specific.

Table 1.3, Induction of CPR activity in *Aspergillus ochraceus* TS.
1): CPR activities in microsomal fractions (nmol.min^{-1}.mg protein^{-1})
- : Not determined

Inducer (100 µM)	A[1)]	B[1)]	C[1)]	Average activity[1)]	Relative activity
Control	-	24	30	27	1.0
Benz<a>pyrene	46	-	45	46	1.7
3-Methylcholanthrene	45	40	42	42	1.6
Phenobarbital	150	120	150	140	5.2
ß-Naphtoflavone	59	-	59	59	2.2
Benzanthracene	49	-	-	49	1.8
PCB (Arocolor)	115	90	115	107	4.0
Pyrene	59	-	-	59	2.2
Naphthalene	49	-	48	49	1.8
Anthracene	50	-	-	50	1.9
Progesterone	78	74	78	77	2.9
19-Nor-testosterone	-	105	-	105	3.9
Testosterone	-	72	-	72	2.7
Nicotinic acid	-	130	-	130	4.8
Xylene	-	147	-	147	5.4
Toluene	-	101	-	101	3.7
Phenanthrene	-	-	49	49	1.8

A) Ghosh et al. 1983, B) Samanta and Ghosh 1987, C) Dutta et al. 1983

CPR activity in microsomes isolated from phenobarbital treated mycelium from *Aspergillus ochraceus* (Ghosh et al. 1983) and *A. parasiticus* (Bhatnagar et al. 1982) was increased three to five fold (Table 1.3) while phenobarbital treatment did not affect *cprA* mRNA levels in *A.*

niger (van den Brink et al. 1995) or CPR activity in *A. fumigatus* (Baillie 1993). The same strain specificity was observed by these authors for other compounds like benz<a>pyrene. An approximately 4 fold increase of CPR activity in *Aspergillus niger* was found after exposure to benzoate, which also results in a strong induction of the fungal *bphA* gene (*cyp53*) (van den Brink et al. 1995).

Currently the knowledge about regulation of internal CPR levels in different organisms is scarce and fragmentary. Most of the data have been obtained in studies aimed at the investigation of cytochrome P450 gene expression whereas studies on coregulation of *cpr* gene expression were performed only incidentally and non-structured.

4.3 cytochrome b_5

Recent studies indicate that another important element in the electron donating system of some, but not all, cytochrome P450 enzymes, is cytochrome b_5. Urban et al. (1990) were the first to show that overexpression of cytochrome b_5 in *S. cerevisiae* resulted in an increase of P450 activity. Vergères and Waskell (1992) confirmed these results by overexpression of rat cytochrome b_5 in *S. cerevisiae*. Later a number of contradictory results were reported showing very different effects of cytochrome b_5 overexpression on a number of different P450 activities. Positive effects on P450 activities by addition of endogenous cytochrome b_5 were reported for mouse CYP1A1 (2 fold increase) and human CYP3A4 (6 fold increase) while negative effects were reported for human CYP21A1 (2 fold decrease; Urban et al. 1990 and references therein). Studies by Truan et al (1993) in which the gene encoding cytochrome b_5 was co-expressed with the human genes encoding CYP3A4 and CYP1A1 also resulted in contradictory results.

The effect of cytochrome b_5 on cytochrome P450 activities is not only dependent on the cytochrome P450 gene expressed but also on the expression system used. Studies of the effects of cytochrome b_5 on activation of one particular cytochrome P450 in different expression systems are often contradictionary. For exemple in a yeast *cyp1A1* expression system increasing the cytochrome b_5 levels resulted in two fold elevated activity levels (Urban et al. 1990) while co-expression in insect cell lines, using the baculovirus system, of cytochrome b_5 with the same cytochrome P450 gene did not result in any increase in CYP activity (Buters et al. 1995). Using the same baculovirus expression system Lee et al. (1995) could not detect any effect of additional cytochrome b_5 on activity of CYP3A4, in contrast to the six fold increase in activity reported by Urban et al. (1990) in *S. cerevisiae*.

The exact mechanism by which cytochrome b_5 is able to stimulate activity levels of some cytochrome P450s remains unclear. However, some interesting experiments have been carried out to unravel the mechanism. Truan et al. (1994) were able to clone the cytochrome b_5 gene of *S. cerevisiae* by complementation of the ketoconazole resistance phenotype of a *cpr* deletion strain, using a genomic library. Knocking out the cytochrome b_5 encoding gene (*Cyb5*) in a wildtype strain surprisingly resulted in an increase in the overall cytochrome P450 amounts but not in any significant phenotypical changes. In contrast, disruption of the *Cyb5* locus in a Δ-*cpr* strain was lethal. These results indicate that CPR and cytochrome b_5 may act as complementing electron donor systems for, at least some, vital P450 activities. Using independent expression systems, Patten and Koch (1995) and Shet et al (1995) showed a clear effect of cytochrome b_5 on the formation of H_2O_2 and NADPH consumption. The effect of cytochrome b_5 on the interaction between CPR and P450s was studied in a titration experiment. Using invariable CYP2E1 and increasing CPR concentrations, cytochrome b_5 did not influence the titration effect (Patten and Koch 1995), showing that cytochrome b_5 does not influence the interaction between the other components of the enzyme system. Following a completely different approach, two research groups independently showed the formation of transient complexes between P450 and both CPR and cytochrome b_5 (Yamada et al. 1995; Jansson and Schenkman 1995). Cross linking experiments reported in both papers clearly showed interactions between different P450s (CYP1A2 and CYP2B4) and cytochrome b_5. Only CYP2B4 was found to cross link to CPR as well, suggesting that either cytochrome b_5 is the major electron donor for CYP1A2 or that cytochrome b_5 functions as an intermediate between the CYP1A2 and CPR molecules.

The importance of cytochrome b_5 in the electron donation pathway of some cytochrome P450 enzymes seems to be clear. However, where CPR activity seems to be a prerequisite for activation of all endoplasmic cytochrome P450s, the activating effect of cytochrome b_5 seems to be additional and depending on the cytochrome P450 species involved.

5. Outline of this thesis

Cytochrome P450 enzymes are involved in complex bioconversion reactions in eukaryotic organisms. Cytochrome P450 systems have been extensively studied at the molecular level in mammalian systems. However, molecular biological studies on cytochrome P450 mediated systems in filamentous fungi are scarce.

The aim of the research presented in this thesis was to provide insight in ways to improve cytochrome P450 activities in *A. niger* by co-overexpression of the CPR encoding gene together with the cytochrome P450 gene of interest. A second point of interest was to study the complex mechanisms underlying the co-regulation of CPR and cytochrome P450 gene expression.

In chapter 2 of this thesis the cloning and characterization of the *Aspergillus niger* gene encoding cytochrome P450 reductase (*cprA*) is described. Some data are presented on the response of *cprA* gene expression to different inducers of cytochrome P450s known from mammalian systems. The construction of a *cprA* overexpressing *A. niger* strain is described as well.

In chapter 3 construction of strains with multiple copies of *cprA* and/or of the gene encoding the fungal sterol 14α-demethylase (*cyp51*) is described. The transformants were analyzed for resistance to a number of commercially available sterol 14α-demethylase inhibitors (DMI's), which can be used as a measure for sterol 14α-demethylase activities in these strains.

Chapter 4 describes the construction of *A. niger* strains containing multiple copies of the *cprA* gene and/or the *A niger* gene encoding the cytochrome P450 enzyme benzoate para-hydroxylase (*bphA*). The strains generated were analyzed for BPH activity levels to evaluate the effect of *cprA* overexpression on BPH activity levels.

Regulation of *cprA* gene expression was studied in more detail in chapter 5. The transcription control region of *cprA* was analyzed using a promoter-*uidA* fusion construct and detailed mRNA analysis. Aspects of co-regulation of *cprA* gene expression with gene expression of a cytochrome P450 encoding gene were studied using the *bphA* system.

In chapter 6 a general discussion of the results presented in this thesis is given. Furthermore, a hypothesis is presented describing the possible mechanisms for regulation of *cprA* gene expression in response to the presence of benzoate

DNA Cell Biol. ***14(8)***, *719-729 (1995).*

Chapter 2

Cloning and Characterisation of the NADPH Cytochrome P450 Oxidoreductase Gene from the Filamentous Fungus *Aspergillus niger*

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Cees A.M.J.J. van den Hondel and Robert F.M. van Gorcom.

Abstract

In this chapter we describe the cloning and molecular characterisation of the *Aspergillus niger* cytochrome P450 reductase gene, *cprA*. Attempts to clone the *cprA* gene by heterologous hybridisation techniques were unsuccessful. Using PCR with degenerate primers based on conserved regions found in *cpr* genes from other organisms, we were able to isolate a fragment that contained part of the gene. With the aid of this fragment, a genomic fragment, containing the entire coding region and 5'- and 3' untranslated ends of the *cprA* gene, was isolated and sequenced. The *cprA* gene was introduced in multiple copies in *A. niger* strain N402 using the *amdS* transformation system. One of the resulting transformants, AB2-2, showed a 14 fold increase in CPR activity, indicating that the cloned *cprA* gene is functional. We analysed the induction of *cprA* gene expression by several generally used cytochrome P450 inducers but did not find any induction of *cprA* gene expression. However, *A. niger cprA* gene expression could be induced by benzoic acid, which is the substrate of the highly inducible *A. niger* cytochrome P450 gene *bphA* (*cyp53*).

Based on a comparison of the deduced protein sequence of the *A. niger cprA* gene with CPR proteins isolated from other organisms, the structure-function relationship of some conserved regions is discussed.

Introduction

Members of the cytochrome P450 enzyme superfamily are found throughout nature and are involved in a wide range of very different biotransformation reactions. Most of these cytochrome P450 reactions involve the addition of one atom of molecular oxygen to the substrate molecule, the second atom being converted to water. The electrons needed for this reaction mechanism are usually donated by special electron transfer proteins. In general, substrates of cytochrome P450 enzymes are lipophilic substances.

To date more than 500 cytochrome P450 genes have been cloned (Nelson et al., 1996). However, only few fungal cytochrome P450 genes have been characterised so far. One of these fungal genes is the *bphA* gene of *Aspergillus niger* (cyp53) which is involved in the conversion of benzoic acid to para-hydroxy-benzoic acid (van Gorcom et al., 1990). In addition, the *pda* gene (cyp57) of *Nectria haematococca* (Weltring et al., 1988) was isolated, which is involved in demethylation of pisatin, an anti-fungal compound secreted by some plant species. Recently, a cytochrome P450 (like) gene, called $P450_{nor}$ (cyp55), was cloned from the fungus *Fusarium oxysporum* (Kizawa et al., 1991). $P450_{nor}$ is involved in the reduction of nitrate. This cytochrome P450 is very different from all other eukaryotic cytochrome P450 enzymes since it is a soluble protein (the first soluble eukaryotic cytochrome P450 reported) and uses a substrate that is not lipophilic. Another unique feature of $P450_{nor}$ is its reaction mechanism. $P450_{nor}$ is able to reduce its substrate independently of any electron transfer protein and in the absence of oxygen.

The cytochrome P450 superfamily can be divided in three major groups, based on the type of electron transfer system used. The first group is able to reduce substrates independently of an electron transfer system. Thus far $P450_{nor}$ is the only reported member of this group. The second group, which comprises almost all prokaryotic cytochrome P450s and eukaryotic mitochondrial cytochrome P450s, is dependent on the activity of a two component electron transfer system. In prokaryotes electrons are transferred from NAD(P)H via ferredoxin reductase (in mitochondria adrenodoxin reductase is used), a flavin containing enzyme, to ferredoxin (adrenodoxin in the mitochondrial system), an iron-sulphur protein, and then to the cytochrome P450. The best studied example of this group is the soluble cytochrome P450 camphor hydroxylase ($P450_{cam}$) from *Pseudomonas putida* (Poulos and Raag, 1992). The third group comprises all eukaryotic non-mitochondrial cytochrome P450 enzymes. These enzymes, anchored in the membrane of the endoplasmic reticulum, require the activity of only one electron donating protein, the NADPH-cytochrome P450 oxidoreductase (CPR). CPR is a

flavoprotein containing 1 mol each of the prosthetic factors FAD and FMN per mol protein.

In *Bacillus megaterium* an exceptional cytochrome P450, BM_3, was found. This protein is a soluble self sufficient cytochrome P450 that proved to be a fusion of a cytochrome P450 protein with a CPR-like protein (Ruettinger et al., 1989). It is the only natural cytochrome P450 - CPR fusion protein known thus far and contains an eukaryotic-like electron transfer system.

In the last several years the isolation of *cpr* cDNA or genomic DNA clones has been reported from *Saccharomyces cerevisiae* (Yabusaki et al., 1988), *Candida tropicalis* (Sutter et al., 1990), *Schizosaccharomyces pombe* (Miles et al., 1993), *Vigna radiata* (Shet et al., 1993), *Catharanthus roseus* (Meijer et al., 1993), rat (Porter and Kasper, 1985), mouse (Ohgiya et al., 1992), rabbit (Katagiri et al., 1986) and man (Haniu et al., 1989). From CPR of trout (Urenjak et al., 1987) and pig (Haniu et al., 1986) the primary structure of the purified protein was determined. Comparison of the (deduced) amino acid sequences of these proteins shows several conserved regions involved in specific interactions.

Introduction of multiple copies of cytochrome P450 reductase genes into a cytochrome P450 overproducing *S. cerevisiae* strain resulted in a considerable increase in cytochrome P450 enzymatic activity, indicating its importance for cytochrome P450 dependent activities (Murakami et al., 1990). Construction of cytochrome P450-CPR fusion proteins also proved to be a valuable tool for increasing cytochrome P450 related enzymatic activities in yeast (Sakaki et al., 1990).

In previous work in our laboratory we cloned and overexpressed a cytochrome P450 gene (*bphA*) from the filamentous fungus *A. niger,* encoding benzoate para-hydroxylase (van Gorcom et al., 1990). Surprisingly, overexpression of this gene did not result in a significant increase in BPH activity. One of the reasons for this hampered increase in cytochrome P450 enzyme activity might be a shortage of the second component of the cytochrome P450 system, the NADPH cytochrome P450 oxidoreductase. Therefore we decided to attempt cloning and overexpressing of the gene encoding CPR of *A. niger*.

In this chapter we describe the cloning and molecular characterisation of a functional copy of the cytochrome P450 reductase gene (*cprA*) from *A. niger*, and the effect of different cytochrome P450 inducing substances on *cprA* gene expression.

Materials and Methods

Strains and plasmids

A. niger strains N402 (a derivative of ATCC 9029, *cspA*1; Bos, 1986), AB4.1 (N402 *pyrG*$^-$; van Hartingsveldt et al., 1987) and N204 (ATCC 1015, *csp21, met21*; Boschloo et al., 1990) were used. *Escherichia coli* K12 strain JM109 (Yanisch-Perron et al., 1985) was used for construction and propagation of vector molecules.

For subcloning, plasmids pUC19 (Yanisch-Perron et al., 1985) and pBluescript SK II(+) (Stratagene, La Jolla) were used. Probes to be used in heterologous hybridisation experiments were isolated from plasmids pTS1 (Sutter et al., 1990), and pTS20 (Sutter and Loper, 1989).

Probes used in Northern analysis were a 1.4 kb *Eco*RI-*Bam*HI fragment isolated from plasmid pCPR1 (*cprA*-specific probe, this chapter) and a 1.5 kb *Hin*d III fragment from plasmid pAB5-2 (*A. niger gpdA*; Punt et al., unpublished).

Polymerase chain reaction

Primers used in PCR reaction were MBL997 (CCG GAA TTC CA(G/A) ACN GGN ACN GCN GA(G/A) GA; 1024 times degenerated), MBL998 (CCG GAA TTC GGN GAN CCN ACN GA(T/C) AA(T/C) GC; 1024 times degenerated), MBL999 (CGC GGA TCC GGN CCN A(C/T)N A(G/T/A)(G/T/A) ATN AC; 4608 times degenerated), MBL1000 (GCG GGA TCC T(C/G)(C/T) TGN AC(G/A) TAN AC(C/T) TT; 256 times degenerated) and MBL1001 (CGC GGA TCC GGN CC(G/T/A) ATC AT(G/T/A) ATN AC; 144 times degenerated).

A. niger N402 chromosomal DNA (100 ng) was used as a template and denatured for 10 min at 94 °C, followed by 25 cycles (1 min 94°C, 1 min 43 °C, 2 min 72°C) in a Perkin-Elmer DNA thermocycler. PCR reactions were carried out with *Tth* DNA polymerase (Sphaero Q, Leiden, the Netherlands).

Preparation of A. niger cDNA library and determination of intron sequences

Total RNA was isolated from *A. niger* strain N402 and poly-A$^+$ RNA was isolated using oligo-dT spin columns (Pharmacia). A cDNA library was prepared from 5 μg mRNA using the λ-ZAP cDNA cloning system (Stratagene, La Jolla). To determine the putative intron

sequence 1 µg DNA was isolated from this library and used as template in a PCR reaction. Primers used to determine the intron sequence were CPR11 (ACG GCT TGA CTT ACT C) and CPR20 (CGT CAC CAC AGA CGT A). PCR reaction conditions were: 5 min 94 °C, followed by 30 cycles (1 min 94°C, 1.5 min 43 °C, 2 min 72 °C).

The positions of the primers are indicated in Figure 2.2.

Isolation of A. niger cpr λ-clones

PCR reaction products were separated by electrophoresis on a 1% TAE-agarose gel. A product of the expected size was isolated from gel and cloned in pUC19. The cloned fragment was labelled with ^{32}P-dCTP using the Amersham multiprime labelling kit and used as a probe to screen 40,000 plaques (approximately 20 x genome size) from a library of *A. niger* N204 genomic DNA in λEMBL3 (van Gorcom et al., 1990). The final washing was performed at 65 °C in 0.2 x SSC. Individual plaques were isolated after serial dilution and rescreening.

Induction experiments

For induction experiments mycelium from strain AB4-1 was cultured in complete medium (Pontecorvo et al., 1953) for 18 hr. Mycelium was harvested by filtration through Miracloth, washed extensively with 0.9 % NaCl and transferred to induction medium (minimal medium with 0.05% glucose and inducing substances as C-sources). After a 3-hr induction period, mycelium was harvested by filtration through Miracloth and frozen in liquid nitrogen.

Other molecular biological methods

DNA manipulation and bacterial transformation were performed using standard methods (Sambrook et al., 1989). DNA sequences were analysed using Genetics Computer Group programmes (Devereux et al., 1984). Total fungal RNA was isolated using the RNAzol™ kit from CINNA/BIOTECX.

Primer extension experiments were performed using primers PE1 (GAG TTG CGC CAT GGT G) and PE50 (CCA CGC TAC CCA C) and approximately 5 µg mRNA as a template.

Transformation of A. niger

Fungal transformations were performed as described before (Yelton et al., 1984) using plasmid pCPR2amdS (Figure 2.3). After transformation, protoplasts were selected by growth on acetamide as described (Kelly and Hynes, 1985). Plates were incubated at 35 °C for 10 days.

RNA colony hybridisation

For RNA colony hybridisation of *A. niger* transformants a protocol, adapted from Stepien and Butow (1992), was used. In this protocol nucleic acids are blotted to a sheet of Hybond-N membrane without denaturing double stranded DNA. Thus, only RNA will hybridize to the probe.

Spores of transformants were inoculated on minimal medium agar plates, covered with a Hybond-N filter, and incubated at 25°C until mycelium was just visible. Filters were lifted and placed upon a 500 µl drop of sorbitol-buffer (1.2 M sorbitol, 0.1 M sodiumcitrate/pH 5.8, 0.1 M EDTA, 50 mM ß-mercaptoethanol) for 5 min and subsequently dried on a sheet of Whatmann 3MM filtration paper. Protoplasts were formed from the mycelium by incubation of the filter in a petri dish containing 500 µl of protoplasting-buffer (sorbitolbuffer with 10 mg/ml Novozym 234 (Novo Nordisk)), at 35 °C for 1 hour. Protoplasts were lysed by incubating the filter for 5 min on 500 µl lysis buffer (2% SDS, 7.3% formaldehyde, 50 mM Tris-HCl/pH 7.5, 10 mM EDTA). Subsequently filters were dried by blotting on filtration paper. This step was repeated once. To blot the RNA the filter was transferred to filtration paper after a 2 min incubation on a 700 µl drop of 6xSSC, 0.1% SDS. RNA was fixed to the Hybond N filter by illuminating for 3 min with a UV-illuminator. Filters were hybridised overnight at 65 °C with a ^{32}P-dCTP labelled *cprA* probe. Washing was carried out at the same temperature using 0.2xSSC, 0.1% SDS in the final wash step. Positive transformants could be identified after overnight exposure at -70 °C.

CPR activity assay

Mycelium was ground to a fine powder in liquid nitrogen using a Dismembrator II apparatus (Braun) for 2 min. Mycelial powder was dissolved in 1 ml cold extraction buffer (50 mM sodium phosphate buffer/pH 7.8, 20 % glycerol, 1 mM EDTA, 1 mM DTT, 4 mM PMSF, 0.2% sodiumdeoxocholate), mixed immediately and stored on ice for approximately 15 min.

After centrifugation for 15 min at 4 °C, 1000 g, CPR activity was measured in the supernatant by following the reduction of cytochrome c spectroscopically at 550 nm at room temperature for 2 min as described by Madyastha et al.(1979).

Protein concentrations were determined using the Bio Rad protein assay kit with Bovine Serum Albumin (Sigma) as a standard.

Results

Isolation of the A. niger cprA gene

In an attempt to isolate the *A. niger cprA* gene, heterologous hybridisation experiments were carried out, using the *cpr* genes of *C. tropicalis* (3.7 kb *Spe*I fragment isolated from plasmid pTS1; Sutter et al., 1990) and *S. cerevisiae* (3.3 kb *Pvu*II fragment, isolated from plasmid pTS20; Sutter and Loper, 1989) as a probe. In a Southern blot experiment, using 10 µg of *A. niger* chromosomal DNA, no specific hybridising bands were obtained (hybridisation at 48 °C, followed by a final washing step at 6xSSC, 0.1% SDS). Since these experiments were not successful, a PCR approach was used to isolate *cpr* specific fragments from the *A. niger* genome.

Degenerated PCR primers were designed based on conserved regions present in characterized CPR genes from other organisms. An *Eco*RI site and a *Bam*HI site were present at the 5' ends of the forward and reverse primers, respectively, to facilitate subcloning of the PCR fragment.

A. niger N402 chromosomal DNA (100 ng) was used as a template. PCR reactions resulted in many aspecific fragments of small size, due to the high degree of degeneracy in the PCR primers. However, using primer combination MBL997-MBL1001 a fragment of about the expected size (1.4 kb) was found. No larger fragments were detectable. This fragment was isolated from gel and subcloned in pUC19, resulting in plasmid pCPR1. A partial sequence analysis of the cloned insert of pCPR1 revealed sequence similarity to sequences of known *cpr* genes.

Approximately 40,000 plaques from an *A. niger* N204 genomic library (van Gorcom et al., 1990) were screened using the *cpr*-specific *Eco*RI-*Bam*HI fragment of pCPR1 as a probe. After rescreening, 4 positive clones were isolated and characterized by restriction analysis. All clones were closely related although not identical. Phage λ19-1, expected to contain the

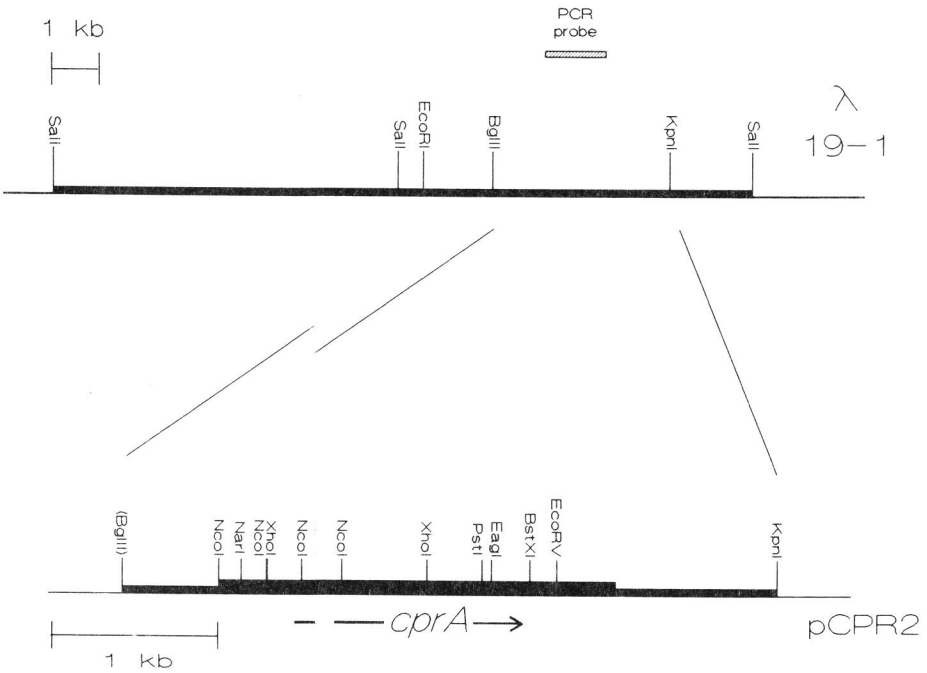

Figure 2.1, Partial restriction map of phage λ19-1. The bar indicates the position of the PCR derived *cpr* specific probe. The 3.7 kb *Bgl*II-*Kpn*I fragment of phage λ19-1, comprising the complete coding region of the *A. niger cprA* gene, was subcloned in pBluescript, resulting in plasmid pCPR2.

complete coding region of the *cpr* gene, was used in further experiments. A restriction map of clone λ19-1 is shown in Figure 2.1. A 3.7 kb *Bgl*II-*Kpn*I fragment of clone λ19-1 was subcloned in pBluescript SK II(+) resulting in plasmid pCPR2.

Characterisation of the A. niger cprA gene

The nucleotide sequence of the 3.7 kb insert of plasmid pCPR2 was determined independently on both strands (Figure 2.2). The insert contains an open reading frame of 2150 bp interrupted by one intron of 71 bp. The presence of this intron was confirmed by PCR and sequence analysis. Primers, flanking the intron sequence were used in PCR reactions using cDNA (derived from an *A. niger* cDNA library) and genomic DNA (plasmid pCPR2) as a template. A clear difference in size of the generated fragments was found (not shown). The presence of the intron was proven by subcloning and sequence analysis of the derived PCR-fragment.

The *A. niger cprA* gene encodes a protein of 693 amino acids. Its deduced amino acid sequence contains all conserved regions found in other *cpr* genes and is around 30%-40% identical to *cpr* genes of other organisms described so far.

Three transcription start points were found in primer extension experiments using primers PE1 and PE50 (results not shown). A major transcription start point was present at 338 bp upstream of the predicted translation start codon. Two minor starts were found 281 bp and 81 bp upstream of the ATG codon.

The presence of additional *cpr*-like sequences in the genome of *A. niger* was investigated by Southern analysis of *A. niger* chromosomal DNA using heterologous hybridisation conditions and a *cprA* specific probe. Hybridisation at 48 °C followed by a final washing step at 6xSSC, 0.1% SDS, revealed no additional hybridising bands.

CHEF gel analysis revealed that the *cprA* gene of *A. niger* is located on chromosome VIII (Verdoes et al. 1994a).

Table 2.1, NADPH:cytochrome c reductase activity of a wildtype *A. niger* strain, having only one copy of the *cprA* gene (N402), and of a *cprA* multiple copy strain (AB2-2). CPR activity was measured as described in the **materials and methods** section. One unit corresponds to the reduction of 1 mmol cytochrome c per milligram protein per minute at room temperature.

Strain	*cprA*-copy number	CPR-activity (units)
N402	1	1.05
AB2-2	10	14.64

Functional characterisation of the A. niger cprA gene

To prove the functionality of the cloned *cprA* gene, plasmid pCPR2 was introduced in multiple copies in *A. niger* strain N402. For selection of transformants the *A.nidulans amdS* gene, present on a *Not*I fragment, was cloned into the *Not*I site of plasmid pCPR2, resulting in plasmid pCPR2amdS. After transformation with plasmid pCPR2amdS (Fig. 2.3), transformants were selected as described by Verdoes et al. (1993). Transformants were subsequently screened by RNA colony hybridisation for the presence of increased *cprA*

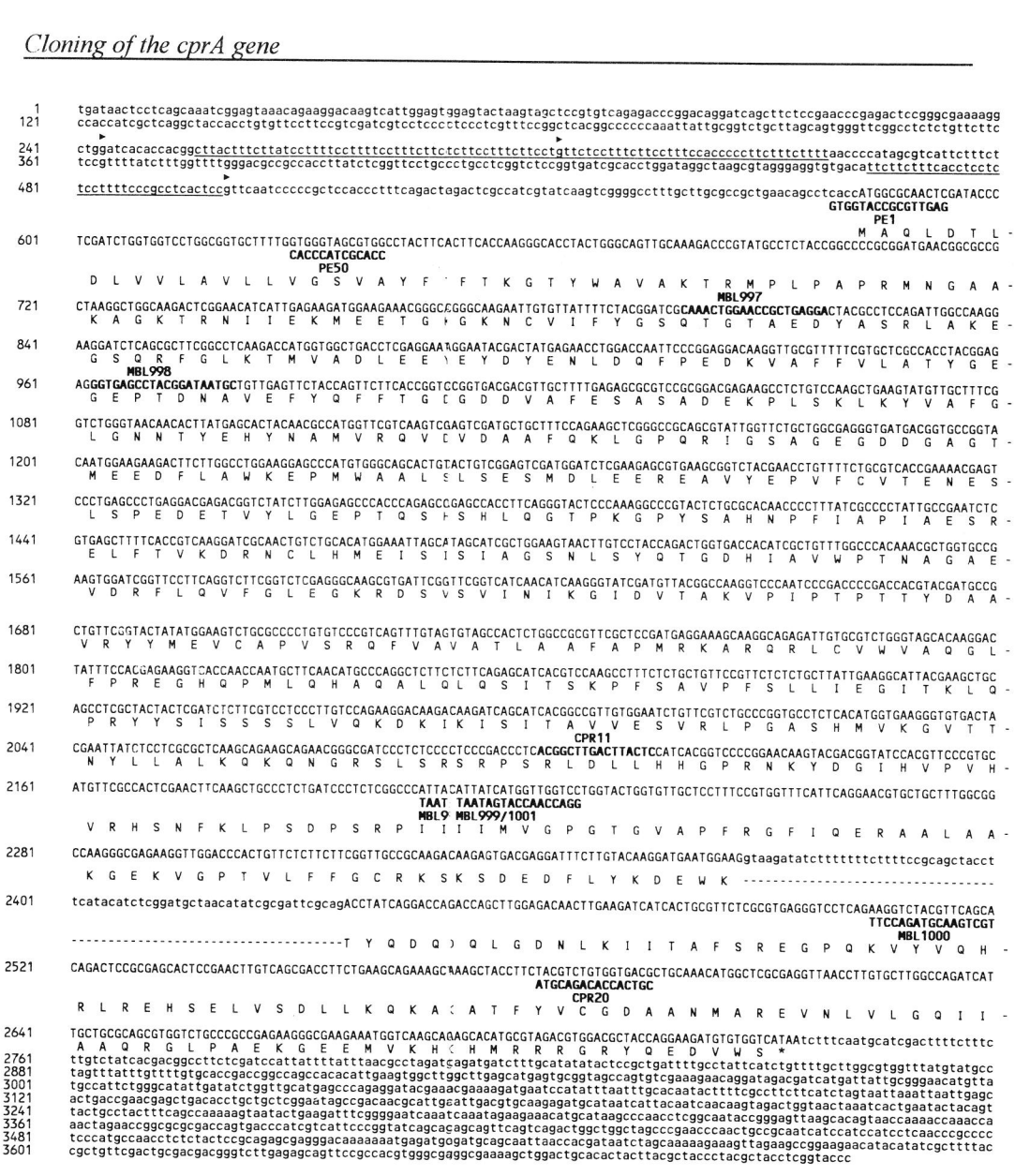

Figure 2.2, Complete nucleotide sequence of the *Bgl*II-*Kpn*I fragment of phage λ19-1, comprising the complete coding region of the *A. niger cprA* gene, preceded by a 581 bp untranslated sequence. Sequence analysis was performed independently on both strands. Indicated are primers used for cloning the gene (MBL997, MBL998, MBL999, MBL1000 and MBL1001), primers used to determine the exact position of the intron (CPR11 and CPR20) and primers used in primer extension experiments (PE1 and PE50).
(▸) indicates transcription starts.
The CT rich domains, probably involved in transcription initiation are underlined.
The sequence of the *cprA* gene is available from the EMBL gene bank under accession number Z26938.

mRNA levels. Transformants with increased *cprA* mRNA levels were analysed by Southern analysis (results not shown). Transformant AB2-2, in which the *cprA* mRNA level was increased more than 10 fold, was selected for further experiments. NADPH:cytochrome c reductase activity was measured as described (Table 2.1). Introduction of additional copies of the *cpr*A gene resulted in a 14 fold increase in NADPH:cytochrome c reductase activity whereas NADH:cytochrome c reductase activity was not significantly increased in transformant AB2-2. These results clearly indicate that a functional *A. niger cprA* gene was cloned.

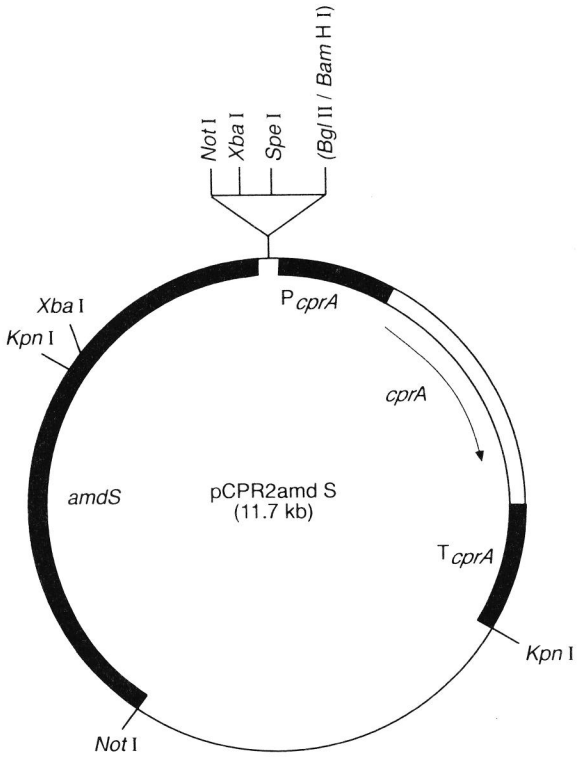

Figure 2.3, Plasmid pCPR2amdS constructed by cloning a 5.1 kb *Not*I fragment, containing a functional *A. nidulans amds* gene, in the unique *Not*I site of pCPR2.

Inducibility of the cprA gene

A. niger mycelium was transferred to different media containing some well-known general inducers of mammalian cytochrome P450 genes and to a medium containing benzoic acid. To reduce glucose repression effects, only a limited amount of glucose was added to the medium. In these experiments, none of the inducers used was able to increase expression of the *cprA* gene significantly, except for benzoic acid (Figure 2.4). To analyse induction of *cprA* gene expression by benzoic acid in more detail, a time-course experiment was carried out. CPR activity rapidly increases upon addition of benzoic acid. One hour after transfer to induction medium, a clear increase of CPR activity was detectable (Figure 2.5). A clear increase in mRNA level was observed already

after 30 min (not shown).

After prolonged induction, CPR activity decreased (not shown), probably due to stress caused by depletion of carbon sources in the medium or by toxicity of the benzoic acid.

Discussion

Cloning and physical characterisation of the cprA gene

Using highly degenerated primers, based on conserved sequences in *cpr* genes, on *A. niger* chromosomal DNA, we were able to generate a fragment of expected size that showed clear sequence similarity to other *cpr* genes. Using this PCR derived fragment we were able to isolate a clone from a genomic library comprising the complete coding region of the *A. niger cprA* gene. Sequence analysis of this clone revealed that the *cprA* gene contains all conserved regions characteristic for *cpr* genes. The DNA sequence of the *cprA* gene was only 52% identical to the *S. cerevisiae cpr* gene and 57% identical to the *C. tropicalis cpr* gene. This low identity could explain the lack of hybridization observed in Southern blot experiments using *cpr*-specific DNA fragments from these organisms as a probe.

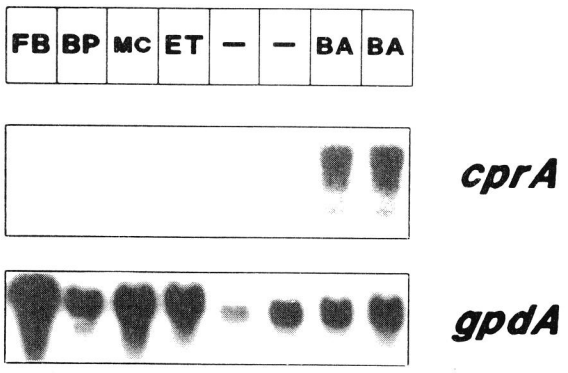

Figure 2.4, Induction of *cprA* gene expression by different inducers. Mycelium was transferred to minimal medium with 0.05% glucose and inducers. After 3 hr mycelium was harvested and RNA isolated. **FB**, 100 µM Fenobarbital; **BP** 100 µM benzo<a>pyrene; **MC**, 100 µM 3-methyl-cholanthrene; **ET**, 5% Ethanol; **BA**, 0.1% benzoic acid; -, no inducer.

The sequences surrounding the translation start (AUG) at position 582 of the sequence resembles the consensus sequence for higher eukaryotes and fungi (Kozak, 1986; Gurr et al., 1988). The presence of CT rich regions, found immediately upstream of one of the transcription starts is a feature found in many fungal promoters. These pyrimidine rich regions seem to be involved in the determination of the transcription initiation sites and are expected to have a function comparable to CAAT and TATA boxes in other eukaryotic promoters (Punt and van den Hondel, 1992). However, as found in several other fungal promoters, the main transcription

start of the *cprA* gene is not preceded by any of these sequence motifs.

To exclude the presence of additional *cpr* genes in the genome of *A. niger*, as was recently found in the plant *A.thaliana* (two functional *cpr* genes were cloned with 63% identity at the protein level; EMBL Gene Bank accession numbers X66016 and X66017), a low stringency hybridisation experiment was performed using the cloned *cprA* gene as a probe. No second hybridising band was detectable in this experiment. However, the presence of a second, functional, *cpr* gene in the *A. niger* genome cannot be completely excluded, because the homology between nucleotide sequences of the known *cpr* genes is low.

cprA gene expression

To date only little is known about regulation of *cpr* gene expression in different organisms. Because some cytochrome P450 genes are highly regulated (e.g. the *A. niger bphA* gene; van Gorcom et al. 1990 and the *N. haematococca pda* gene; Weltring et al. 1988), it seems likely that also *cpr* gene expression is regulated to some extend, although a basal level of *cpr* gene expression will be needed.

Under noninduced conditions *cprA* gene expression is relatively low. Only weak signals could be identified by Northern blot analysis. Total cytochrome P450 contents in *A. niger* microsomes are also low compared to those of other fungi (Faber and Kelly; personal communication). These results suggest a close correlation between the expression levels of both components of the cytochrome P450 system in the endoplasmic reticulum in *A. niger*.

To study *cprA* gene expression in more detail, plasmid pCPR2amdS was introduced in multiple copies in a wildtype *A. niger* strain. Transformants were screened for overexpression of the introduced *cprA* gene using a newly developed technique for filamentous fungi, RNA colony hybridisation. This technique offers advantages over standard techniques currently used for screening of large numbers of transformants. Standard techniques used are either based on DNA hybridisation (DNA colony hybridisation) or enzymatic activities in plate screening tests. For many genes no simple plate screening test, detecting differences in enzymatic activity, is available. In these cases DNA colony hybridisation was the only alternative strategy to test a large number of transformants for integration of multiple copies of the gene of interest. However, using DNA colony hybridisation, transformants can be selected that do contain many (in)complete copies of the gene of interest without having a significantly increased expression level. The use of the RNA based screening technique we describe here could overcome this disadvantage.

Structure of the cpr genes

The predicted amino acid sequences of several *cpr* genes have been elucidated in the last years, including those of mammals, plants and yeasts. Some authors have tried to identify regions of CPR proteins involved in interactions with prosthetic groups, NADPH and cytochrome P450, based on alignment studies of (deduced) protein sequences from different organisms and comparison of CPR sequences with structures of other FAD and FMN binding proteins with known 3-dimensional structures (Porter and Kasper, 1985, 1986; Nadler and Strobel, 1991; Meijer et al., 1993; Shet et al., 1993). The availability of the predicted amino acid sequence of a CPR from a filamentous fungus provides the opportunity to extend the characterisation of the regions involved in these interactions by comparison of the *A. niger* CPR with CPR sequences from other organisms and by comparison of the *A. niger* CPR sequence with the sequences of the crystallized proteins flavodoxin from *Desulfovibrio vulgaris* (FMN binding; Watt et al., 1991) and spinach ferredoxin-NADP$^+$-reductase (FNR; FAD-binding; Karplus et al., 1991).

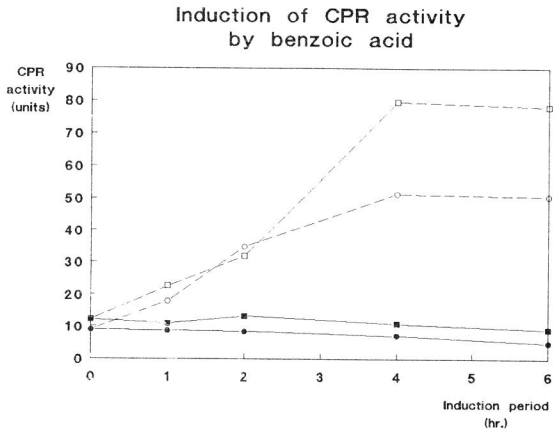

Figure 2.5. Time course of induction of CPR activity by benzoic acid. In two independent experiments mycelium was transferred to medium with or without 0.1% benzoic acid; samples were taken at different time points and CPR activity was determined.
(□ , O) 0.1% glucose, 0.1% benzoic acid; (■, ●) 0.1 % glucose without benzoic acid.

FMN binding region: In *A. niger* CPR two regions can be identified which are homologous to the regions in rat CPR postulated by Porter and Kasper (1985) to be involved in binding of the FMN prosthetic factor (FMN-1 and FMN-3; see Figure 2.6). These regions are well conserved between the *A. niger* CPR and other known CPR proteins, consistent with the theory of Porter and Kasper. In the FMN-1 region four amino acids (S-x-T-G-T; amino acids 72-76 of the *A. niger* CPR) are supposed to be involved in the formation of hydrogen

bonds with the 5' pyrophosphate of FMN. Watt et al. (1991) showed that in flavodoxin the same amino acids form hydrogen bridges with the FMN group.

Region FMN-3 of *A. niger* CPR (a.a. 164-182 of the *A. niger* CPR) is homologous to part of the second FMN binding region proposed by Porter and Kasper (1985). Since in the C-terminal region of the originally proposed FMN-3 region no homology between different CPR sequences is found this part of the FMN-3 region (a.a 183-196 in *A. niger* CPR) is probably not important for interaction with the FMN prosthetic factor). In region FMN-3 of *A. niger* CPR a G-N-x-x-Y-E-Sc-x-N sequence is found (a.a. 168-177 of the *A. niger* CPR; Sc= H/F/Y) which is partly conserved between CPRs. This sequence is homologous to a G-D-x-x-Y-E-Y-x-C sequence which is in close contact with FMN in flavodoxin. A conserved Gly residue found 26 amino acids C-terminal from this sequence in flavodoxin, is also found in *A. niger* CPR (Gly-191) and is completely conserved between CPR proteins from different organisms.

Watt et al. suggested the sequence S-T-W-G-D of the flavodoxin molecule to be involved in interaction with the FMN isoallexin ring by formation of hydrogen bonds. A similar amino acid sequence is present in CPR molecules (A/S-T-Y-G-D/E; a.a 123-127 of the *A. niger* CPR). At the third position in this sequence the presence of an aromatic side chain is conserved (W in flavodoxin, Y in CPR and F in rat nitric oxide synthase). Therefore we propose an additional region, FMN-2 (a.a. 123-139 of the *A. niger* CPR), to be involved in interaction with the FMN prosthetic factor in CPR.

FAD binding region: The FAD-1 domain of *A. niger* CPR (a.a. 289-324) is homologous to the first region proposed to be involved in binding of the FAD prosthetic factor by Porter and Kasper (1985). The G-D-H sequence (a.a. 314-316 in the *A. niger* CPR) found in this domain, which is almost completely conserved between different CPR molecules, was proposed to be in interaction with the OH-group of the ribose sugar.

Comparison of *A. niger* CPR with the sequence of ferredoxin-$NADP^+$-reductase, revealed two additional sequences possibly involved in interaction with the FAD prosthetic factor. Region FAD-2 (a.a. 448-457, a.a. 465 in the *A. niger* CPR) is supposed to interact with the FAD isoallexin group. Studies with crystallized ferredoxin-$NADP^+$-reductase (FNR;

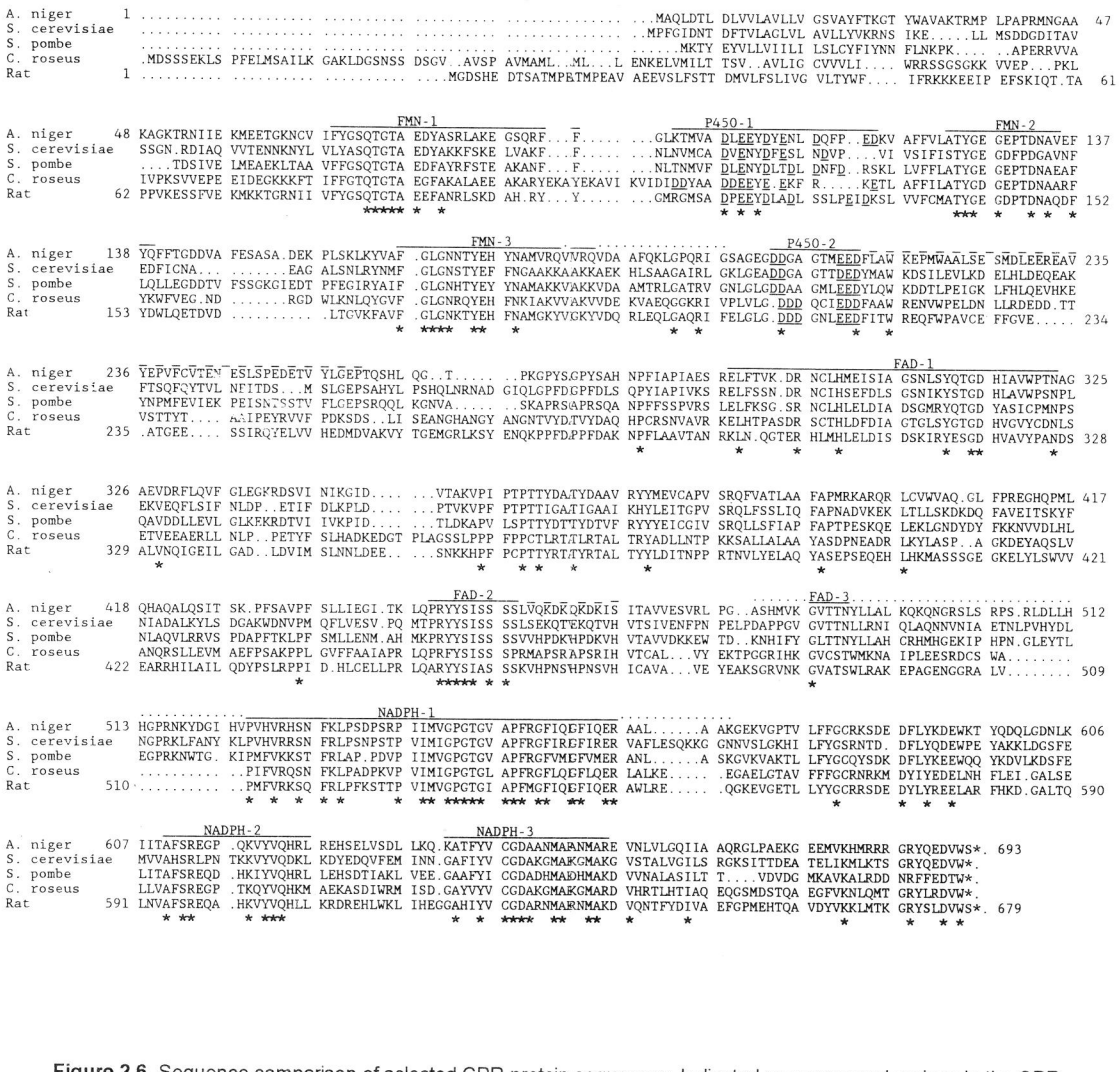

Figure 2.6, Sequence comparison of selected CPR protein sequences. Indicated are conserved regions in the CPR protein. Asterisks, conserved residues; Overline, position of the proposed domains; Overlayed dots, previously suggested domains as discussed in the text; Underlined residues in domains P450-1 and P450-2, negatively charged residues.

Karplus et al. 1991) showed close interaction of the completely conserved Arg residue (Arg-449 in the *A. niger* CPR) in this region with the phosphate group of FAD.

By comparison of *A. niger* CPR with FNR a third region can be identified that is important for interaction with the FAD domain. The sequence G-V-T-T-N (a.a 484-488) is very homologous to the G-V-T-N-N sequence found in FNR. This sequence was found to be in close interaction with the phosphate group of the FAD molecule in FNR (Karplus et al. 1991). Some variation is found between CPR proteins from different organisms.

The C-terminus of *A. niger* CPR resembles another domain of the crystallized FNR protein which was shown to be in close interaction with the FAD molecule (G-R-Y-x-x-D-V-W-S). The completely conserved Gly en Tyr residues are in close contact with the FAD molecule. This sequence is well conserved between different CPR molecules.

NADPH binding region: Three regions can be identified in *A. niger* CPR that are involved in the binding of NADPH. The first region suggested by Porter and Kasper (1985) resembles a.a. 478-534 of the *A. niger* CPR. However, this sequence is interrupted by an insertion in the CPR sequences of *A. niger*, *S.pombe* and *S.cerevisiae*. Therefore it is more likely that only parts of this region are involved. Since the amino proximal part of this region comprises part of the proposed FAD binding region and the homologous region in the crystallized FNR molecule does not interact with the NADPH group (Karplus et al., 1991), it seems reasonable to assume that only the C-terminal part of this region is involved in NADPH binding (forming part of region NADPH-1, a.a. 525-562 of the *A. niger* CPR). In this region a sequence, G-T-G-x-A-P (position 549-554 in *A. niger* CPR), is present which is homologous to the canonical dinucleotide binding loop G-x-G-x-x-G (Powel, 1991) and corresponds with the FNR G-T-G-x-x-P found to be in direct interaction with the NADP ribose group.

A second NADPH binding domain (NADPH-2; a.a. 610-625 in *A. niger* CPR) is supposed to be important for discrimination between NADPH and NADH. The Ser-Arg sequence (a.a. 612-613 in the *A. niger* CPR) and the Lys residue (a.a. 618 in the *A. niger* CPR) of the FNR protein are directly involved in binding of the 2' phosphate moiety of the NADP. The importance of the Arg residue in CPR proteins for discrimination between NADP and NAD was experimentally shown by Sem and Kasper (1993). The Ser-Arg and Lys residues are conserved in NADPH binding proteins (including *A. niger* and other CPR molecules) and absent in NADH binding proteins thus supporting the hypothesis.

The Cys-645 residue, which is found in region NADPH-3 (a.a. 639-653 of the *A. niger*

CPR) is fully conserved between different CPR proteins. At the corresponding position of the FNR molecule a Cys residue is found that is supposed to be in close interaction with the NADP ribose group.

Interesting is the position of the fully conserved Tyr at the C-terminus of CPR proteins. In the crystallized FNR molecule this residue seems to cover the NADPH binding pocket and thus seems to be important for protection of the FAD binding pocket in the absence of NADPH (Karplus et al., 1991).

Binding of cytochrome P450: Two very negatively charged regions can be identified in the *A. niger* CPR sequence (region P450-1; a.a. 95-115 and region P450-2; a.a. 192-201 in *A. niger* CPR). According to Nadler and Strobel (1991) such negatively charged regions might be involved in the interaction between CPR and cytochrome P450 proteins. Although in both regions a lot of sequence variation is found, a clearly negatively charged region can be found in the corresponding sequences of all CPR proteins. Computer analysis showed that region P450-1 in *A. niger* CPR is likely to be exposed at the exterior of the protein. Since many negatively charged amino acids are found C-terminal from the proposed P450-2 binding site, the importance of these residues for interaction with cytochrome P450 enzymes can not be excluded. Exact localisation of the P450 binding sites will depend on further experimental data.

In conclusion, we have cloned a functional *cpr* gene from the filamentous fungus *A. niger*. To our knowledge, this is the first *cpr* gene isolated from filamentous fungi. The *A. niger cprA* gene is clearly related to *cpr* genes isolated from other organisms. Increasing the copy number of this gene results in considerable increase in the NADPH:cytochrome c reductase activity. Gene expression of the *cprA* gene is clearly induced by addition of benzoic acid to the medium whereas some other, general, cytochrome P450 inducers do not affect *cprA* gene expression levels.

Since in many cytochrome P450 dependent processes improvement of the CPR activity is necessary for improvement of cytochrome P450 specific activities, the *cprA* gene may be useful in applications of cytochrome P450 expression systems in filamentous fungi. Our next goal will be to improve cytochrome P450 activities in filamentous fungi by overexpression of the *A. niger cpr*A gene in combination with overexpression of cytochrome P450 genes of interest.

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Chapter 3

Increased Resistance to 14α-Demethylase Inhibitors (DMIs) in *Aspergillus niger* by Coexpression of the *Penicillium italicum* Eburicol 14α-Demethylase (*cyp51*) and the *A. niger* Cytochrome P450 Reductase (*cprA*) Genes

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Abstract

In this chapter we describe the effects of overexpression of the *Penicillium italicum* gene encoding eburicol 14α-demethylase (*cyp51*), in *Aspergillus niger* strains with one or multiple copies of the gene encoding cytochrome P450 reductase (*cprA*), on the eburicol 14α-demethylase activity. Eburicol 14α-demethylase activity was determined by measuring the resistance of transformants against some eburicol 14α-demethylase inhibitors (DMIs). DMIs are widely used as fungicides in crop protection and human and veterinarian health care.

DMI resistance in a transformant overexpressing both CPR and CYP51 was increased 5-30 fold compared to DMI resistance in the wild type strain, depending on the test compound used. Resistance in this strain was approximately 2-5 fold increased compared to DMI resistance in a transformant that was overexpressing the *cyp51* gene but had only the wild type copy of the *cprA* gene and approximately 3-12 fold increased compared to a strain overexpressing the *cprA* gene (and having only the wild type copy of the *cyp51* gene). These results show the importance of CPR overexpression for increasing cytochrome P450 activities in filamentous fungi.

Introduction

Eburicol 14α-demethylase (14DM), involved in the removal of the 14α-methyl group from eburicol, is a key enzyme in the biosynthesis of ergosterol in fungi. Ergosterol is important for maintenance of membrane integrity and fluidity. The function of ergosterol is comparable to that of cholesterol in membranes of higher eukaryotes. Inhibition of the synthesis of C_{14}-desmethyl sterols by eburicol 14α-demethylation inhibitors (DMIs) results in depletion of ergosterol, accumulation of abnormal sterol intermediates and thus in severely retarded growth of the fungus. Eburicol 14α-demethylase inhibitors are widely used as agricultural fungicides for control of plant diseases (Vanden Bossche 1988).

The gene encoding lanosterol 14α-demethylase has been cloned from *Saccharomyces cerevisiae* (*14dm*; Kalb et al 1986, 1987) and recently the gene coding for eburicol 14α-demethylase was cloned from the filamentous fungus *Penicillium italicum* (*cyp51*; van Nistelrooy et al 1996). Both genes belong to one of the cytochrome P450 subfamilies (the CYP51 family) and are members of the class of cytochrome P450 enzymes that are attached to the membrane of the endoplasmic reticulum. This class of cytochrome P450 enzymes needs the activity of a second enzyme, the electron donating enzyme NADPH cytochrome P450 reductase (CPR) to become catalytically active (Gonzalez & Korzekwa 1995).

Overexpression in *S. cerevisiae* of the *14dm* gene has been achieved by fusing the *14dm* gene with strong yeast promoters on high copy number vectors. Using this strategy lanosterol 14α-demethylase protein levels could be increased more than 10 times compared to the wild type strain (Weber et al 1990, 1992). Overexpression of the *P. italicum cyp51* gene in *Aspergillus niger* resulted in a 2.5-7 times increase in resistance to different DMIs (van Nistelrooy et al 1996).

Using other cytochrome P450 genes it was shown in *S. cerevisiae* that activity levels in cytochrome P450 overproducing strains could be significantly increased by introduction of additional copies of the yeast *cpr1* gene encoding NADPH cytochrome P450 reductase (Sakaki et al 1990, Truan et al 1993). Since we recently cloned the *A. niger cprA* gene, encoding NADPH cytochrome P450 reductase (van den Brink et al 1995), we were able to use the same strategy for improvement of 14α-demethylase activity in *A. niger*. For this purpose we overexpressed the *P. italicum cyp51* gene together with the *A. niger cprA* gene.

Materials and Methods

Strains and plasmids

A. niger strain N402 (a derivative of ATCC9029, *cspA*1; Bos 1986) and transformant AB2-2 (derivative of N402, containing multiple copies of the *cprA* gene; van den Brink et al 1995) were used for transformations. *Escherichia coli* K12 strain JM109 (Yanisch-Perron et al 1985) was used for construction and propagation of vector molecules.

Constructions of YEpW5B, (van Nistelrooy et al 1996), pCPR2amdS (van den Brink et al 1995) and pAN7-1 (Punt et al 1987) have been described elsewhere.

Chemicals

Benomyl was obtained from Du Pont de Nemours & Co (Wilmington, De., USA). Etaconazole, imazalil and fenarimol were generous gifts of CIBA Geigy AG (Basel, Switzerland), Janssen Pharmaceutica (Beerse, Belgium) and DowElanco (Indianapolis, USA), respectively. All other chemicals were of the highest purity commercially available.

Transformation of A. niger

A. niger strain N402 and transformant AB2-2 were grown in complete medium (Pontecorvo et al 1953) and harvested by filtration over Miracloth filtration wrap (Calbiochem). Fungal transformations were performed as described before (Punt & van den Hondel 1993) using either plasmid pCPR2amdS or plasmids YEpW5B and pAN7-1. After transformation protoplasts were selected for growth on plates with 100 µg/ml hygromycin (pAN7-1 transformants) or for growth on acetamide as sole nitrogen source (*amdS* transformants; Verdoes et al 1993). Plates were incubated at 35 °C for 10 days.

Selected transformants were tested for increased mRNA production using an RNA colony hybridization approach as described before (van den Brink et al 1995).

Other molecular biological methods

DNA manipulation and bacterial transformation were performed using standard methods (Sambrook et al 1989). Total fungal RNA was isolated using the RNAzol™ kit from CINNA/BIOTECX.

Probes used in Northern analysis experiments were respectively a 1.6 kb *Bam*HI

fragment isolated from plasmid YEpW5B (*P. italicum cyp51* specific probe), a 1.2 kb *Eco*RI-*Bam*HI fragment from pCPR1 (*A. niger cprA* specific probe; van den Brink et al 1995) and a 1.5 kb *Hin*dIII fragment from pAB5-22 (*A. niger gpdA* specific fragment; Verdoes et al 1994b).

Toxicity assays

Inhibition of fungal radial growth by the fungicides was determined in Petri dishes containing PDA amended with fungicides at various concentrations (Kapteyn et al 1992). Inverted 5 mm agar discs with young mycelium were placed on the agar surface (in triplicate). After 3 days of incubation at 25 °C radial mycelial growth was measured.

Results and Discussion

Construction of strains

In a co-transformation experiment using plasmids pAN7-1 and YEpW5B, multiple copies of the *P. italicum cyp51* gene encoding eburicol 14α-demethylase were introduced in *A. niger* strain N402. Hygromycin resistant transformants were screened for their resistance to different concentrations of hygromycin (100-500 µg/ml). Highly resistant transformants were analysed by Southern analysis using a *cyp51* specific probe. Transformant AB-D1 was selected for further experiments. Additional copies of the *A. niger cprA* gene were introduced in transformant AB-D1 (van Nistelrooy et al 1996) in a transformation experiment using plasmid pCPR2amdS (van den Brink et al 1995). Transformants were selected for high copy numbers of the *amdS* selection marker as described by Verdoes et al (1993) and subsequently screened by RNA colony hybridization for presence of increased *cprA* mRNA levels. Transformant AB-D1.15 was used in further experiments. The number of *cprA* and *cyp51* copies integrated in the genome of the described transformants were determined by Southern analysis and subsequent scanning of the autoradiographs.

Functional evaluation of transformants

CPR activities were measured in all selected strains. Activity levels in the *cprA* multiple copy strains AB2-2 and AB-D1.15 were strongly increased compared to CPR activities in strains N402 and AB-D1 (Table 3.1).

Table 3.1 NADPH:cytochrome c reductase activities in selected transformants. CPR activities were measured in total mycelial extracts for 2 minutes at room temperature as described by van den Brink et al (1995). 1 unit CPR activity corresponds to the reduction of 1 mmol cytochrome c per milligram microsomal protein per minute at room temperature. All activities were determined at least three times. Approximate gene copy numbers were determined using an Ultroscan XL Densitometer (Pharmacia LKB, Uppsala, Sweden).

Transformant	*cyp51* copy -number		*cprA* copy - number	CPR activity (units)
	A. niger	*P. italicum*		
N402	1	-	1	1.1 ± 0.3
AB2-2	1	-	6-12	14.6 ± 5.4
AB-D1	1	6-12	1	1.5 ± 1.1
AB-D1.15	1	6-12	15-20	41.6 ± 5.1

Although qualitative analysis of 14α-demethylase activity is possible *in vitro* using TLC or Radio-HPLC based assays (Ballard et al 1990; Stehmann et al 1994), these assays were found to be not suitable for quantification of 14α-demethylase activity. Therefore 14α-demethylase activity was assayed indirectly by measuring inhibition of radial growth of transformants on agar plates amended with DMIs at various concentrations (etaconazole, fenarimol and imazalil). As a control benomyl was included in the radial growth experiments. This fungicide inhibits fungal growth by a 14α-demethylase independent mechanism. DMI resistance was clearly increased in the *cyp51* multiple copy transformant AB-D1 (3-5 fold increase compared to strain N402, Figure 3.1; van Nistelrooy et al, 1996). Transformant AB2-2 in which only the copy number of the *cprA* gene, encoding the second component of the 14α-demethylase system, was increased was also more resistant to different DMIs than the wild type N402 strain. However, resistance in this transformant was less than the resistance of the *cyp51* multiple copy strain AB-D1 (1.5-2.5 fold increase compared to the wild type strain). Most interesting was the effect of introducing multiple copies of both components of the 14α-demethylase system in the fungus as in transformant AB-D1.15. Resistance to DMIs in this transformant was increased by 4.5-30 fold, depending on the test compound used. The results observed clearly show a synergistic effect of CPR and 14α-demethylase towards DMI resistance. These results indicate the importance of overexpression of the *cprA* gene for increasing cytochrome P450 activities. Wild type levels of CPR seem to be insufficient to fully support all CYP51 molecules present in a *cyp51* multiple copy strain. The increase in resistance

in transformant AB-D1.15 can not be explained by simple addition of independent effects of 14α-demethylase and CPR since EC_{50} values in transformant AB-D1.15 are up to 6 fold the EC_{50} values in strain AB-D1 and since the effect of the increase of CPR alone (AB2-2) on DMI resistance is relatively minor. No significant differences in resistance to benomyl could be observed for different transformants, indicating that the results described here are CYP51 specific.

Control experiments with other *cprA* and/or *bphA* multicopy transformants, obtained from the same transformation experiments as the selected transformants, gave comparable

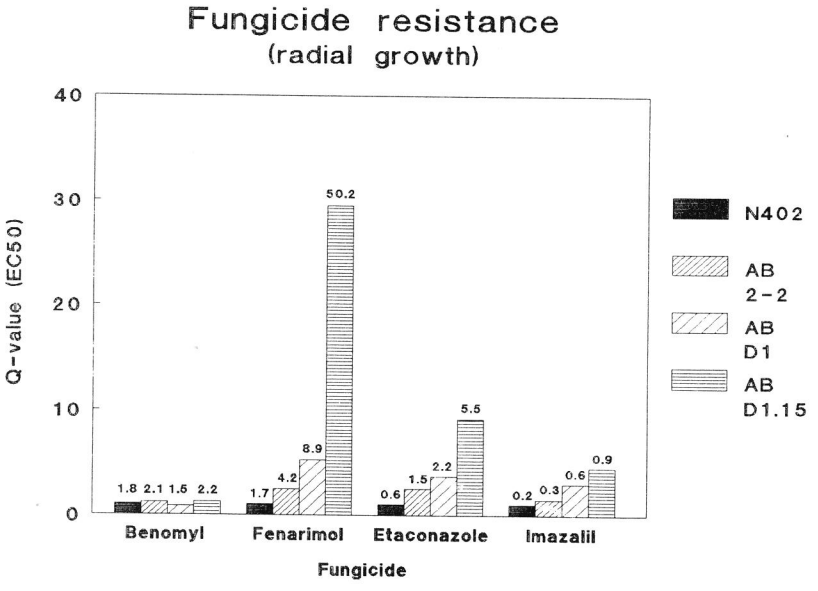

Figure 3.1, Relative resistance of transformants to different test compounds.
Transformants were grown in triplicate on plates with increasing concentrations of fungicides. Radial growth was determined. Q-values were determined for each fungicide tested (Q value is the EC_{50} concentration of a transformant relative to the EC_{50} concentration of *A. niger* N402). Absolute EC_{50} concentrations (µg/ml) are indicated on top of the bars.

results (not shown). To investigate the effect of the used selection markers (*amdS*, *hph*) on DMI resistance, transformants were tested which had only multiple copies of the selection markers integrated in their genome. DMI resistance of these transformants was not

significantly different from the wild type N402 strain (results not shown).

Relative *cprA* and *cyp51* mRNA levels were determined by comparison of mRNA levels to the mRNA level of the constitutive *A. niger gpdA* gene in a Northern analysis experiment (Figure 3.2). Introduction of multiple copies of the *cprA* or *cyp51* gene was found to have no effect on the relative mRNA expression levels of the other component of the cytochrome P450 system (*cprA* or *cyp51*, respectively).

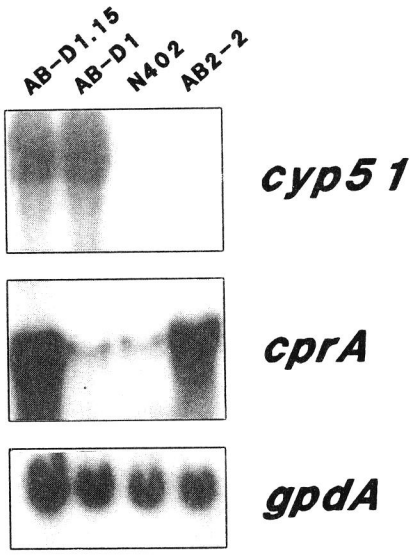

Figure 3.2,
Northern blot containing RNA samples of N402, AB2-2, AB-D1 and AB-D1.15.
Total RNA was isolated from mycelium obtained after overnight growth in complete medium. Approximately equal amounts of RNA (10 µg) were separated on a 1% agarose/formaldehyde gel, blotted to Hybond N$^+$ and probed with different probes.
Panel **a**) *cprA* specific probe
Panel **b**) *cyp51* specific probe
Panel **c**) *gpdA* specific probe

The results presented here show that increase of 14DM activity in filamentous fungi can be achieved by increasing the gene copy number of the *cyp51* gene. However, it seems that wild type CPR levels in *A. niger* are insufficient to fully support all cytochrome P450 enzymes present in the ER membranes of *cyp51* multiple copy strains, resulting in low average conversion rates per molecule cytochrome P450. Simultaneous overexpression of both CPR and cytochrome P450 encoding genes is necessary to fully activate all cytochrome P450 enzymes present in multiple copy strains. These results correspond with results obtained by overexpression of cytochrome P450 genes together with *cpr* genes in *S. cerevisiae* (Sakaki et al 1990, Truan et al 1993).

Since resistance towards DMI based fungicides is an increasing problem in agriculture, the use of 14α-demethylase overproducing fungi might be an important tool for studying resistance mechanisms and for the development of stronger, more specific DMIs. Further increase of 14α- demethylase activity levels could be achieved by replacing the relatively inefficient promoters of both genes by strong promoters like the *A. niger gpdA* promoter (Punt et al 1988).

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Chapter 4

Optimization of the Benzoate-Inducible Benzoate *p*-Hydroxylase Cytochrome P450 Enzyme System in *Aspergillus niger*

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Abstract

Introduction in the fungus *Aspergillus niger* of multiple copies of the *A. niger bphA* gene, encoding the cytochrome P450 enzyme Benzoate Para-Hydroxylase, did not result in increased BPH enzymatic activities (van Gorcom et al., Mol Gen Genet 1990), probably due to low expression levels of the gene encoding the second component of the microsomal cytochrome P450 enzyme system, cytochrome P450 reductase (CPR). For improvement of this and other cytochrome P450 dependent reactions, *A. niger* strains were constructed in which the gene copy number of the *A. niger cprA* gene (encoding CPR) or the gene copy numbers of both *cprA* and the cytochrome P450 encoding gene were increased. Expression of both genes was controlled by their own transcription control regions. BPH activity of different transformants was determined in microsomal fractions using a newly developed indirect *in vitro* assay. In transformants containing multiple copies of both genes, benzoate para-hydroxylase activity was significantly higher as compared to the wildtype strain or to transformants in which the gene copy number of only one of the genes was increased. These results clearly indicate the importance of co-expression of CPR for achieving maximal cytochrome P450 activities in cytochrome P450 overproducing filamentous fungi.

Introduction

Cytochrome P450 enzymes are found throughout nature and are responsible for a wide range of very different biotransformation processes, including hydroxylation and demethylation of steroid-like molecules, processing of PAH molecules and terminal hydroxylation of aliphatic compounds. All of these processes involve the addition of one atom of molecular oxygen to the substrate molecule, the second atom being converted to water. Cytochrome P450 substrates are very different in structure and biological function but are usually lipophilic.

At this moment over 500 cytochrome P450 genes have been cloned (Nelson et al. 1996), most of them from mammalian species. Only few fungal cytochrome P450 genes have been characterized so far. Fungal cytochrome P450 genes described at this moment include the *bphA* gene of *Aspergillus niger* (*cyp53*) the gene product of which is involved in the conversion of benzoic acid to para-hydroxy-benzoic acid (van Gorcom et al. 1990). The gene product of the *pda* gene (*cyp57*) of *Nectria haematococca* (Weltring et al. 1988) is involved in the demethylation of pisatin, a phytoalexin secreted by some plant species. The $P450_{14dm}$ (*cyp51*) gene of *Penicillium italicum* (van Nistelrooij et al. 1996) encodes eburicol 14α-demethylase which is the key enzyme in the synthesis of the membrane compound ergosterol. The *Tri4* gene, encoding a cytochrome P450 enzyme involved in the trichothecene biosynthesis pathway, was cloned from *Fusarium sporotrichiodes* (Hohn et al. 1995). Recently a soluble cytochrome P450 gene, called P450nor (*cyp55*), was cloned from the fungus *Fusarium oxysporum* (Kizawa et al. 1991).

Cytochrome P450 enzymes need the electron donating activity of a second enzyme system to become catalytically active. In mitochondria and prokaryotes this electron providing enzyme system consists of two proteins while in the endoplasmic reticulum electrons are donated by one enzyme, cytochrome P450 reductase (CPR). It is generally accepted that CPR is capable of supporting all different types of cytochrome P450 molecules present in the microsomal compartment.

A few years ago the *A. niger* cytochrome P450 gene *bphA* was cloned in our laboratory. Multiple copies of this benzoate inducible gene were introduced in *A. niger* and benzoate para-hydroxylase activity was determined. To our surprise no increase of BPH activity was measured in multicopy *bphA* transformants although increase of the gene copy number of the *bphA* gene did result in substantially increased mRNA and protein levels, as compared to the wildtype strain (van Gorcom et al. 1990). One of the proposed explanations

for this surprising result was that in *bphA* multiple copy strains depletion occurred of the second component of the enzyme system, cytochrome P450 reductase. Support for this hypothesis came from experiments in *S. cerevisiae* showing that overexpression of a *cpr* gene together with several different cytochrome P450 genes was necessary for achieving maximum expression levels (e.g. Sakaki et al. 1990; Truan et al. 1993). However, in contrast with our results with the *bphA* gene, each author reported at least some increase in activity when the cytochrome P450 gene of interest was overexpressed in *S. cerevisiae* without simultaneous *cpr* overexpression. Recently we cloned and characterized the gene coding for cytochrome P450 reductase (*cprA*) from *A. niger* (van den Brink et al. 1995). With the availability of this gene we were able to test our hypothesis.

In this chapter we describe the effects of introduction of extra copies of the *cprA* gene on BPH activity, in strains which contain one or multiple copies of the *bphA* gene.

Material and Methods

Strains and plasmids

A. niger strains N204 *(csp21, met21*; Boschloo et al. 1990), T18 (derivative of N271 *csp21, fwn21, pdx21*, multiple copies of the *bphA* gene; van Gorcom et al. 1990) and ΔBPH (derivative of N245, *csp21, met21, pyrG, bphA::hph*, van Gorcom et al. 1997) were all derivatives of *A. niger* ATCC1015. *Escherichia coli* K12 strain JM109 (Yanisch-Perron et al. 1985) was used for construction and propagation of vector molecules.

Construction of pCPR1 and pCPR2 (van den Brink et al. 1995) and of pAN7-1 (Punt et al. 1987) were described previously. As *cprA* specific probe a 1.2 kb *Eco*RI-*Bam*HI fragment isolated from plasmid pCPR1 was used, the *bphA* specific probe was a 2.7 kb *Bg*lII fragment isolated from plasmid pAB8-41 (van Gorcom et al. 1990). A 1.5 kb *Hind*III fragment comprising part of the coding region of the *A. niger gpdA* gene was isolated from plasmid pAB5-22 (van Gorcom et al. unpublished).

Transformation of A. niger

A. niger strain N204 was cultivated on complete medium (Pontecorvo et al. 1953) and harvested by filtration over Miracloth filtration wrap (Calbiochem). Fungal co-transformations were performed as described before by Punt and van den Hondel (1993) using plasmids pCPR2

and pAN7-1. Transformants were selected on agar plates amended with 100 µg/ml hygromycine. Plates were incubated at 35 °C for 10 days.

Preparation of microsomal fraction

Mycelium of transformants was cultured by inoculating 500 ml complete medium (Pontecorvo et al. 1953) with 1.10^6 spores per ml. After 18 hours cultivation at 30 °C, 300 rpm. in a rotary shaker, mycelium was harvested by filtration over Miracloth filtration wrap, washed extensively with 0.9% NaCl and transferred to 500 ml induction medium (minimal medium with 0.1% benzoic acid and 0.1% glucose as sole C-sources). After a 3 hours induction period mycelium was harvested. Mycelium (20 gram wet weight) was disrupted in a Bead-Beater 60 ml chamber (Biospec Products, Bartlesville, OK) cooled with ice-water and filled with 20 grams of glass pearls (0.5 mm). The Bead-Beater was operated four times at full speed for 30 seconds using 30 second intervals. Following the cell disruption, microsomes were prepared as described by Ballard et al. (1990).

Activity assays

BPH activity was determined in microsomal fractions by measuring the benzoate dependent consumption of NADPH in 500 µl reaction buffer (100 mM Tris-HCl/pH 7.8, 10 mM $MgCl_2$, 0.2 mM NADPH) at 340 nm. NADPH consumption was recorded after addition of microsomes (typically 25 µl was used) until a stable baseline of NADPH consumption was obtained. To determine specifically BPH activity 20 µl of a 20 mM benzoate solution was added and the NADPH consumption was measured for 5 minutes. One unit of BPH activity corresponds to the benzoate dependent consumption of 1 µmol NADPH per mg microsomal protein per minute at room temperature.

CPR activity was determined as described (van den Brink et al. 1995; Madyastha et al. 1979) using NADPH as electron donor.

Other molecular biological methods

DNA manipulation and bacterial transformations were performed using standard methods (Sambrook et al. 1989). Western blot analysis was performed using antibody α-SP32 as described before (Gerritse et al. 1990).

Results

Construction and analysis of cprA multiple copy strains

In a co-transformation experiment strain N204 (wildtype for *bphA* and *cprA*) and strain T18 (wildtype for *cprA*, multiple copies of the *bphA* gene) were transformed with a mixture of plasmids pAN7-1 and pCPR2. The latter plasmid contains the *cprA* coding region proceeded by 582 bp upstream from the ATG codon and having 967 bp downstream of the stop codon. To select for integration of multiple copies of the plasmids approximately 20 hygromycine resistant transformants were tested for their resistance to increasing concentrations of hygromycine. Highly resistant transformants were expected to contain multiple copies of pAN7-1 integrated in their genome. From transformants obtained from each parental strain seven transformants with strongly increased hygromycine resistance were analysed for integration of multiple copies of plasmid pCPR2 by Southern analysis with a *cprA* specific probe. Transformants with multiple copies of the *cprA* gene were tested for an increase of CPR activity and of the transformants with maximal CPR activity two transformants ABW-13 (wildtype for *bphA*, approximately 10 copies of the *cprA* gene) and T18-5 (approximately 10 copies of the *bphA* and 15 of the *cprA* gene), were used for further analysis of BPH and CPR activities (comparable results were obtained using other transformants with maximal CPR activity).

Analysis of CPR activity in cprA multicopy strains

Crude microsomal fractions were prepared from benzoate induced mycelium and enzymatic activities were determined. CPR activities were found to be 2.5-3 fold higher in microsomes of the *cprA* transformants ABW-13 and T18-5 as compared to the *cprA* wildtype strain N204 (Figure 4.1), indicating the presence of increased amounts of CPR protein. Surprisingly, CPR activity in *bphA* multicopy transformant T18 was only half of the CPR activity in strain N204.

In addition the effect of benzoate induction on CPR activity was studied in microsomal fractions of transformants. Microsomes were isolated from mycelium obtained after a 3 hr. induction period in the absence (-BA) or presence (+BA) of benzoate and CPR activity was determined. A clear inducing effect of benzoate in CPR activity was shown in the wildtype strain. No benzoate dependent induction of CPR activity was found in transformants ABW-13

and T18. CPR activity in transformant T18-5 (multicopy for both genes) decreased after benzoate induction.

Analysis of BPH activity in cprA multicopy strains

Determination of BPH activity by measuring the conversion of benzoate to para-hydroxy benzoate *in vivo* is not reliable since para-hydroxy benzoate can be further metabolized (Boschloo et al. 1990). Therefore BPH activity was determined *in vitro* by using a novel, indirect, activity assay. This assay is based on substrate dependent consumption of NADPH by the cytochrome P450/CPR enzyme complex and might be of general use for determination of other cytochrome P450 activities for which no direct assay is available. Using the NADPH consumption assay, BPH activity was found to be considerably increased in microsomes isolated from transformant T18-5 (multiple copies of the *bphA* and of the *cprA* gene) as compared to the wildtype N204 strain. In microsomal fractions obtained from transformants ABW-13 and T18 in which the expression of only one element of the BPH enzyme complex was increased, a clear increase of BPH activity was found compared to the wildtype N204 strain, although BPH activity in these strains was much lower than BPH activity in transformant T18-5 (Figure 4.1). In non-induced mycelium no BPH activity was detectable. In microsomal fractions of a control strain, Δ-BPH, in which the *bphA* gene was disrupted by insertion of a *hph* selection marker, also no BPH activity was detectable (less than 1 unit), thus showing the specificity of this NADPH consumption assay.

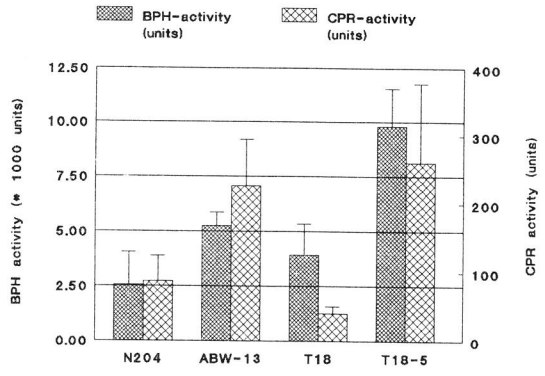

Figure 4.1, Enzymatic activities in selected transformants. Enzymatic activities were determined in microsomal fractions isolated from mycelium after a 3 hours induction period (0.1% benzoic acid). 1 unit CPR activity corresponds to the reduction of 1 mmol cytochrome c per milligram microsomal protein per minute at room temperature. 1 unit BPH activity corresponds to the benzoate dependent conversion of 1 µmol NADPH to $NADP^+$ per milligram microsomal protein per minute at room temperature.

Analysis of BPH protein levels

To determine the BPH protein level in different transformants, western analysis of solubilized microsomal fractions were performed. As shown in Figure 4.2 no difference in BPH protein levels between the parental strain N204 and the multicopy *cprA* strain ABW-13 was found. In agreement with our previous results an increase of BPH protein level was detected in the *bphA* multicopy strains T18 and T18-5. BPH protein levels in transformants T18 and T18-5 were comparable. No BPH was detectable in the Δ-*bphA* strain.

Discussion

The results presented in this paper support our hypothesis that substantial increase of cytochrome P450 activity can only be achieved if both components of the system, the specific cytochrome P450 and the general cytochrome P450 reductase, are overproduced. Introduction of multiple copies of the cytochrome P450 *bphA* gene only (transformant T18) results in a significant increase of BPH protein levels (Figure 4.2) but in only a minor increase in BPH activity (Figure 4.1). Introduction of multiple copies of the *cprA* gene only (transformant AB-W13) results not only in a twofold increase of CPR activity but also in a twofold increase in BPH activity. Since the BPH protein levels of wildtype N204 and AB-W13 are comparable (Figure 4.2), this result suggests that CPR activity is a limiting factor for BPH activity in the wildtype situation. Introduction of multiple copies of the *cprA* gene in the multiple copy *bphA* strain T18 (resulting in transformant T18-5) results in a sixfold increase of the CPR activity and in a fourfold increase of BPH activity, although BPH protein levels of T18-5 remain comparable to those in T18 (Figure 4.2). This result clearly

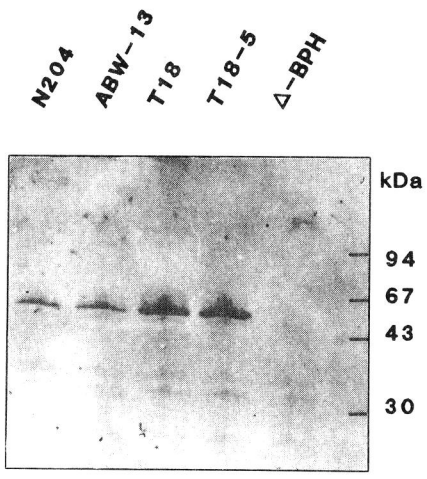

Figure 4.2 Western analysis of microsomal fractions of selected transformants.
Approximately 5 µg microsomal protein was solubilized on ice by incubating in the presence of 1% sodium deoxycholate for 15 minutes. Solubilized proteins were separated on a Phast system 10-15% SDS-polyacrylamide gel (Pharmacia), transferred to nitrocellulose and incubated with a BPH specific antibody (α-SP32).

indicates that, in line with our hypothesis, an increase of CPR activity is required for achievement of increased BPH activity by overexpression of the *bphA* gene. Our results are consistent with the results obtained in *S. cerevisiae* where overproduction of cytochrome P450 reductase was shown to be necessary for high cytochrome P450 activities.

The relative BPH activities of strain T18 compared to the wildtype strain, as presented in this article, are not completely in line with data which have previously been published by our laboratory (van Gorcom et al. 1990). In our previous paper BPH activity was determined in total cell free extracts which resulted in very high standard deviations. A clear improvement of accuracy was achieved by using microsomal fractions in the BPH assay. Nevertheless, relative BPH activities determined in total mycelial extracts of the selected strains were on average comparable to results obtained with microsomal fractions.

Table 4.1 Induction of CPR activity by benzoic acid. CPR activity was determined in microsomal preparation obtained from selected transformants after a 3 hr. induction period with or without benzoic acid. Experiments were repeated at least three times. 1 unit CPR activity corresponds to the reduction of 1 mmol cytochrome c per milligram microsomal protein per minute at room temperature.

Strain	CPR activity - Benzoic acid Units	CPR activity + Benzoic acid Units
N204	38 ± 1	88 ± 37
ABW-13	211 ± 17	226 ± 69
T18	32 ± 8	40 ± 10
T18-5	441 ± 33	261 ± 12

As presented in Table 4.1 CPR activity in the wildtype strain N204 is clearly inducible by benzoic acid. This supports our previous observation that CPR activity and *cprA* mRNA levels are induced by benzoate induction (van den Brink et al. 1995). Surprisingly no increase in CPR activity could be found in strains with multicopy of the genes encoding one of the elements of the BPH enzyme system. In strain T18-5, which is multicopy for both genes, CPR activity even decreased after benzoate induction. These results suggest a negative effect of overproduction of one or both components of the BPH enzyme system on benzoate

inducibility. Wether this effect is due to physical problems like changes in the membrane structure of the endoplasmic reticulum, or is caused by limitations at the regulatory level will be subject for further research.

In conclusion we have constructed a set of *A. niger* transformants in which copy numbers of the genes encoding both components of the endoplasmic BPH (cytochrome P450) system independently or both components together were increased. Only in transformants with increased expression levels of both components BPH activity was significantly increased, thus showing the importance of co-overexpression of the gene encoding cytochrome P450 reductase for improvement of cytochrome P450 activities in filamentous fungi.

A further increase of cytochrome P450 activities might be achieved by overproduction of both the *cprA* gene and the gene encoding cytochrome b_5 together with the cytochrome P450 gene of interest. In *S. cerevisiae* it was shown that coordinate overexpression of CPR and cytochrome b_5 results in a more efficient electron flow to some cytochrome P450 molecules (Truan et al. 1993).

In the near future we will investigate the use of *cprA* overexpressing *A. niger* strains for overexpression of other cytochrome P450 genes.

manuscript in preparation

Chapter 5

Regulation of Expression of the *Aspergillus niger* Gene Encoding Cytochrome P450 Reductase (*cprA*)

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Abstract

In the filamentous fungus *Aspergillus niger* a two component endoplasmic cytochrome P450 enzyme system, carrying out the para-hydroxylation of benzoate, is present. The genes encoding both components of this system, the cytochrome P450 gene encoding benzoate para-hydroxylase (*bphA*) and the gene encoding cytochrome P450 reductase (*cprA*) were cloned and gene expression of both genes was shown to be inducible by the substrate of the system, benzoate.

In this chapter the mechanism underlying benzoate inducibility of the *cprA* gene was studied in more detail. Northern analysis revealed a clear discrepancy between relative induction at the activity level (approx. 4 fold) and at the level of transcription (>20 fold). The majority of the transcripts observed after benzoate induction (*cprA*β) was of a larger size compared to the constitutively expressed *cprA*α transcript. The difference in size between the *cprA*α and *cprA*β transcript was caused by differential promoter use as was shown in RACE and primer extension experiments. Besides use of different transcriptional start points a second mechanism of regulation was found to be important. In a deletion study of the *cprA* TCR a 0.2 kb DNA sequence, located upstream from both transcription start sites, was identified which was shown to be involved in benzoate induction. Cloning of this DNA fragment upstream from a minimal promoter conferred benzoate inducibility on this promoter.

A deletion analysis of the *bphA* TCR revealed a 0.2 kb DNA sequence involved in benzoate induction of *bphA* expression. Sequence comparison of the *cprA* and *bphA* BRRs revealed a completely conserved 9 bp sequence. EMSA analysis with oligonucleotides comprising the element gave indications for a common regulatory mechanism resulting in benzoate dependent regulation of expression of both genes.

Introduction

Eukaryotic cytochrome P450 enzymes can be divided into two major classes. The first class of cytochrome P450s is localized in the mitochondrial membrane and requires the activity of a two component electron donating system to become catalytically active. The second class of cytochrome P450 enzymes is anchored to the endoplasmic membrane with the enzymatic part of the enzyme protruding in the cytosol. Members of this class obtain their electrons from one electron donating enzyme, cytochrome P450 reductase (CPR), which is also anchored in the endoplasmic membrane (Ortiz de Montellano 1986, Black and Coon 1987).

In higher eukaryotes many different endoplasmic cytochrome P450 species have been characterized, involved in very different biocatalytical processes. Many of these P450s are involved in the degradation (and sometimes activation) of xenobiotics. The expression of most of these xenobiotic metabolizing P450s (like CYP1A1, 2B1) is highly increased in the presence of the substrate (Nebert and Gonzalez 1987, Waxman and Azaroff 1992). Increase of cytochrome P450 activity levels usually takes place at the level of transcription although regulation by changes in mRNA stability or at the translational level has been reported as well (Porter and Coon 1991). The xenobiotics mediated increase of expression of mammalian cytochrome P450 genes has been extensively investigated. Since the substrate mediated increase in expression levels of these P450s is considerable, it seems obvious that expression of the gene encoding CPR (*cpr*) is also regulated to some extent, in order to obtain a fully active enzyme system. This would imply a highly complex but very interesting regulatory mechanism, since CPR expression levels would have to adapt to the expression levels of a number of different P450 genes, each responding to different inducers. Understanding the regulation of *cpr* gene expression might provide insight in the way eukaryotic cells deal with complex changes in their environment.

Surprisingly, thus far no detailed study has been undertaken to dissect the mechanisms of regulation of *cpr* gene expression in higher eukaryotes, although some reports describe induction of *cpr* at the transcriptional level in response to certain compounds like phenobarbital and *trans*-stilbene oxide (O'Leary et al. 1994, Gonzalez and Kasper 1982), dexamethasone and β-naphtaflavone (Gonzalez and Kasper 1982, Gonzalez et al. 1982, Sheppard et al. 1982), pregnelone (Simmons et al. 1987) and 2-acetylaminofluorene (Gonzalez et al. 1982) as well as high doses of DTT (Balakrishnan et al. 1985). Some data are also available on the regulation of *cpr* gene expression in lower eukaryotes. In the yeast *Saccharomyces cerevisiae* Stansfield et al. (1991) showed a clear decrease in *cpr* transcript level when *S. cerevisiae* was grown

under (semi) anaerobic conditions. In the *n*-alkane-assimilating yeasts *Candida maltosa* and *Candida tropicalis*, each containing a family of closely related *cyp* genes involved in the *n*-alkane assimilating pathway (Sanglard and Fiechter 1989), the expression of the *cpr* gene was shown to be increased 6-8 fold by the addition of *n*-alkanes to the medium (Ohkuma et al. 1995, Sutter et al. 1990).

Increase of CPR activity in response to the presence of substrates of a number of cytochrome P450 enzymes in filamentous fungi is described for several species but seems to be highly strain specific. CPR activity in microsomes isolated from phenobarbital treated mycelium from *Aspergillus ochraceus* (Ghosh et al. 1983) and *A. parasiticus* (Bhatnagar et al. 1982) was increased three to five fold while treatment with phenobarbital did not affect *cprA* mRNA levels in *A. niger* (van den Brink et al. 1995) or CPR activity in *A. fumigatus* (Baillie 1993).

In our laboratory we are analysing the regulation of expression of the two genes encoding the components of a cytochrome P450 enzyme system (BPH) from the filamentous fungus *Aspergillus niger* which is involved in the hydroxylation of benzoic acid. We cloned the genes encoding the benzoate para-hydroxylase (*bphA*; van Gorcom et al. 1990) and cytochrome P450 reductase (*cprA*; van den Brink et al. 1995) from *A. niger*. Transcription of both genes appeared to be up regulated by the addition of the substrate of the enzyme system, benzoate. The availability of this highly controlled P450 enzyme system in a relatively simple eukaryotic organism, for which advanced molecular genetic techniques are available, allows the study of the regulation of both partners of this P450 enzyme system in more detail.

In this chapter we describe experiments using both *in vivo* and *in vitro* techniques to study the mechanisms underlying the benzoate dependent induction of *cprA* (and *bphA*) gene expression in *A. niger*.

Materials and Methods

Strains and Plasmids

A. niger strain AB4.1 (*pyrG*$^-$, derivative from strain N402; van Hartingsveldt et al. 1987) was used for fungal transformations.
Construction of plasmids pNOM102 and pGUS54 (Roberts et al. 1989, Verdoes et al. 1994b) were described previously. Construction of plasmid pAB94-83, which contains a 2 kb *Bam*HI fragment, comprising the complete *bphA* transcription control region (TCR), fused to the *lacZ* reporter gene and using an *A. niger pyrG* allele with a defined mutation for selection (*pyrG**) was described by van Gorcom et al. (1993).
E. coli strains JM109 and DH5α were used for cloning and propagation of vector molecules.

RNA isolation and northern analysis

Total fungal RNA was isolated using the RNAzol^{TM} kit from CINNA/BIOTECX. Approximately 10 μg of total RNA was separated on a formaldehyde-agarose gel and transferred to Hybond N-membrane. Filters were hybridized with the appropriate probe at 65 ^{0}C overnight and washed twice with 2xSSC and twice with 0.2xSSC at 65 ^{0}C.

Determination of transcription start points

For rough determination of transcription start points a RACE reaction was performed using the MARATHON kit (Clontech). After a nested PCR on the cDNA pool (using primers CPR8/AP1 in the first round and primers pE3/AP2 in the second round), specific PCR products were obtained. Primers AP1 and AP2 are nested primers specific for the adaptor sequence and were supplied with the MARATHON kit. The PCR fragments were digested with *Not*I (introduced in the adaptor sequence) and *Spe*I (introduced in primer pE3) and subsequently cloned in *Not*I/*Xba*I digested pBluescript SK-II+ (Stratagene, La Jolla, CA). Transcription start points were determined by sequence determination of the inserts of independent clones.

Exact determination of transcription start points was achieved in primer extension experiments. Total RNA was isolated and used in a primer extension reaction which was carried out at 42 ^{0}C using AMV and ^{32}P-kinated primer pE3. After completion of the reaction the mixture was loaded on a 6% polyacrylamide sequencing gel.

Name	Sequence (5' -> 3')	Position
CPR8	GGC TCT GGG TGG GCT C	+ 774 R
CPR38	GAC TCA CAG GGA CT	- 645 F
CPR39	CAC ACT ACC ACT GA	- 679 R
CPR40	AAC TCG GCA GCG GT	- 1109 F
CPR44	CGC *GAA TTC* ACG AGC TGC ATT ATC C	- 1533 F
CPR45	CGC *GAA TTC* AGA CAG CTG ACA GAA TT	- 1443 F
CPR46	CGC *GAA TTC* TTT AAG TTC TTG TAC CTG	- 1333 F
CPR48	CGG *GGT ACC* G TAA ACC GCT GCC CCG A	- 1090 R
CPR49	CGG *GGT ACC* GG GTG CAA CT TCT CCA GG	- 1589 F
CPR51	CGG *GGT ACC* A TCT TTA AGT TCT TGT ACC TG	- 1333 F
CPR55	CGG *GGT ACC* GAG AAA ACG TAG GGA TGA TTA TG	- 793 R
BPH30	ACG ACG *GGA TCC* GAA TTC GTC GAC	+ 25 R
BPH31	GCG *GGA TCC* GGC ACA TTG GAA GAC GCC CTT TT	- 1080 F
BPH32	GCG *GGA TCC* GTG GAT TGA GCG ATG GGA GGC G	- 879 F
BPH33	GCG *GGA TCC* TTT GGG TGA GGA TCT CCC TCC A	- 503 F
CPR60	GTA CTA CTA A*TA GGT AGC* CAT CTT TAA GT	- 1373 F
CPR61	ACT TAA AGA T*GG CTA CCT* ATT AGT AGT AC	- 1344 R
CPR60mut	GTA CTA CTA A*TA G<u>TC CA</u>C* CAT CTT TAA GT	- 1373 F
CPR61mut	ACT TAA AGA T*GG <u>TGG A</u>CT* ATT AGT AGT AC	- 1344 R
BPH40	CAG TGA ACG *TAG GTA GCC* GGA AAA GTC	- 925 F
BPH41	GAC TTT TCC *GGC TAC CTA* CGT TCA CTG	- 896 R
BPH40mut	CAG TGA ACG *TAG <u>TCC A</u>CC* GGA AAA GTC	- 925 F
BPH41mut	GAC TTT TCC *GG<u>T GGA</u> CTA* CGT TCA CTG	- 896 R
pE3	CCC *ACT AGT* CTG TTG GCA GTA GTA GTG T	- 641 R

Table 5.1, Primers used in this study. Primers named CPR are based on the sequence of the *cprA* gene of *A. niger*, primers named BPH on the sequence of the transcription control region of the *A. niger bphA* gene (BPH30 is based on the fusion site between the *bphA* transcription control region and the *lacZ* reporter gene on plasmid pAB94-83). Positions indicated are relative to the predicted translation start point (+1). Primers based on the coding strand of the genes are indicated with **F**(orward) and primers based on the non-coding strand with **R**(everse).
Italics indicate restriction sites introduced in the primers for cloning purposes (CPR35, CPR36, CPR44, CPR45 and CPR46 have an *Eco*RI site, primers CPR48, CPR49, CPR51 and CPR55 a *Kpn*I site, primer CPR54 a *Xho*I site, pE3 a *Spe*I site and all BPH primers contain a *Bam*HI site).
Primers CPR60, 61, 60mut, 61mut, and BPH40, 41, 40mut, 41mut are used in electrophoretic mobility shift assays. *Italics* indicate the homology box (see text) and <u>underline</u> indicate mutated nucleotides.

Sequence analysis of the cprA transcription control region

To determine the sequence of the transcription control region of the *cprA* gene the 0.4 kb *Eco*RI-*Bgl*II fragment and the 0.6 kb *Bgl*II-*Bgl*II fragment of pCPR7 (comprising parts of the transcriptional control region of *cprA*) were subcloned in pBluescript SK-II+. Sequences of the inserts were determined with the dideoxy chain terminating method (Sanger et al. 1977) using the reverse and universal primers and gene specific primers CPR38, CPR39 and CPR40 (Table 5.1). The complete sequence of the *cprA* 5' non coding region is shown in Figure 5.1.

Construction of reporter plasmids

cprA reporter constructs: Constructs pGUS1.7 and pCPRGUS1 (Figure 5.2) were used to construct a number of plasmids with different deletions in the transcription control region (TCR) of the *cprA* gene. Deletion of a 0.6 kb and a 1 kb *Eco*RI-*Bgl*II fragment from the *cprA* TCR present on pGUS1.7 followed by insertion of the *pyrG** selection marker resulted in plasmids pCPRGUS2 and pCPRGUS3 respectively. For smaller deletions specific PCR fragments were generated using forward primers CPR44, CPR45, CPR46 and reverse primer CPR48 which was localized downstream from the *Bgl*II site in the *cprA* TCR (Table 5.1). By adding an *Eco*RI site to the upstream primers, PCR fragments could easily be used to replace the 0.4 kb *Eco*RI-*Bgl*II fragment of the *cprA* TCR present on pGUS1.7. This resulted, after introduction of the *pyrG** selection marker, in plasmids pCPRGUS1Δ100, Δ200 and Δ300.

For construction of pCPRGUS2.1 plasmid pGUS1.7 was digested with *Eco*RI and *Xho*I (870 bp upstream from the translation start point). Blunt ends were generated using the Klenow fragment of *E. coli* DNA polymerase. After gel purification of the fragment the vector was recircularized with an excess of *Eco*RI adaptor (GGAATTCC) to facilitate insertion of the *Eco*RI fragment containing the *pyrG** selection marker.

bphA reporter constructs: For construction of plasmids with progressive deletions of the transcription control region of the *bphA* gene the 2 kb *Bam*HI fragment of plasmid pAB94-83, comprising the complete transcription control region of the *bphA* gene, was replaced by smaller fragments of the *bphA* TCR. For construction of pPBPH1.4 a 1.5 kb *Bgl*II-*Bam*HI fragment isolated from pAB94-83 was used. For construction of pPBPH1.7 the 2.0 kb *Bam*HI

```
   1  GAATTCGTCC GCGAGGAAGG TTGCCCGTGT ATGCAGGACA TTTGGTGCAC TAACCTGTAC
  61  ACAAAGTGCG GAACGGGTGC AACTTCTCCA GGAGCgGAAA ACAgGCAGAG GAGGTGCCGC
 121  AGTCACGAGC TGCATTATCC AAGGTCAGCC AGCCTGCCAG GCTACTGAGT CcATACGCAC
 181  GGGGgcGGAT TGAGCAGaTC GGGTCTTTCA GGGATGCTGC AGACAGCTGA CAGAATTGGC
 241  AGCCGGTGTA GCGTGATTAG AAGTCGGTAT ACGAAGCATA GTAAGGATCG GATATGCATG
 301  GACTACGAAG TACTACTAAT AGGTAGCCAT CTTTAAGTTC TTGTACCTGA AACCATTCTT
 361  CTTCCCTTAA TGGCAATTGA AATAGGAAGA TGATTTgcAG TTCATCTAAC AGGTACTCCA
 421  GATCTCCGGA CTCTCCGGTC TTGCCACCTC CACGGGGTCT CGGGAGACCG GCACAGAGAG
 481  GCCTTCATAT GCCTATCTAT CAAGAATTAC TGAAAGAGGC AGGGTTCGAA AGGCGGACTC
 541  GGTTAGCAAG AGGAACTCGG ggCAGCGGTT TACGGAAGAT CCCCAGATGA AACAGAGGAA
 601  CAACTGGTCA TTCCCGGCTG CGCTTTCATG GCTCAAGGGC CTCCGCCGCC ACTTGCCATC
 661  GTCTCCAAGC CAAGCCAGGG CCGAAGTTTC CTTCCGAACC AGACGCCATC TCGGTCAGTG
 721  GTCGGCCAAT GGGGAATCCC CACCTCGAGG GAGGCAATGG TATCTAGATA GATAAAAGGA
                                                    →  →   ▸
 781  GAGAGAGGGT GACCATCATG ATACTCTGGT TATTTCTTGA CTTCAATTTT AAGAGTTGTA
        →
 841  CTACACATAA TCATCCCTAC GTTTTCTCGG AAGGTTTGAG TCACAAGATA CGTTGTTGTT
 901  GTTGTTGTTG TTGTTGTTGT TGTTGTTGGA AACCcTCCGG CAACCGCTGC TCTGCTAGTG
 961  AACCTCCCCA TCAGTGGTAG TGTGTCTAAT AGTAGTTCTA CACTACTACT GCCAACAGAC
1021  TCACAGGGAC TATATCAGAT AGTCCCCAAC ACGCACAAGG GTCCCTGACC TTGACACGAG
1081  AGATCTGATA ACTCCTCAGC AAATCGGAGT AAACAGAAGG ACAAGTCATT GGAGTACTAA
1141  GTAGCTCCGT GTCAGAGACC CGGACAGGAT CAGCTTCTCC GAACCCGAGA CTCCGGGCGA
1201  AAAGGCCACC ATCGCCTCAG GCTACCACCT GTGTCTTCGC TCTCTCTCCT CTCCGTCGAT
1261  CGTCCTTCCC TCGTCTTTCC GGCTCACGGC CCCCCAAATT atgcggtctg cttacgagtg
                                                    ▸
1321  ggttcggcct ctctgttctt cctggatcac accacggctt actttcttat ccttttcctt
                                      ▸                             ▸
1381  ttcctttctt cctttcttcc tgttctcctt tcttctttcc acccccttct ttcttttaac
1441  cccatagCGT CATTCTTTCT TCCGTTTTAT CTTTTGGTTT TGGGACGCCG CCACCTTATC
1501  TCGGTTCCTG CCTCGGCTCC GGTGATCGCA CCTGGGACAG GCTAAGCGTA GGGAGGTGTG
1561  ACATTCTTCT TTCACCTCCT CTCCTTTTCC CGCCTCACTC CGTTCAATCC CCCGCTCCAC
1621  CCTTTCAGAC TCGCCATCGT ATCAAGTCGG GGCCTTTGCT TGCGCCGCTG AACAGCCTCA
1681  CCATGGCGCA
```

Figure 5.1, Complete sequence of the *cprA* transcription control region.
The sequence of only 582 upstream from the ATG codon of the *cprA* gene was determined previously (van den Brink et al. 1995). The DNA sequence of a larger part (1682 bp upstream from the translational start) of the transcription control region was determined. Characteristic features of the TCR are indicated.

Underlined	CT rich sequences probably involved in transcriptional regulation
Double underlined	Putative Benzoate Regulatory Region
Boxed	Conserved box
▸	Major transcription start points
→	Minor transcription start points
ATG	Coding region of *cprA* is indicated in **BOLD**
lower case italics	uORF

fragment of pAB94-83 was replaced by a 715 bp *Spe*I-*Bam*HI fragment and plasmid pPBPH1.8 was constructed by religation of *Bam*HI digested plasmid pAB94-83 to obtain a *bphA* promoter-less construct. Plasmids pPBPH1.10, pPBPH1.11 and pPBPH1.12 were

bphA promoter-less construct. Plasmids pPBPH1.10, pPBPH1.11 and pPBPH1.12 were obtained by insertion of PCR fragments (using reverse primer BPH30 and forward primers BPH31, BPH32 and BPH33 respectively; Table 5.1) in the unique *Bam*HI site of plasmid pPBPH1.8. For this purpose all oligonucleotides were designed to have *Bam*HI sites. For all constructions care was taken to exactly restore the fusion sites of the *lacZ* gene and the fragment comprising the *bphA* transcription control region. Positive clones were tested for correct orientation of the inserts by restriction analysis.

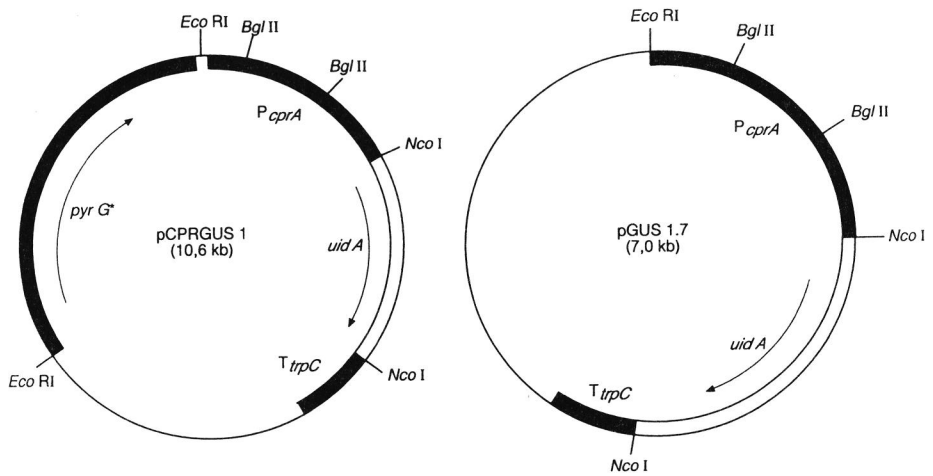


Figure 5.2, Restriction map of plasmids pGUS1.7 and pCPRGUS1.
For construction of pCPRGUS plasmids a 7 kb *Eco*RI-*Sal*I fragment, comprising the complete coding region of the *cprA* gene preceded by 1.7 kb 5' non-translated region and comprising 3.3 kb 3' non-translated sequence was isolated from phage λ19-1 (van den Brink et al., 1995) and subcloned in pBluescript SK II + (Stratagene, La Jolla, CA) resulting in pCPR7. A 1.7 kb *Eco*RI-*Nco*I fragment, on which the complete *cprA* transcription control region is present, was isolated from pCPR7. This fragment was used to replace a 2.3 kb *Eco*RI-*Nco*I fragment of plasmid pNOM102 (Roberts et al. 1989), thus replacing the *gpdA* transcription control region by the *cprA* transcription control region. Since the *Nco*I site in the resulting plasmid pGUS1.7 spanned the ATG codon used for translation start, an exact translational fusion between the *uidA* reporter gene and the *cprA* transcription control region was obtained. A 3.7 kb *Eco*RI fragment comprising an *A. niger pyrG* gene with a defined mutation (*pyrG**; Verdoes et al. 1994a) was cloned in the *Eco*RI site of pGUS1.7, resulting in plasmid pCPRGUS1. Relevant restriction sites are indicated.

Introduction of regulatory elements in minipromoter vector: Fragments comprising (parts of) the *cprA* TCR were generated by PCR using primer combinations CPR49-CPR48 (pAN5mini2.3), CPR51-CPR48 (pAN5mini2.5) and CPR55-CPR49 (pAN5mini2.6) (Table

5.1). After digesting plasmid pAN5mini2.0 (Figure 5.6A) with *Not*I, blunt ends were generated using the Klenow fragment of DNA polymerase. PCR fragments were digested with *Kpn*I (a *Kpn*I restriction site had been introduced in the primers) and were made blunt using T4-DNA polymerase. Transformants were tested for correct orientation by restriction and PCR analysis.

Fungal transformations

Fungal transformations were carried out as described by Punt and van den Hondel (1993). After selection for growth on agar-plates containing *Aspergillus* minimal medium without uridine, indicating a restoration of the mutated *pyrG* gene of strain AB4.1, transformants were tested for correct integration of one copy of the plasmid at the *pyrG* locus by Southern analysis.

Analysis of reporter activity in fungal transformants

Fungal transformants were grown overnight at 30 °C in complete medium (Pontecorvo et al. 1953), harvested by filtration over Miracloth filtration wrap (CalBiochem), washed extensively with 0.9% NaCl and transferred to benzoate medium (+BA; minimal medium with 0.1% glucose and 0.1% benzoic acid as sole C-sources) or control medium (-BA; minimal medium with 0.1% glucose as sole C-source). After cultivation for 3 hr at 30 °C mycelium was harvested and frozen in liquid nitrogen. Reporter activities were determined as described before (Roberts et al. 1989; Punt et al. 1990). Variation of reporter activities between various experiments was as reported before.

Electrophoretic mobility shift assays (EMSA)

Protein extracts were prepared from approximately 10 grams of dry weight mycelium. After filtration through Miracloth filtration wrap (Calbiochem), mycelium was grinded in liquid nitrogen and dissolved in extraction buffer (25 mM Tris-HCl, 5 mM Mg-Acetate, 3 mM DTT, pH 7.5). After centrifugation at 25000 g and for 30 minutes at 4 °C the resulting supernatant was centrifuged for 1.5 hr at 100000 g and 4 °C. The supernatant was applied to a 5 ml Sepharose-Heparin CL-6B column (Pharmacia) equilibrated with extraction buffer.
After addition of the proteins the column was washed with extraction buffer until no protein was detectable in the eluate, as determined by measuring OD_{280nm}. Bound proteins were eluted from the column in extraction buffer containing respectively 50 mM , 100 mM, 200 mM and 300 mM KCl. If no more protein could be detected in the eluate a higher salt concentration

was applied to the column. When extraction buffer with 400 mM or 1000 mM KCl was applied to the column, only minimal amounts of protein were eluted. Fractions comprising maximal amounts of protein, as determined by measuring OD_{280nm}, were pooled, precipitated with 80% ammonium sulphate and dialysed overnight at 4 ^{0}C against extraction buffer without KCl.

As probes equimolar amounts of oligonucleotide combinations CPR60-CPR61, CPR60mut-CPR61mut, BPH40-BPH41 and BPH40mut-BPH41mut were annealed and 4 pmol of each double stranded oligonucleotide were labelled with γ^{32}P-dATP, using T4-polynucleotide kinase.

Nuclear protein extracts (10 µg) were incubated with 20000 cpm of probe (approximately 40 fmol) in retardation buffer (20 mM Tris-HCl, pH7.5, 1 mM EDTA, 5% glycerol, 0.01% Triton X100, 50 mM KCl), supplemented with 1mM DTT, and 10 µg BSA as described by Straney and VanEtten (1994). To prevent non-specific binding 2 µg poly-dIdC (Boehringer) was added to the retardation mixture. The reaction mixture was incubated on ice for 30 minutes and loaded on a 4% polyacrylamide gel, using 0.5*TBE (Sambrook et al. 1989) as runningbuffer. After running the gel for 2-3 hr at 200 V at room temperature the gel was transferred to Whatmann 3MM filtration paper, dried and exposed overnight at -70 °C. In competition experiments an approximately 100 fold molar excess of unlabelled competitor was added to the reaction mixture prior to addition of the specific probe. The reaction mixture was incubated on ice for 15 minutes after which labelled fragment was added and the incubation was continued for an additional 20 minutes.

Other molecular biological methods

DNA manipulation and bacterial transformation were performed using standard methods (Sambrook et al. 1989). DNA sequences were analysed using Genetics Computer Group software.

Results

Benzoate induction of cprA expression

While induction of CPR activity by benzoate is only approximately four fold (van den Brink et al. 1995), northern analysis showed that induction of *cprA* gene expression was much stronger (Figure 5.3A), resulting in a more than 20 fold increase in *cprA* mRNA levels. The majority of the *cprA* transcript after addition of benzoate (*cprAβ*) has an increased size (± 0.5

kb increase) as compared to the *cprA α* transcript observed in control experiments (Figure 5.3A). The smaller *cprA α* transcript could also be observed as a minor transcript in RNA isolated from benzoate induced mycelium.

Characterization of the transcription control region of cprA

The observed difference in size of approximately 0.5 kb between the *cprA α* and *cprA β* transcripts was studied in more detail. To identify the transcription start points used after benzoate induction, a RACE experiment was performed. The DNA sequence of two independent clones, containing *cprA* specific RACE products obtained from benzoate induced mRNA, was determined. The clones analysed contained *cprA* specific sequences starting at respectively 840 bp and 815 bp upstream from the ATG translational start point and approximately 500-550 bp upstream from the previously determined non-induced transcription start points (van den Brink et al. 1995). No intron sequences could be detected in the cloned RACE products.

The transcription start point was determined more precise by primer extension analysis. Using primer pE3 and RNA isolated from benzoate induced mycelium, a major transcription start point could be determined (at -846 relative to the translational start) as well as some minor starts. The determined major transcription start point was comparable to (6 and 31 nt respectively upstream from) the starts found using the RACE strategy (Figure 5.1). If RNA isolated from non-induced mycelium was used, no primer extension product could be observed with primer pE3.

Benzoate responsive regions in the cprA transcription control region

For identification of DNA sequences in the *cprA* transcription control region involved in the benzoate induction of *cprA* gene expression, a deletion study was performed. A translational fusion was made between the *cprA* 5' non coding region and the *uidA* reporter gene (pCPRGUS1). Progressive deletions of the TCR were made and reporter constructs containing these truncated TCRs were targeted in one copy to the *pyrG* locus of *A. niger*. Benzoate induction of GUS reporter activity in pCPRGUS1 transformants reached a maximum of approximately 4 fold after 4 hrs cultivation. This relative induction is comparable to that observed for CPR activity (van den Brink et al. 1995). Western analysis using GUS specific antibodies revealed a comparable, 3-4 fold, increase in GUS protein levels (results not shown), confirming the results obtained at the activity level.

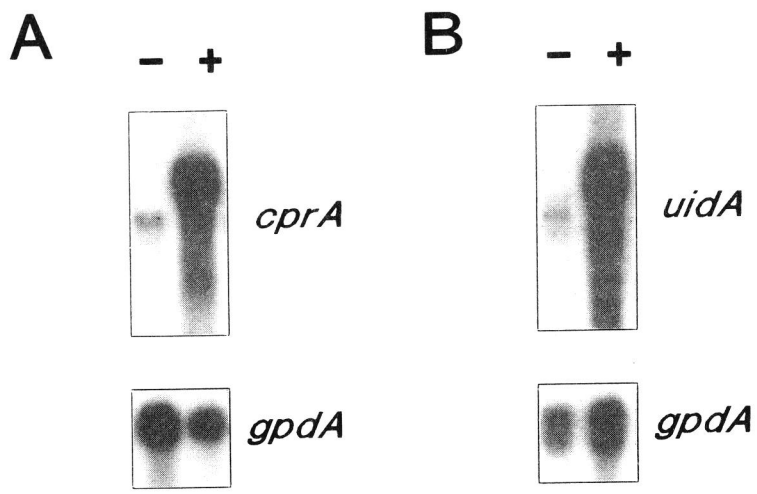


Figure 5.3, Northern analysis of induction of the BPH enzyme system by benzoic acid.
Equal amounts of total RNA, isolated from a pCPRGUS1 transformant of *A. niger* strain AB4.1 after induction with benzoate (+) or without benzoate (-), were separated on gel and blotted to Hybond N membrane. The membranes were probed with a *cprA* **(A)** or *uidA* **(B)** specific probe. As a loading control all filters were hybridised with a probe specific for the constitutively expressed *A. niger gpdA* gene.

Deletion of the most upstream 120 bp of the TCR (pCPRGUS1Δ100) resulted in all experiments in a small decrease of the relative induction without affecting the reporter activity in the control medium (Figure 5.4A). Deletion of another 100 bp (pCPRGUS1Δ200)

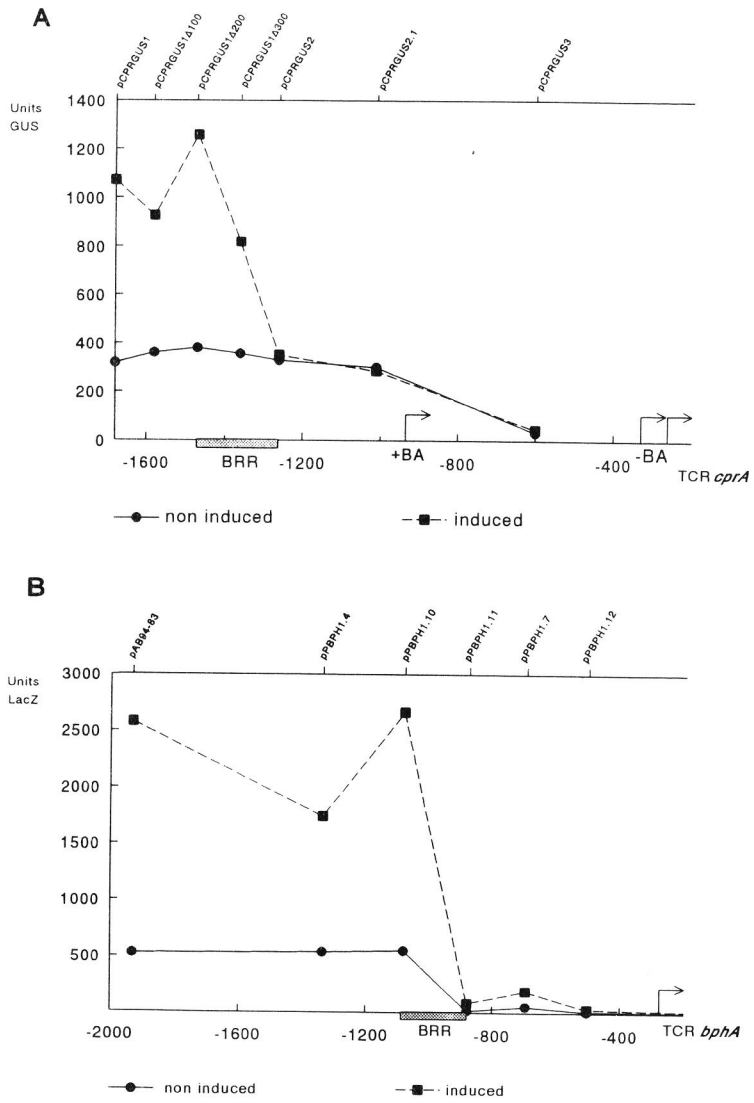


Figure 5.4, Determination of the Benzoate Regulatory Regions of the *cprA* and *bphA* genes.
Indicated on the bottom X-axis are the TCR regions of both genes in bp relative to the translational start point. Transcription start sites are indicated by arrows. The putative BRR is boxed. On the Y-axis reporter activities are indicated. Each value is the mean of at least three independent experiments. Names of constructs used are given on the top X-axis.
A) Deletion study of the *cprA* transcription control region. A translational fusion was made between the *cprA* transcription control region and the *uidA* reporter gene. Different progressive, 5' deletions of the transcription control region were made. Arrows indicate transcription start points. Positions indicated are relative to the translational start. GUS-activities (β-glucuronidase) in selected transformants were determined after 3 hr.

restored this negative effect, suggesting the possible presence of a negatively acting element on this 0.1 kb DNA fragment. Removal of 0.3 kb of the TCR (pCPRGUS1Δ300) resulted in a benzoate induction which was only half of the induction observed in pCPRGUSΔ200 transformants while removal of 420 bp of the transcription control region (pCPRGUS2) completely diminished the effect of benzoate without affecting the activity in control medium (Figure 5.4A). These results indicate the presence of a benzoate regulatory region (BRR) of approximately 0.2 kb located 1 kb upstream from the translation start point of *cprA*. Transformants of plasmid pCPRGUS2.1 (in which 0.7 kb of the TCR was removed) had comparable, non inducible, reporter activities as pCPRGUS2 transformants while deletion of 1 kb of the transcription control region (pCPRGUS3) resulted in a major decrease in general reporter activity (Figure 5.4A).

uidA expression analysis in transformants

The major transcript after benzoate induction, *cprAβ*, has an increased size (± 0.5 kb increase) as compared to the basic *cprAα* transcript (Figure 5.3A). To show that this difference in length is caused by the use of different transcription initiation sites only, RNA was isolated from a transformant of pCPRGUS1. Clear differences in the size of the major *uidA* transcript, the expression of which was controlled by the *cprA* transcription control region, could be observed between benzoate induced and non-induced RNA (Figure 5.3B). As was found for *cprA* mRNA both a longer, major, mRNA species and a minor, basic *uidA* mRNA species could be observed after benzoate induction of pCPRGUS1 transformants (Figure 5.3B). In transformants of plasmid pCPRGUS2, in which the putative BRR was completely removed, both transcripts could be observed as well after benzoate induction, although relatively less of the longer, inducible, transcript was formed. When no benzoate induction was applied only the smaller transcript could be observed in all transformants (Figure 5.5).

induction with benzoate (+ BA) or without benzoate (- BA) and are indicated as units ß-glucuronidase per minute per mg total protein at 37 °C.
B) Deletion study of the *bphA* transcription control region. A translational fusion was made between the *bphA* transcription control region and the *lacZ* reporter gene. Different progressive, 5' deletions of the transcription control region were made. Arrows indicate transcription start points. Positions indicated are relative to the translational start. Reporter-activities (ß-galactosidase) were determined in selected transformants after 3 hr. induction with benzoate (+ BA) or without benzoate (- BA) and are indicated as units ß-galactosidase per mg total protein per minute at 37 °C.

Functionality of the BRR

To analyse the functionality of the 0.2 kb putative benzoate regulatory region (BRR) of *cprA*, three fragments spanning all, or part of, the BRR were generated using PCR and were cloned in the *Not*I site of plasmid pAN5mini2.0, upstream from the *A. nidulans gpdA* mini promoter (Figure 5.6A). The resulting plasmids pAN5mini2.3, 2.5, 2.6 and pAN5mini2.0 (control) were targeted to the *pyrG* locus of *A. niger* strain AB4.1 as described before (van Gorcom et al. 1988). Strains with a correct integration pattern were analysed for β-galactosidase activity with and without benzoate induction. After cultivation in control medium transformants of pAN5mini2.0, 2.3 and 2.5 showed comparable β-galactosidase activities while in pAN5mini2.6 transformants this activity was reduced (Figure 5.6B). Reporter activities in both pAN5mini2.3 and pAN5mini2.6 were considerably induced by benzoic acid (13 and 16 fold respectively), while β-galactosidase activity was only 4.5 fold induced in pAN5mini2.5 transformants. As expected no benzoate mediated induction of reporter activity by benzoate was found in pAN5mini2.0 transformants.

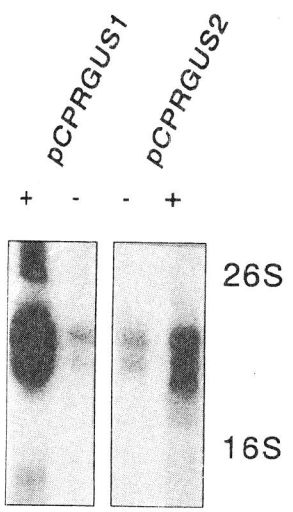
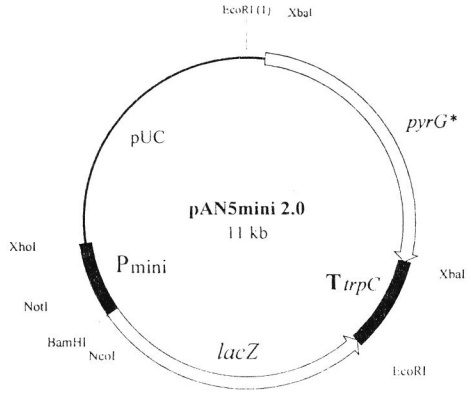


Figure 5.5, Northern analysis of *uidA* transcription in single copy transformants of pCPRGUS1 and pCPRGUS2.
Equal amounts of total RNA, isolated from mycelium of AB4.1 transformants of plasmid pCPRGUS1 and pCPRGUS2, after induction with benzoate (+) or without benzoate (-), were separated on gel and blotted to Hybond N membrane. The blot was probed with a *uidA* specific probe.

Analysis of the bphA transcription control region

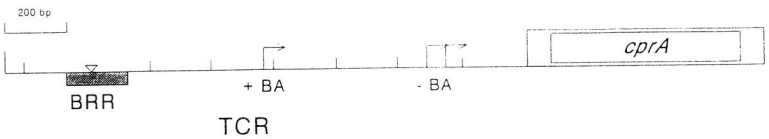
A deletion study was carried out to determine the position of DNA sequences in the *bphA* TCR involved in benzoate mediated induction of transcription. Removal of the most upstream 0.6 kb of the *bphA* TCR (pPBPH1.4), fused to the *lacZ* reporter gene, resulted in all experiments in some decrease of inducibility (Figure 5.4B). Deletion of an additional 254 bp DNA fragment (pPBPH1.10) restored reporter activities, suggesting the presence of a benzoate dependent negatively acting regulatory element on this 0.25 kb sequence. Removal of an additional 0.2 kb almost completely reduced (23 fold reduction) expression of the *lacZ* reporter gene in the presence of benzoate, indicating the presence of a benzoate regulatory region (BRR) on this DNA fragment. Deletion of this putative BRR fragment also reduced the basal, non induced

A



B

	- BA	+ BA	%
pAN5mini2.0	116 1057	308 1029	97
pAN5mini2.3	410 1009	2504 13483	1336
pAN5mini2.5	302 1255	1530 5628	448
pAN5mini2.6	101 563	485 8841	1570



TCR

Figure 5.6, Functionality study of the *cprA* BRR
A) Restriction map of plasmid pAN5mini2.0.
For construction of pAN5mini2.0, a 3.6 kb *Xba*I fragment of plasmid pAN5mini (Punt et al. 1995) comprising the *A. nidulans argB* selection marker, was replaced with a 3.7 kb *Xba*I fragment isolated from plasmid pAB94-11 (van Gorcom et al. 1988) which comprised the mutated *A. niger pyrG** gene.
B) Effects of introduction of BRR elements on benzoate inducibility of the constitutively expressed *gpdA* promoter on plasmid pAN5mini2.0.
Different DNA fragments were isolated from the *cprA* transcription control region and introduced in the right orientation upstream from the minimal *A. nidulans gpdA* promoter. Reporter (ß-galactosidase) activities were determined in selected one copy transformants of *A. niger* AB4.1 after a three hours incubation in minimal medium with benzoate (+ BA) or without benzoate (- BA). Indicated are units of ß-galactosidase activity per mg total protein per minute at 37 °C. Each value is the mean of at least three independent experiments, standard deviations are indicated in small numbers above each value. Relative benzoate inducibility is indicated as the percentage of the activity observed in the non-induced situation.

expression levels.

Sequence comparison of putative benzoate regulatory regions

Sequence comparison of the putative benzoate regulatory regions (BRRs) in the transcription control regions of the *cprA* and *bphA* genes, as identified in the transcription control region deletion studies, revealed a completely identical 9 bp sequence TAGGTAGCC) starting at -1355 bp for the *cprA* and at -1611 bp for the *bphA* transcription control region (relative to the translational start point).

Electrophoretic mobility shift assay

To determine if the 9 bp homology box, identified by sequence comparison of both BRRs was involved in benzoate inducible transcriptional activation of *cprA* and *bphA* gene expression, electrophoretic mobility shift experiments were performed (Figure 5.7). Double stranded oligonucleotides were designed based on the sequences of the *cprA* and *bphA* transcription control regions. These 27-mers comprised the identified 9 bp homologous boxes surrounded by *bphA* or *cprA* specific sequences. As a control oligonucleotides were designed in which 4 nucleotides of the homology box were mutated (G/C ↔ A/T transitions). To prove the specificity of the binding reactions purified extracts from both induced and non-induced mycelium were obtained. Binding of all four double stranded oligonucleotides was found using the protein fraction from benzoate induced mycelium. The strongest binding was observed in the fraction eluted with 100 mM KCl while weak binding was found in the 200 mM eluate. Using the BPH oligonucleotide one retarded complex was observed in the induced protein fraction while no retardation could be found using protein extracts isolated from non-induced mycelium. The CPR oligonucleotide was retarded by both fractions, resulting in two complexes, the electrophoretic mobility of one of them being comparable to the complex formed with the BPH oligonucleotides. Retardation by the non-induced fraction was clearly less efficient as compared to retardation by proteins from benzoate induced mycelium. Surprising was the fact that both mutated oligonucleotides were able to form retarded complexes with about the same efficiency as wildtype oligonucleotides.

Cross competition experiments were performed to show that all oligonucleotides were recognized by the same protein factor. Binding of both the BPH and the CPR specific oligonucleotide could be competed for by addition of either a 100 fold molar excess of unlabelled BPH or a 100 fold molar excess of unlabelled CPR specific oligonucleotides.

Discussion

Benzoate mediated regulation of *cprA* gene expression seems to be a complex event. Comparison of the relative induction rate at the activity level (approximately 4 fold) and at the level of transcription (over 20 fold) revealed a clear discrepancy. This discrepancy was also found in transformants containing plasmid pCPRGUS1 in which the *cprA* TCR was fused to a *uidA* reportergene, indicating that the differences in relative induction levels were caused by sequences present in the *cprA* TCR region. This observation prompted us to a more detailed analysis of the *cprA* TCR and of the mechanism underlying the benzoate mediated induction of *cprA* expression.

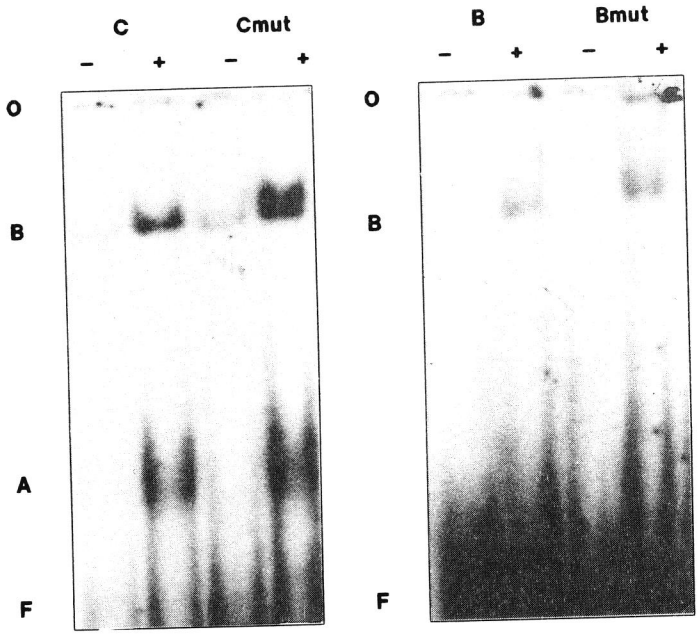


Figure 5.7, EMSA experiments
EMSA experiments were performed using protein extracts isolated from both benzoate induced (+) and non-induced (-) mycelium. Oligonucleotides used were based on the conserved box and flanking sequences of the *cprA* TCR (CPR) and *bphA* TCR (BPH). Oligonucleotides CPRmut and BPHmut are identical to oligonucleotides CPR and BPH respectively, except for mutation of four basepairs in the conserved sequence (Table I). Complexes formed are indicated with **A** and **B**, **F** indicates unbound labelled nucleotides while **O** indicates the origin of loading.

Benzoate dependent transcriptional activation results in the synthesis of a larger *cprA* mRNA (*cprA*β) as the major mRNA species, in addition to the constitutively expressed smaller *cprA* mRNA (*cprA*α) as was shown in Northern experiments (Figure 5.3A). The exact transcription start points of both mRNAs were determined by RACE and primer extension analysis. This analysis revealed the presence of an additional 500-550 bp on the leader of the *cprA*β mRNA. Since there is a clear discrepancy between the relative induction levels of *cprA* mRNA and CPR activity it seems likely that sequences within the additional leader sequence of the *cprA*β transcript are involved in down regulation of

translation. On this additional leader sequence an upstream open reading frame (uORF) of 48 codons is present. Some studies have been published in which effects of uORFs on the efficiency of translation of fungal mRNAs are described. A well studied example is the *A. nidulans brlA* gene, involved in developmental regulation. In this gene two mRNA species can be formed, the translation of one of them being down regulated by the presence of an uORF (Prade and Timberlake 1993, Han et al. 1993). Other examples of uORFs involved in regulation of expression are the *A. nidulans stuA* gene (Miller et al. 1992), the *Neurospora crassa arg-2* gene (Luo and Sachs 1996) and the *S. cerevisiae GCN4* gene (reviewed in Geballe and Morris 1994). In the leader sequence of the benzoate inducible *A. niger bphA* gene two small uORFs are present as well. However, deletion of these uORFs did not affect benzoate inducible reporter activity driven by the *bphA* TCR (van Gorcom et al 1997). The uORF identified in the leader sequence of *cprAβ* might be involved in down regulation of translation of this larger mRNA, in a way comparable to the regulatory mechanisms involved in regulation of *brlA* gene expression. This could explain the discrepancy observed between induction at the mRNA and at the activity level. More experimental data are needed to confirm this hypothesis.

Differential promoter use seems to be a major mechanism in controlling intracellular CPR levels. Another important feature involved in regulation of *cprA* gene expression is the Benzoate Regulatory Region identified in this study. Cloning of BRR comprising fragments from the *cprA* TCR upstream of a constitutively expressed, minimal promoter (the *A. nidulans gpdA* promoter) conferred benzoate inducibility to this promoter, indicating that all sequence elements needed for regulation of benzoate inducibility are present on this BRR fragment (pAN5mini2.3; Figure 5.6B). Deletion of half of the *cprA* BRR (as in pCPRGUS1Δ300 and pAN5mini2.5) resulted in a reduction, but not loss, of benzoate inducibility. This result indicates that multiple sequence elements within the *cprA* BRR are involved in benzoate dependent induction of transcription, some of which still are present while others have been removed from the TCR fragment of plasmid pCPRGUS1Δ300.

Some indications were found for a benzoate dependent negatively acting DNA fragment present in the TCR of *cprA*. This fragment is found directly upstream of the BRR. The *in vivo* function of this negatively acting sequence remains unclear but it is tempting to suggest a function in control of gene expression in response to the conversion products of the BPH enzyme system (e.g. para-hydroxy benzoate), effectively creating a negative feed back

regulatory mechanism (addition of para-hydroxy benzoate to the induction medium was shown to result in reduction of benzoate inducibility of reporter activity in pCPRGUS1 and pCPRGUS1Δ200 transformants while this negative effect was not present anymore in pCPRGUS1Δ300 transformants; unpublished results).

Since benzoate induction stimulates a switch in the major transcription start point used it was expected that some interaction between this upstream, inducible transcription initiation site and the BRR would be found. However, this study revealed no evidence for such an interaction. Cloning of the BRR upstream from a minimal promoter was sufficient to confer benzoate inducibility to this minimal promoter (pAN5mini2.3). Cloning of a larger fragment, including the induced transcription start point upstream from the mini-promoter (pAN5mini2.6) did not change the relative induction levels achieved, showing that both elements (BRR and induced tsp) have independent modes of action. Northern analysis of RNA isolated from benzoate induced mycelium of pCPRGUS1 and pCPRGUS2 transformants revealed additional evidence for this independent character. The larger, major *uidA* transcript observed in pCPRGUS1 transformants after benzoate induction, was observed in induced mRNA isolated from pCPRGUS2 transformants as well. Since the complete BRR is absent in plasmid pCPRGUS2 this supports the observation that the *cprA* BRR is not involved in selection of the transcription start used. Taken together, these results suggest the presence of a benzoate dependent, enhancer like element on the *cprA* TCR. After benzoate induction this enhancer like element probably is recognized by a *trans*-acting factor resulting in stimulation of transcription from the most nearby transcription start point. Since the use of this alternative transcription start point is benzoate dependent, and BRR independent, this suggests that a second benzoate

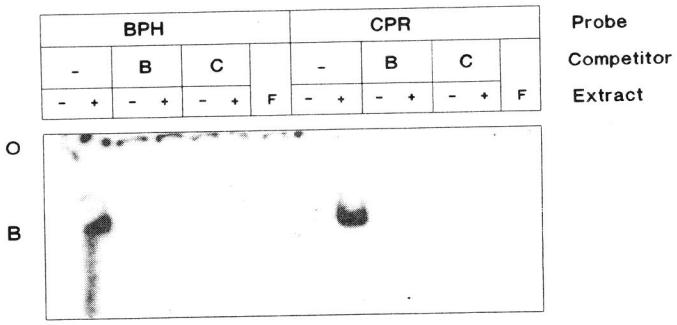


Figure 5.8 Competition experiments.
EMSA experiments were carried out as in figure 5.7 except that competitor oligonucleotides were added in a 100 fold molar excess to the protein extracts. The mixture was incubated for 15 minutes prior to addition of the radio labelled oligonucleotide.

dependent *trans*-acting factor is involved in the activation of the alternative transcription start point.

Regulation of gene expression of the *bphA* gene encoding the second component of the BPH enzyme system, seems to be less complex than regulation of *cprA* gene expression although clear similarities are found. In a deletion study of the TCR of the *bphA* gene of *A. niger* a 0.2 kb DNA fragment involved in the benzoate dependent regulation of gene expression (benzoate regulatory region, BRR) was identified. As was found for the *cprA* TCR, directly upstream from the *bphA* TCR a sequence element was found which had a negative effect on benzoate induced gene expression. Besides sequence elements involved in benzoate dependent regulation, the 0.2 kb BRR of the *bphA* gene comprises sequence elements involved in the general regulation of transcription, since removal of the BRR diminished non-induced reporter activities as well.

Regulation of expression of the genes encoding benzoate para hydroxylase (*bphA*) and cytochrome P450 reductase (*cprA*) by benzoate seems to be a coordinate event, reaching maximal activity levels after 4 hours of induction. Since both genes involved in this system have been shown to be located on different chromosomes (Verdoes et al. 1994a), it seems likely that *trans*-acting regulatory factors, recognizing specific sequences in the TCR of both genes, are involved in this coordinate regulation of expression of both genes.

Sequence comparison of both BRRs revealed a conserved box of nine base pairs. In plasmids pCPRGUS1Δ300 and pAN5mini2.5 this box is almost completely deleted. Reporter activities in these transformants still are benzoate inducible but induction levels are considerably reduced. These results indicate that the conserved box, although likely to be involved, is not the only sequence element needed for full benzoate inducibility of *cprA* gene expression.

Based on the sequences surrounding the conserved box in the TCRs from both *cprA* and *bphA*, double stranded oligonucleotides were designed for EMSA studies. Complexes were detected with both CPR and BPH oligonucleotides. The complexes formed have approximately the same electrophoretic mobility which might implicate that both oligonucleotides are recognized by the same DNA binding protein. Support for the presence of such a common *trans*-acting regulatory factor came from cross-competition experiments. Competition for binding of both BPH and CPR oligonucleotides in EMSA experiments could

be achieved by addition of excess of unlabelled BPH and of excess unlabelled CPR oligonucleotides, suggesting that both oligonucleotides are recognized by the same *trans*-acting factor (Figure 5.7). Surprisingly, introduction of mutations in the conserved core sequence of both CPR and BPH oligonucleotides did not affect binding. Therefore it seems likely that the flanking elements of both boxes, and not the conserved core sequence, are important for recognition of the oligonucleotides. This is unexpected since no major sequence homology between these flanking regions could be observed. Preliminary experiments, in which the mutated CPR oligonucleotide was cloned upstream from the minimal promoter present on pAN5mini2, showed that the presence of this oligonucleotide was sufficient to confer benzoate inducibility (unpublished results), thus providing *in vivo* confirmation of the results obtained in *in vitro* with EMSA experiments.

General discussion

A complex mechanism is involved in the benzoate dependent regulation of *cprA* gene expression, resulting in a discrepancy in the relative induction rates of CPR activity and *cprA* mRNA levels. Multiple sequence elements, present on a 0.2 kb DNA region 1 kb upstream from the translation start site, are involved in benzoate induction of *cprA* gene expression. After benzoate induction *cprA*β mRNA is formed which has an unusual long leadersequence of approximately 850 bp (while the *cprA*α transcript has a leadersequence of approximately 0.3 kb). Sequences on the 0.5 kb additional leadersequence of *cprA*β seem to be involved in down regulation of the translation, thus providing an additional regulatory mechanism. Such a complex regulatory mechanism might be important for adaptation of CPR activity levels to the expression levels of a number of different cytochrome P450 genes. However, a second regulatory system is needed to prevent overproduction of CPR. *In vitro* studies have shown severe effects of excessive high levels of CPR on cell viability and integrity of ER proteins. Uncoupling of the CPR-P450 redox chain results in the release of electrons in the cytosol and thus in the formation of activated oxygen molecules (Mukhopadyay and Chatterjee 1994, Ross et al. 1995). To avoid production of oxygen radicals a tight control of CPR levels is of great importance.

Regulation of *bphA* gene expression seems to be less complex. All sequence elements needed for both basal and induced transcription are present on a 0.2 kb DNA fragment. Although uORF sequences are found to be present on the leadersequence of the *bphA* mRNA no effect of deletion of these small uORF sequences on benzoate dependent activity could be

determined (van Gorcom et al. 1997).

From this study it becomes clear that regulation of the general component of this system, the cytochrome P450 reductase (CPR) is highly complex, involving different mechanisms. Like in mammalian species, a number of different cytochrome P450 encoding genes are expected to be present in the *Aspergillus* genome (based on biochemical data), some of which seem to be subject to substrate inducibility (van Gorcom et al. 1990, Samanta and Ghosh 1987, Ghosh et al. 1983). To obtain a functional cytochrome P450 enzyme system in response to these inducers, it seems likely that *cprA* gene expression is induced by these different substrates as well. To completely unravel the regulatory mechanisms involved in *cprA* gene expression, more study is needed to identify regulatory proteins involved in the regulation. Identification of other inducible cytochrome P450 genes from *A. niger* would allow further evaluations of the mechanisms involved in coregulation of *cprA* gene expression with gene expression of different cytochrome P450 encoding genes.

The *A. niger* BPH system provides an interesting model system for studies on regulation of cytochrome P450 gene expression in eukaryotes. Regulatory mechanisms for *cprA* gene expression are already highly complex in a relatively simple eukaryotic organism like *A. niger*. Understanding the regulatory mechanisms involved in regulation of gene expression of a cytochrome P450 enzyme system in *A. niger* might provide important insights in the probably even more complex regulatory pathways involved in the control of expression of P450 enzyme systems in higher eukaryotes.

Chapter 6

General Discussion

The aim of the research described in the preceding chapters of this thesis was to study the use of cytochrome P450 reductase (CPR) overexpressing *A. niger* strains for improvement of cytochrome P450 activities and to get insights in the mechanisms involved in regulation of CPR expression. The basis for both studies has been the cloning of the *A. niger* gene encoding cytochrome P450 reductase (CPR) as was described in chapter 2.

1. Improvement of cytochrome P450 activities in filamentous fungi by CPR overexpression

In chapters 3 and 4 of this thesis research is described aimed at the construction of *A. niger* strains with improved cytochrome P450 activities. To achieve this goal multiple copies of two different cytochrome P450 encoding genes (coding for sterol 14α-demethylase and benzoate para-hydroxylase) were introduced in the *A. niger* genome together with the *A. niger* gene encoding CPR. Subsequent analysis of the strains generated revealed that both cytochrome P450 activities tested could be increased several fold by increasing the expression levels of CPR. Although a clear increase in cytochrome P450 activities could be achieved by coexpression of CPR and cytochrome P450, it is likely that several factors limit cytochrome P450 activity levels in *A. niger*. Some of these putative limiting factors will be discussed in this paragraph.

1.1 Control of cprA expression as a limiting factor

The CPR overproducing strains described in chapters 3 and 4 were constructed using plasmid pCPR2. On this plasmid CPR expression is driven by a relatively small part of the *cprA* transcription control region (TCR), present on a 582 bp fragment. This fragment, comprising part of the *cprA* TCR, is identical to the TCR fragment present on plasmid pCPRGUS3 which was used in the TCR analysis described in Chapter 5. As described in this chapter non-induced *uidA* reporter gene expression in transformants of plasmid pCPRGUS3 was approximately 15 fold reduced as compared to the *uidA* expression driven by the complete 1.6 kb TCR.

Furthermore, important regulatory elements, involved in benzoate induced transcription, were shown to be missing on this smaller TCR fragment, resulting in a loss of benzoate inducibility.

1.2 Formation of reactive oxygen molecules in cprA overexpressing strains

A potential problem, resulting in reduced cytochrome P450 activities in CPR overexpressing strains, is the risk of uncoupling. In uncoupled conditions, in which the generated electrons are donated to O_2 molecules (Figure 6.1), CPR performs its NADPH oxidase reaction in the absence of a redox partner. This results in the formation of oxygen radicals and of H_2O_2, both of which may cause severe cell damage (Mukhopadyay and Chatterjee 1994, Ross et al 1995). *In vitro* studies showed significantly increased radical formation in microsomes isolated from *cprA* overproducing *A. niger* strains compared to wildtype strains or to strains overexpressing only the cytochrome P450 encoding gene *bphA* (unpublished results). This increase in the formation of reactive oxygen molecules forms a potential risk for the integrity and activity of microsomal proteins, like cytochrome P450s, and for cell viability. However, the occurrence of reactive oxygen formation in *cprA* multicopy transformants *in vivo* will require further study to evaluate if, and to which extent, formation of reactive oxygen molecules might provide limitations to the increase of cytochrome P450 activities in *A. niger*.

1.3 Limited cytochrome P450 activities by incorrect heme incorporation

To become catalytically active P450 enzymes need the incorporation of a heme group in their active site. Studies on heme incorporation in the active site of P450 enzymes of filamentous fungi have not been reported yet, while relatively little is known about the heme biosynthesis capacity of filamentous fungi. Studies on the overexpression of other hemeproteins revealed possible limitations in the production and/or incorporation of heme after overproduction of these proteins (e.g. catalase) in *S. cerevisiae* and *A. niger* (Kinoshita et al. 1994, Fowler et al. 1993). However, the fact that introduction of additional CPR molecules in P450 multicopy strains results in a considerable increase in P450 activity implicates that at least a significant part of the cytochrome P450 molecules are potentially active and thus do contain a hemegroup in their active site. Although an important part of the P450 enzymes available presumably does contain a correctly integrated hemegroup, it remains unclear what percentage of the cytochrome P450 enzymes can not be activated because of incorrect, or no, heme incorporation. To evaluate the effects of heme incorporation on cytochrome P450

overexpression it will be of importance to determine the relative amount of cytochrome P450 enzymes with no, or incorrect, heme incorporation in multiple copy *A. niger* strains.

1.4 Electron donation as a limiting factor for cytochrome P450 overexpression

Although it was established that CPR is the main electron donor for microsomal cytochrome P450s, recent studies indicate that another important component of the electron donating system of some, but not all, cytochrome P450 enzymes is cytochrome b_5 (Vergéres and Waskell 1992, Urban et al. 1990, Truan et al. 1993). As was described in chapter 1 of this thesis overexpression of cytochrome b_5 in *S. cerevisiae* had positive effects on the activity of a number of different cytochrome P450s, although cytochrome b_5 did not increase all P450 activities and sometimes even inhibited P450 activities (Urban et al. 1990). The mechanism by which cytochrome b_5 is able to increase activity levels of some cytochrome P450s remains unclear yet. Since deletion of the *Cyb5* locus in a *S. cerevisiae* Δ-*cpr* strain is lethal, it is likely that one of the functions of cytochrome b_5 is to act as an alternative electron donor to vital cytochrome P450 enzymes (Truan et al. 1994).

To explain the effects of CPR and cytochrome b_5 on the P450 reaction mechanism Pompon (1987) suggested that cytochrome b_5 might be involved in stabilization of the cytochrome P450-oxy intermediate (See Figure 1.5), resulting from the activation of the cytochrome P450 active site by CPR. This stabilization would prevent the decay of the P450-oxy intermediate and thus formation of superoxide. Schenkman et al. (1994) propose that cytochrome b_5 might complex with cytochrome P450 and CPR in such a way that CPR can provide two electrons to the cytochrome P450-cytochrome b_5 complex. In this way both electrons could be available for reduction of the cytochrome P450 and subsequently for the reduction of the cytochrome P450-oxy intermediate. Since cytochrome b_5 was shown to cross link both with CPR and with the cytochrome P450, the explanation of Schenkman et al. seems to be the most likely. In this model cytochrome b_5 would facilitate efficient electron flow between CPR and, some, cytochrome P450s, while other cytochrome P450s can efficiently

receive their electrons from CPR without the presence of cytochrome b_5 (Figure 6.1). Another observation that supports this model is the reducing effect of cytochrome b_5 on the formation of oxygen radicals by CPR. CPR mediated oxygen activation is most likely caused by an uncoupling of CPR mediated NADPH oxidation and P450 mediated substrate oxidation (Patten and Koch 1995, Shet et al. 1995). By making the coupling between CPR and the cytochrome P450 more tight, cytochrome b_5 could prevent some of the electron leakage found in the absence of cytochrome b_5. The hypothesis of Schenkman is confirmed by extensive work of Yamazaki et al (1996). Using reconstitution systems containing some or all partners of the CYP3A4 monooxygenase system, they were able to show that, although most of the electrons needed were donated by CPR, some electron donation by cytochrome b_5 could take place. However, acceleration of the electron flow between CYP3A4 and CPR seems to be the major function of cytochrome b_5 in their study. Direct electron donation to P450s by cytochrome b_5, like was found in the *S. cerevisiae Δ-cpr* strain, seems to be only a minor activity.

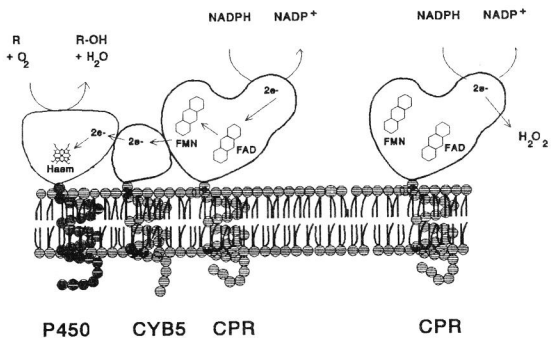


Figure 6.1, model for the electron donating system of cytochrome P450 enzymes in the ER.
CPR= cytochrome P450 reductase, CYB5=cytochrome b_5, R=substrate.

The importance of cytochrome b_5 in the electron donation pathway of some cytochrome P450 enzymes is clear. However, where CPR activity seems to be a prerequisite for activation of all endoplasmic cytochrome P450s, the activating effect of cytochrome b_5 is additional and depending on the cytochrome P450 species involved.

gedefinieerd te worden als het bezit van het vermogen tot het zelfstandig produceren van een functioneel eiwit.

8.

De conclusie van Perret et al. dat zeer attractieve vrouwengezichten systematisch afwijken van het gemiddelde is, alhoewel correct, in tegenspraak met de getoonde onderzoeks resultaten.

Perret, D.I. (1994) et al. Nature 368, 239-242.

9.

Het Nederlandse namensysteem waarin voorletters niet automatisch corresponderen met de roepnaam en waarin veelvuldig tussenvoegsels gebruikt worden leidt tot een achterstand voor Nederlandse onderzoekers in citatie onderzoeken.

10.

Het introduceren van *in vivo* ontdooitechnieken voor 1.5 ml reactievaatjes, gebruikmakend van de lichaamswarmte achter de oorschelp, leidt tot een aanzienlijke tijdsbesparing voor moleculair biologisch onderzoekers en dus tot een effectiever gebruik van publieke middelen.

11.

De beschrijving 'berenklauw' voor een gefrituurd produkt uit snackbars geeft een even accurate indicatie van de gebruikte ingrediënten als de beschrijving 'vleeskroket'.

12.

De keuze van Amsterdam als hoofdstad van het Koninkrijk der Nederlanden is niet gebaseerd op de aanwezigheid van regeringsmacht of op de woonplaats van het Koninklijk huis noch op het inwonertal. Derhalve dient de keuze van een andere hoofdstad serieus overwogen te worden.

STELLINGEN

Behorende bij het proefschrift:

Cytochrome P450 reductase of Aspergillus niger*: a molecular biological study.*

1.

Voor het verkrijgen van micro-organismen met optimale cytochroom P450 activiteiten is, naast overexpressie van het gen coderend voor het cytochroom P450 enzym, overexpressie van het gen coderend voor cytochroom P450 reductase noodzakelijk.

Dit proefschrift

2.

De grote nadruk in de moderne biologie op transcriptionele regulatie van genexpressie doet onrecht aan het belang van post-transcriptionele regulatie voor het nauwkeurig controleren van intra-cellulaire enzym niveaus.

Dit proefschrift

3.

Het indelen van nieuw geïdentificeerde eiwitten in de cytochroom P450 superfamilie op basis van alleen sequentie gegevens, zonder biochemische onderbouwing, is gezien de geringe verwantschap op aminozuur niveau tussen leden van de superfamilie onverantwoord.

Nebert, D.W. et al. (1991), DNA Cell Biol. 10, 1-14.
Nelson, D.R. et al. (1996), Pharmacogen. 6, 1-42.

4.

De algemeen aanvaarde veronderstelling dat $P450_{nor}$ uit *Fusarium oxysporum* een lid is van de cytochroom P450 superfamilie is slechts gebaseerd op een geringe sequentie homologie, en wordt niet onderbouwd door een piek bij 450 nm in een gereduceerd CO spectrum terwijl remming van de enzymatische activiteit door CO slechts in één van de beschreven experimenten kon worden aangetoond. Derhalve dient de indeling van NOR in de cytochroom P450 superfamilie als voorbarig beschouwd te worden.

Nakahara, K. et al. (1993), J. Biol. Chem. 268, 8350-8355
Shoun, H. et al. (1991), J. Biol. Chem. 266, 11078-11082

5.

Het bestuderen van inductie van *S. cerevisiae cyp51* gen expressie door het vergelijken van *cyp51* mRNA data en 7-ethoxycoumarine O-deethylase activiteit, zoals beschreven door Del Carratore et al., is gebaseerd op de foutieve gedachte dat alle cytochroom P450 enzymen EROD activiteit bezitten.

Del Carratore, R. et al. (1992), Carcinogenesis 13, 2175-2177.

6.

De mogelijkheid *Aspergilli* genetisch te modificeren met behulp van RNA in plaats van het gebruikelijke DNA biedt grote kansen voor de moderne biotechnologie.

Zucchi, T.M.A.D. et al. (1996), Cell. Mol. Biol. 42, 235-240.

7.

De vaak gehanteerde omschrijving van het begrip leven, waarin het bezit van het vermogen tot voortplanting als uitgangspunt wordt genomen, geldt niet voor elk individu. Leven dient

2. Regulation of *cprA* gene expression

Induction of *cprA* gene expression by benzoate was shown to be a complex event (chapter 5). In a deletion study of the *cprA* TCR a 0.2 kb DNA fragment was identified which was involved in the benzoate dependent induction of *cprA* gene expression (benzoate regulatory region; BRR). Deletion of this fragment from the *cprA* TCR resulted in a loss of benzoate inducibility while cloning of the BRR upstream from the constitutively expressed *A. nidulans gpdA* mini-promoter made this minimal promoter benzoate inducible. Since deletion of part of the BRR did not result in a complete loss of benzoate inducibility it was proposed in chapter 5 that more sequence elements in the BRR are important for benzoate induction of gene expression.

In a deletion study of the *bphA* TCR a 0.2 kb DNA fragment (BRR) involved in benzoate inducibility of the *bphA* gene was identified. Sequence comparison of the BRRs from the *cprA* and the *bphA* TCRs revealed a common, completely conserved, DNA sequence element of 9 basepairs. Double stranded oligonucleotides were designed, comprising the conserved 9 bp box and the flanking regions present on the *cprA* and *bphA*. In electrophoretic mobility shift assays (EMSA) clear retardation of both BPH and CPR derived oligonucleotides was observed using protein extracts obtained from benzoate induced mycelium while no (BPH) or less efficient binding (CPR) was observed using the non-induced extracts. The electrophoretic mobility of retardation complexes with both CPR and BPH oligonucleotides was approximately the same, indicating that a common factor was bound to both oligonucleotides. This possibility was supported by the observation that binding of radio-labelled CPR and BPH oligonucleotides was inhibited by both unlabelled CPR and unlabelled BPH oligonucleotides, as was shown in cross-competition experiments. However, the exact function of the 9 basepair sequence elements remains unclear. Partial mutagenesis of the 9 bp conserved box (replacement of 4 out of 9 conserved residues) did not alter the retardation efficiency, indicating that other DNA sequences, present on both oligonucleotides, are important for recognition of the oligonucleotides by the protein factors involved.

The oligonucleotides used in the EMSA studies were specifically recognized by protein extracts obtained from benzoate induced mycelium and are thus likely to be involved in benzoate induction. However, since the *in vivo* results obtained with the *cprA* TCR deletion study indicated the presence of multiple sequence elements involved in the benzoate dependent regulation of *cprA* gene expression, the sequence element present on the CPR oligonucleotide

is probably not the only one involved.

Besides regulation via the BRR elements, a second regulatory mechanism was found to be involved in the benzoate mediated induction of *cprA* gene expression. After benzoate induction an alternative promoter (Pβ) is used in the *cprA* TCR. This results in the transcription of a major species of mRNA (*cprAβ*) which is approximately 0.5 kb longer than the *cprAα* transcript found under non-induced conditions. No intron sequences could be detected on the *cprAβ* transcript which makes it unlikely that, due to alternative splicing mechanisms, this longer transcript would be translated in a different protein. An upstream open reading frame (uORF) of 48 codons was found to be present in the *cprAβ* leader sequence. The presence of such a long uORF is often associated with translational regulation (Geballe and Morris 1994, Mueller and Hinnebusch 1986).

Since benzoate induction stimulates a switch in the major transcription start point (tsp) used it seems likely that some interaction occurs between this upstream, inducible transcription initiation site and the BRR. Therefore some experiments were performed to analyse this putative interaction. Cloning of the BRR, and not the Pβ promoter, upstream from a minimal promoter was sufficient to confer benzoate inducibility to this minimal promoter. Cloning of a larger fragment, which comprised both the BRR and the Pβ promoter, upstream from the minimal promoter did not change the relative induction levels achieved, showing that both elements (BRR and induced tsp) have independent modes of action. Northern analysis revealed additional evidence for this independent character. The larger, major, *uidA* transcript observed in pCPRGUS1 transformants after benzoate induction, was observed in induced mRNA isolated from pCPRGUS2 transformants as well. Since the complete BRR is absent in plasmid pCPRGUS2 this supports the observation that the *cprA* BRR is not involved in selection of the transcription start used. These results suggest that although the switch of promoters used is depending on benzoate induction this event is independent from the presence of a BRR.

By comparing the effect of benzoate induction on the levels of *cprA* mRNA and CPR activity a considerable discrepancy was found, CPR activity being induced only 4-5 fold while mRNA induction is 20 fold or more. To establish a clear correlation between activity and mRNA levels determination of protein levels is inevitable. Because no CPR specific antibodies were available, transformants of plasmid pCPRGUS1 in which *uidA* expression was controlled

by the *cprA* TCR, was used for a detailed analysis. As expected, in these transformants a high increase in *uidA* mRNA levels was observed upon benzoate induction, as well as an increase in transcript size comparable to the increase in size observed for the *cprA* transcript. In addition both GUS activity levels and GUS protein levels (as detected on Western blots) were increased only 4-5 fold. Based on these results it can be concluded that although mRNA levels are greatly increased by benzoate induction, protein levels are much less increased. This indicates a second mode of regulation of *cprA* (*uidA*) expression, presumably at the level of translation.

2.1 Model for benzoate dependent regulation of cprA gene expression

The data presented in chapter 5 of this thesis clearly indicate a complex regulatory mechanism involved in the control of gene expression of the *A.niger cprA* gene. A model for this complex regulation of *cprA* gene expression is presented in Figure 6. 2. Under normal, non-induced, conditions only basal expression of *cprA* is needed. In this situation relatively low amounts of a short transcript (*cprA*α) are produced. Upon induction of expression of a cytochrome P450 encoding gene, e.g. induction of the BPH system by benzoic acid, a *trans*-acting regulatory factor is activated. Interaction of this activated factor (or multiple factors) with regulatory elements on the *cprA* TCR fragment results in induction of *cprA* gene expression at the level of transcription. An alternative, upstream, Pβ promoter is used in this induced situation besides the Pα promoter, resulting in two transcripts (*cprA*α and *cprA*β). To avoid too high CPR levels in the cell, which may cause cell damage by oxygen radical formation (Mukhopadyay and Chatterjee 1994, Ross et al. 1995), a second control mechanism is regulating translation from the longer *cprA*β transcript. It is hypothesized that this control mechanism acts via the upstream open reading frame (uORF) which is present on the *cprA*β transcripts, resulting in reduced translation efficiency of this transcript. Such uORFs present in 5'-leader sequences of mRNAs can, in some cases, have a major inhibiting effect on the translation of the gene involved (reviewed in Geballe and Morris 1994). The importance of uORFs has been shown clearly for the regulation of translation of the yeast transcription factor *GCN4* (Altmann and Trachsel 1993, Mueller and Hinnebusch 1986). Expression of at least two genes of *A. nidulans*, both involved in developmental control pathways, has been shown to be affected by uORFs. Expression of the *A. nidulans brlA* gene is regulated in a way which is comparable to the *A. niger cprA* system. As was observed for benzoate induced *cprA* gene expression, two different *brlA* transcripts can be formed, *brlA*α and *brlA*β (Prade and

Timberlake 1993). The *brlA*α transcript codes for a 455 amino acid protein, the expression of which is completely regulated at the level of transcription. In contrast to the situation found for *cprA*, differential splicing of the larger *brlA*β transcript results in a protein which differs from the *brlA*α encoded protein by an additional 23 N-terminal residues. The *brlA*β transcript is the most predominant in the vegetative state in which condition its translation is repressed by the presence of an upstream uORF (Han et al. 1993). Expression of *brlA*β is de-repressed by deletion of the uORF. A similar regulatory mechanism is described for the *A. nidulans stuA* gene which is involved in developmental regulation. Two different *stuA* transcripts were found. A unique uORF, present on the leader sequence of the *stuA*α transcript only, is involved in positive regulation of expression of *stuA* (Miller et al. 1992).

2.2 Why is such a complex regulatory mechanism needed ?

BPH expression is only needed after induction by specific environmental stimuli. In contrast to this, CPR expression is always needed at a basal level to support constitutively expressed cytochrome P450s like sterol 14α-demethylase, but its expression levels should also be adapted to the expression levels of a number of different inducible cytochrome P450 genes, responding to different environmental changes. A complex regulatory mechanism, resulting in a rapid and strong increase of transcription in response to the availability of the inducing compound, is required to meet these changes. However, uncontrolled overexpression of CPR activity can result in uncoupling of the system and thus in the production of harmful oxygen radicals. This risk makes the maintenance of a careful balance between CPR and P450 levels necessary. To achieve this balance a second regulatory mechanism, e.g. on the level of translation, moderates the effect of the transcription induction.

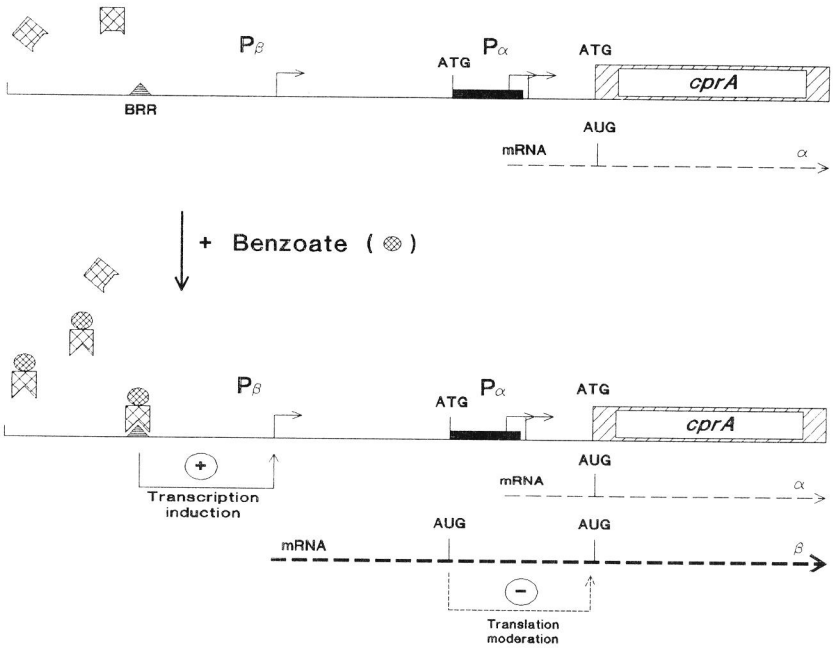


Figure 6.2, Model for the benzoate dependent regulation of expression of the *A. niger cprA* gene (details are explained in the text).
Indicated are the transcription control region of the *cprA* gene, the promoters used in the presence and absence of benzoate induction (Pα and Pβ respectively) and the uORF present between the two transcription starts (black bar).
Induction with benzoate (hatched circles) activates the Benzoate Regulatory Factor(s) (hatched block). After interaction of the activated protein(s) with sequence elements present on the BRR, transcription is induced and mRNA levels are highly increased, the majority of the mRNA formed belonging to the larger *cprAβ* species. Translation from this larger size mRNA is downregulated, probably via initiation control by the upstream AUG codon present on the 5'-leader sequence of the inducible *cprAβ* transcript.

3. Future prospects

3.1 Improvement of cytochrome P450 activities in filamentous fungi

In the studies presented in this thesis CPR overproducing strains were constructed with plasmids in which CPR expression was driven by a small part of the *cprA* TCR. The use of this fragment resulted in relatively low, constitutive expression levels. The use of a longer *cprA* TCR fragment would result in higher and inducible CPR activity levels. However, if higher

CPR expression levels result in imbalance of the ratio CPR / cytochrome P450s, the risk of uncoupling of the system increases. Therefore controlled overexpression of CPR, which might be achieved by the use of other TCRs, is necessary. For example, replacing the *cprA* TCR by the TCR of the *A. niger gpdA* gene (Punt et al. 1988) would result in relatively high, constitutive CPR expression while the use of *A. niger glaA* TCR would result in low constitutive expression which could be strongly induced by the addition of maltodextrine to the culture medium (Verdoes et al. 1993). By a careful choice of the expression signals used to drive CPR expression, optimal adjustment of CPR activity levels to the P450 levels may be achieved.

Simultaneous overexpression of cytochrome P450 and CPR encoding genes has been shown to be an effective strategy to achieve an increase of P450 activities in microorganisms (this work, Pompon et al. 1995, Sakaki et al. 1991, Picataggio et al. 1992, Sengstag et al. 1994). However, further improvements can be made. In *Bacillus megaterium* a cytochrome P450 molecule was found with an extremely high turnover rate (Narhi and Fulco 1986). This cytochrome $P450_{BM-3}$ was shown to be a fusion between an eukaryotic-like CPR domain and a cytochrome P450 domain, interspaced by a small linker sequence (Ruettinger et al. 1989). A comparable protein was found in the fungus *Fusarium oxysporum*, involved in fatty acid hydroxylation (Nakayama et al. 1996). In analogy to these two systems different studies in *S. cerevisiae* employed fusion proteins, composed of a P450 domain and a CPR domain, separated by a linker sequence, to achieve largely increased specific activities (Shibata et al. 1990, Sakaki et al. 1990). The closer, permanent contact between both domains resulted not only in increased conversion rates but might also be important for reduction of electron leakage, thus preventing cell-damage by reactive oxygen species. A disadvantage of the fusion protein strategy in some cases was the instability of the protein, resulting in relatively low protein levels per cell (Shibata et al. 1990).

Since CPR is not the only electron donor in some P450 enzyme systems, further improvement of activities might be achieved by co-expression of cytochrome b_5. However, the effects of cytochrome b_5 overproduction seem to depend on the P450 system studied and sometimes cytochrome b_5 overproduction even resulted in decreased P450 activities.

3.2 Expression of heterologous cytochrome P450

Many mammalian cytochrome P450 enzymes perform chiral specific bioconversion reactions that could be of great potential use for productions of pharmaceutically active

compounds or other complex biochemicals. To obtain highly active conversion systems the use of microorganisms overexpressing the specific mammalian cytochrome P450 encoding genes seems to be the best choice. Although *S. cerevisiae* has been the organism of choice until this moment, filamentous fungi might be attractive hosts as well for expression of these heterologous cytochrome P450 genes since they posses a functional and compatible electron donating system and extensive internal membranes. The compatibility of a fungal CPR has been shown *in vitro* by Scala et al. (1988). CPR enzymes isolated from a number of different fungi were able to activate a purified P450 enzyme from the fungus *N. haematococca* (PDA) in reconstitution experiments, though activation was becoming less efficient (or even not detectable) when CPR enzymes from more distantly related organisms were used (Scala et al. 1988). In *S. cerevisiae*, it was shown that endogenous CPR was able to activate P450 enzymes from many different mammalian species (Reviewed in Yabusaki 1995). However, to obtain optimal P450 activity some authors prefer coexpression of a mammalian CPR encoding gene (Pompon et al. 1995, Eugster et al. 1992). The potential use of *A. niger* strains for overexpression of cytochrome P450 genes, together with the endogenous *cprA* gene or with a mammalian CPR encoding gene, will have to be evaluated.

3.3 Regulation of cprA gene expression

For a complete understanding of the regulatory mechanism underlying benzoate induction of both the *cprA* and *bphA* genes, isolation of the transcription factor(s) involved is of great importance. To be able to isolate such factors clear determination of the exact sequence elements involved in benzoate induction is necessary, implicating the need to perform extensive electrophoretic mobility shift analyses and *in vitro* DNA footprinting experiments for evaluation of the entire BRR and of sequences flanking the benzoate dependent Pβ promoter. The cloning and genomic inactivation, of genes encoding the transcription factors involved would allow detailed study of coregulation of cytochrome P450 enzyme systems.

A complete understanding of the regulatory mechanisms involved in *cprA* gene expression can only be achieved if coregulation of *cprA* gene expression with the expression of a number of other cytochrome P450 genes is studied. However, at present no other inducible cytochrome P450 genes are known in *A. niger*. Studying the coregulation of *cprA* gene expression with inducible cytochrome P450 genes in closely related organisms (e.g. the sterigmatocystin biosynthesis gene cluster in *A. nidulans*; Brown et al. 1996) might offer

interesting alternatives.

It is known that the availability of transcription factors can be the limiting factor for overexpression of inducible genes in filamentous fungi. Introduction in *A. niger* of more than 20 copies of the *A. niger* glucoamylase encoding gene did not result in any further increase of glucoamylase activity, probably due to titration of a transcription factor (Verdoes et al. 1993). Since it seems that benzoate inducible gene expression of both *cprA* and *bphA* is regulated by a common transcription factor, titration of this factor might cause problems in multicopy strains. Therefore isolation of the genes encoding transcriptional regulators of *cprA* gene expression is not only important for understanding of the mechanism involved but might also prove to be useful for improvement of CPR and P450 activities in *A. niger*.

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Summary

Many different members of the cytochrome P450 superfamily have been identified from organisms throughout nature. Cytochrome P450 enzymes are involved in very different, often complex, biotransformation reactions. Because of their capability to catalyse the conversion of complex substrate molecules, cytochrome P450s are of great interest for biotechnological purposes. To become catalytically active, cytochrome P450 enzymes need a supply of electrons which is donated by a second enzyme system. In the case of endoplasmic cytochrome P450s this enzyme system comprises only one major component, cytochrome P450 reductase (CPR).

The research described in this thesis focussed on cytochrome P450 enzyme systems in the filamentous fungus *Aspergillus niger*, using molecular genetical techniques.The availability of two fungal cytochrome P450 encoding genes and the cloning of the CPR encoding gene *cprA*, as reported in Chapter 2 of this thesis, supplied us with the major tools for the study of cytochrome P450 enzyme systems in *A. niger*. The *cprA* gene was isolated from the *A. niger* genome by a PCR mediated approach and further characterization showed that it contains regions of considerable homology with CPR encoding genes from other organisms. Careful comparison of these conserved regions enabled a detailed characterization of putative functional domains involved in binding of NADPH and of the cofactors FAD and FMN.

Overexpression of either the *A. niger cprA* gene or the $P450_{14DM}$ encoding gene (*cyp51*) from *Penicillium italicum* in *A. niger* resulted in a considerable increase in resistance to a number of sterol 14α-demethylase inhibitors (DMIs) in the transformants (chapter 3) due to increased $P450_{14DM}$ activity. This increase in resistance was much more pronounced in strains in which both genes were overexpressed, indicating the importance of having sufficient amounts of both components of the cytochrome P450 enzyme system present at the ER membrane.

In a similar experiment, described in chapter 4 of this thesis, *A. niger* transformants were generated in which the gene copy numbers of either the *cprA* gene or the *A. niger bphA* gene, encoding the cytochrome P450 enzyme benzoate para-hydroxylase (BPH), or of both genes were increased. Using a novel enzyme activity assay, based on the benzoate stimulated consumption of NADPH by CPR, it was shown that maximal BPH activity could only be achieved by co-expression of both genes, supporting the conclusions from the $P450_{14DM}$ study in chapter 3.

A number of reports have described effects of cytochrome P450 inducers on the expression of *cpr* genes, using a variety of experimental systems. Surprisingly, no detailed study of the mechanisms resulting in this co-regulation was performed yet. As described in chapter 2 of this thesis, mRNA expression levels of the *A. niger cprA* gene could be highly induced by addition of benzoate to the culture medium. Benzoate is also the major inducer of the only *A. niger* cytochrome P450 gene identified to date, the *bphA* gene. The presence of a cytochrome P450 enzyme system, of which expression of both partners is induced by the same, simple, inducer, in a model organism which is easily accessible for molecular genetical studies provided a good opportunity to study these mechanisms in more detail. As is described in chapter 5 of this thesis, a deletion study of the transcription control region of both genes was performed to identify DNA sequences involved in the benzoate mediated induction of gene expression of both genes (Benzoate Regulatory Region, BRR). Comparison of the DNA sequences of both identified BRRs resulted in the identification of a completely conserved 9 bp DNA fragment. In electrophoretic mobility shift assays it could be shown that oligonucleotides, based on the conserved element and flanking sequences from both genes, were recognized by nuclear proteins isolated from benzoate induced mycelium while no (or much reduced) binding was observed using proteins isolated from non-induced mycelium. Since binding of these oligonucleotides could be competed for by excess of either non-labelled oligonucleotide it seems likely that regulation of gene expression of the *cprA* and of the *bphA* gene is mediated via a common regulatory protein.

A remarkable observation was made in chapter 2. Induction of *cprA* mRNA levels by benzoic acid was highly efficient, resulting in an increase of transcript levels of more than 50 fold. However, this large increase in mRNA levels resulted only in a relatively small increase, of up to 4 fold, of CPR activity. In chapter 5 this discrepancy was studied in more detail. This analysis revealed that after benzoate induction the majority of the mRNA formed was considerably larger compared to the mRNA formed in control situations, due to the benzoate induced use of an independent upstream promoter. The expression level of the smaller mRNA was not affected by benzoate induction. Sequence analysis revealed the presence of an upstream open reading frame (uORF) on the larger sized mRNA which might be involved in the post-transcriptional regulation of CPR expression levels in the presence of benzoate.

A model describing the mechanisms resulting in benzoate mediated induction of *cprA* gene expression in *A. niger* is described in chapter 6. According to this model benzoate induction results in activation of a regulatory factor which, following interaction with the BRR,

stimulates transcription of the *cprA* gene from the upstream promoter. The resulting, longer, transcript comprises an uORF. Translation of this uORF provides a negatively acting regulatory mechanism which is important for control of intracellular CPR levels. This model would provide the cell with a very sensitive control mechanism which would prevent overproduction of CPR while meanwhile the CPR level can be adapted to intracellular cytochrome P450 levels.

Samenvatting

Een groot aantal verschillende leden van de cytochroom P450 superfamilie zijn momenteel bekend, afkomstig uit een breed scala aan organismen. Cytochroom P450 enzymen zijn betrokken bij zeer verschillende, vaak complexe omzettingen. Vanwege hun vermogen om complexe verbindingen als substraat te gebruiken vormen P450s een interessante groep enzymen voor de moderne biotechnologie.

Voor het uitvoeren van hun monooxygenase reactie hebben P450s elektronen nodig. Vrijwel alle P450s hebben een tweede enzym systeem nodig dat deze elektronen doneert. In het endoplasmatisch reticulum, waar de meeste P450s worden aangetroffen, is één eiwit, het cytochroom P450 reductase (CPR), met name verantwoordelijk voor deze activiteit.

Het onderzoek zoals beschreven in dit proefschrift heeft zich voornamelijk gericht op cytochroom P450 enzym systemen in het ER van de biotechnologisch belangrijke filamenteuze schimmel *Aspergillus niger*. Voor dit onderzoek werd gebruik gemaakt van moleculair genetische technieken.

Een gedetailleerde studie van cytochroom P450 enzym systemen in filamenteuze schimmels was mogelijk door de beschikbaarheid van de genen coderend voor twee verschillende P450 enzymen uit schimmels. Om het volledige systeem te kunnen bestuderen werd het gen coderend voor de tweede component van het P450 systeem, het *cprA* gen, geïsoleerd uit de schimmel *Aspergillus niger* (hoofdstuk 2). Voor isolatie van het *cprA* gen uit het *A. niger* genoom werd gebruik gemaakt van een PCR strategie. Door nadere bestudering van de voorspelde eiwitstructuur van het *A. niger* CPR en vergelijking van deze eiwitstructuur met de structuur van CPR eiwitten uit andere organismen konden functionele domeinen in het CPR die betrokken zijn bij binding van NADPH en van de cofactoren FAD en FMN nauwkeurig bepaald worden.

Transformanten waarin het *A. niger cprA* gen óf het gen coderend voor $P450_{14DM}$ (*cyp51*) van *Penicillium italicum* tot overexpressie waren gebracht waren resistent tegen significant hogere doses van een aantal verschillende DMIs (sterol-14α-demethylase inhibitors, een belangrijke klasse van fungiciden). Deze toegenomen resistentie werd veroorzaakt door een toename van de $P450_{14DM}$ activiteit in transformanten (hoofdstuk 3). De toename in resistentie was aanzienlijk groter in transformanten waarin beide genen gezamenlijk tot overexpressie werden gebracht. Uit deze resultaten blijkt het belang van een juiste verhouding van beide componenten van het cytochroom P450 enzym systeem in de ER membraan.

In een vergelijkbaar experiment, beschreven in hoofdstuk 4 van dit proefschrift, werden *A. niger* transformanten geconstrueerd waarin het aantal *cprA* gen-kopieën óf het aantal kopieën van het *bphA* gen, coderend voor het cytochroom P450 enzym benzoaat para-hydroxylase, óf het kopie aantal van beide genen was verhoogd. Gebruikmakend van een nieuw ontwikkelde activiteits bepaling, gebaseerd op de benzoaat afhankelijke consumptie van NADPH door CPR, werd aangetoond dat maximale BPH activiteit alleen werd behaald door co-expressie van beide genen. Deze resultaten vormden een sterke onderbouwing van de conclusies die waren verkregen in de $P450_{14DM}$ studie in hoofdstuk 3.

In een aantal publikaties worden effecten beschreven van cytochroom P450 inducers op de expressie niveaus van *cpr* genen. Verrassenderwijs werd er echter tot nog toe geen gedetailleerde studie verricht naar de mechanismen die ten grondslag liggen aan deze co-regulatie. Zoals beschreven in Hoofdstuk 2, worden mRNA expressie niveaus van het *A. niger* *cprA* gen sterk geïnduceerd door toevoeging van benzoaat aan het kweek medium. Benzoaat is ook de inducer van het enige *A. niger* cytochroom P450 gen dat tot nog toe geïdentificeerd is, het *bphA* gen. De beschikbaarheid van een cytochroom P450 systeem, waarvan de expressie niveaus van beide componenten worden geïnduceerd door hetzelfde, eenvoudige, substraat, in een modelorganisme waarvoor goede moleculair genetische technieken beschikbaar zijn, betekende een goede mogelijkheid om coregulatie van expressie van P450 enzym systemen nader te bestuderen.

In Hoofdstuk 5 van dit proefschrift wordt een deletie studie van de transcriptie controle regio van beide genen beschreven met als doel het identificeren van DNA sequenties die betrokken zijn bij de benzoaat gemedieerde inductie van gen expressie van beide genen (Benzoate Regulatory Region; BRR). Vergelijking van de DNA sequenties van de BRRs van beide genen resulteerde in de identificatie van een volledig geconserveerd 9 bp DNA fragment. Gebaseerd op deze geconserveerde elementen en op de flankerende sequenties in beide genen, werden gen specifieke oligonucleotiden ontworpen. Met EMSA (Electrophoretic Mobility Shift Assay) experimenten kon worden aangetoond dat beide oligonucleotiden worden herkend door nucleaire eiwitten geïsoleerd uit benzoaat geïnduceerd mycelium maar niet (of veel minder goed) door eiwitten uit niet geïnduceerd mycelium. De waarneming dat toevoeging van overmaat ongelabelde oligonucleotiden resulteerde in een sterke afname van binding van beide oligonucleotiden, maakt aannemelijk dat regulatie van expressie van het *cprA* en het *bphA* plaatsvindt via een gemeenschappelijk regulerend eiwit.

Een opmerkelijke waarneming wordt beschreven in hoofdstuk 2. Inductie van *cprA*

mRNA niveaus door benzoaat is zeer sterk, resulterend in een meer dan 50-voudige toename van de hoeveelheid transcript. Echter, deze sterke toename op mRNA niveau resulteerde in een slechts beperkte toename van de CPR activiteit (ongeveer 4 maal). In hoofdstuk 5 wordt deze discrepantie nader bestudeerd. Uit deze analyse bleek dat na benzoaat inductie de meerderheid van het gevormde mRNA groter was dan het mRNA dat wordt gevormd in controle medium. Benzoaat had geen invloed op de expressie van het kleinere mRNA. De benzoaat afhankelijke toename van mRNA grootte bleek het gevolg te zijn van het gebruik van een onafhankelijke promoter. Sequentie analyse resulteerde in de identificatie van een upstream open reading frame (uORF) gelegen op de niet vertaalde 5' sequentie van het langere mRNA. Vermoedelijk is deze uORF betrokken bij post-transcriptionele regulatie van CPR expressie in de aanwezigheid van benzoaat.

De mechanismen die betrokken zijn bij de benzoaat gemedieerde inductie van *cprA* gen expressie worden beschreven in een model dat wordt gepresenteerd in hoofdstuk 6. Volgens dit model reageert de cel op de aanwezigheid van benzoaat door de vorming van een regulerende factor die een interactie aangaat met de BRR. Deze interactie resulteert in een stimulering van het gebruik van de tweede promoter waardoor een grote hoeveelheid van het grotere transcript gevormd wordt. Om te hoge intracellulaire CPR activiteiten, die kunnen leiden tot schade aan de cel, te voorkomen bezit de cel een tweede, negatief werkend, regulatie mechanisme. In het beschreven model speelt de uORF die werd gevonden op het benzoaat induceerbare grotere transcript een belangrijke rol. Een dergelijk complex regulatie mechanisme zou de cel in staat stellen zeer snel en effectief op een verhoging van de intracellulaire cytochroom P450 niveaus te reageren terwijl cel schade door overmatige CPR activiteit grotendeels vorkomen kan worden.

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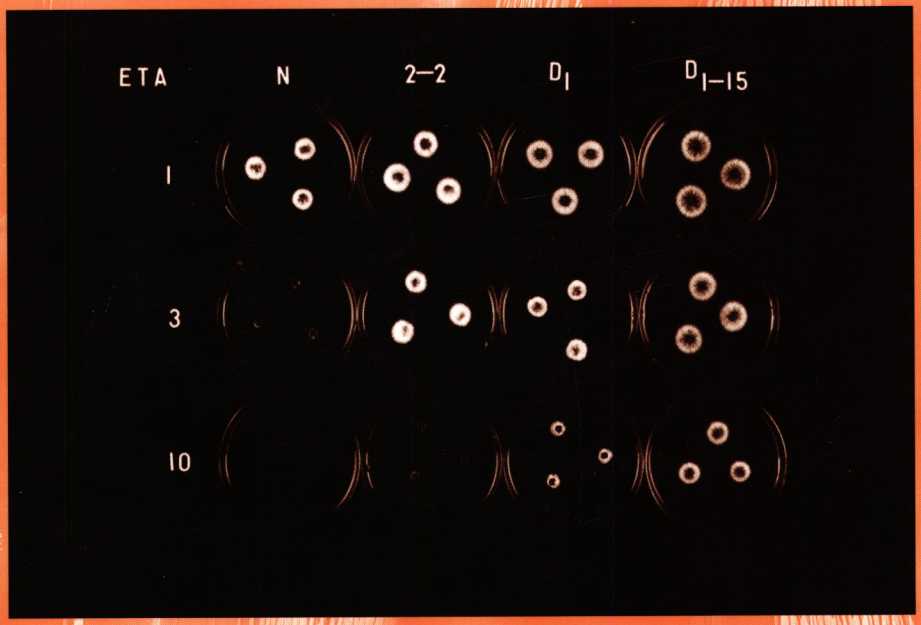
CURRICULUM VITAE

Johannes Maarten (Hans) van den Brink werd op 12 maart 1967 geboren in Barneveld. In mei 1985 behaalde hij het VWO diploma aan het Christelijk Lyceum te Apeldoorn en in september van dat jaar begon hij aan de experimentele studierichting Medische Biologie te Utrecht. Na een bijvak bij de vakgroep Biochemie (faculteit scheikunde) deed hij een 9 maanden onderzoeksstage bij de vakgroep Fysiologische Chemie van de faculteit geneeskunde aan deze universiteit. Op 31 augustus 1990 werd het doctoraal diploma behaald.

Van 15 augustus 1990 tot en met 31 december 1991 werkte hij als erkend gewetensbezwaarde militaire dienst in Rijswijk bij de Aspergillus genetica groep van de afdeling RecDNA van het Medisch Biologisch Laboratorium TNO (de tegenwoordige afdeling Moleculaire Genetica en Gen Technologie van TNO-Voeding) aan een aantal contract research projecten. Vanaf 1 februari 1992 tot 1 mei 1996 werkte hij op deze afdeling aan een promotie onderzoek onder begeleiding van prof. dr. C.A.M.J.J. van den Hondel en dr.ir. R.F.M. van Gorcom. De resultaten van dit onderzoek zijn weergegeven in dit proefschrift. Momenteel is hij werkzaam als post doctoraal onderzoeker bij de Food Specialities Division van Gist-brocades N.V. in Delft.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