First functional expression of cytochrome P450 3A4 in Pichia pastoris

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INTRODUCTION

Cytochrome P450 monoxygenases (CYPs) represent the major enzymatic system involved in the phase 1 metabolism of drugs and xenobiotics in humans [1]. This superfamily of heme-containing enzymes catalyzes the insertion of one atom of molecular oxygen to a non-activated carbon bond in a regio- and stereoselective way. Such metabolites produced by CYPs are often hard to obtain by classical chemical synthesis [2]. However, nowadays there is an increasing need to get access to the respective drug metabolites. They are required as reference compounds for metabolite identification, structure elucidation and in safety tests to investigate their toxicity and biological activity [3]. The fast and reliable production of these compounds on an analytical, but also on a preparative scale is therefore of high importance. Employing the drug metabolizing enzymes themselves as biocatalysts either in isolated form or in whole-cell biotransformations has emerged as an attractive and biological way to reach this goal. A prerequisite for this strategy is the availability of recombinant host systems that efficiently produce the target enzyme in its catalytically active form. Eukaryotic CYPs represent challenging targets for recombinant protein production because of their membrane-bound nature, the requirement for a correctly incorporated heme-cofactor and the need for the presence of their electron transfer partners. If employed as whole-cell biocatalysts, the host systems should also exhibit features advantageous for process relevant parameters such as scalability and down-stream processing.

During the recent years the methylotrophic yeast Pichia pastoris has emerged as an attractive and biological way to reach this goal. A prerequisite for this strategy is the availability of recombinant host systems that efficiently produce the target enzyme in its catalytically active form. Eukaryotic CYPs represent challenging targets for recombinant protein production because of their membrane-bound nature, the requirement for a correctly incorporated heme-cofactor and the need for the presence of their electron transfer partners. If employed as whole-cell biocatalysts, the host systems should also exhibit features advantageous for process relevant parameters such as scalability and down-stream processing.

ABSTRACT

Getting access to drug metabolites has become an important issue within the drug discovery and development process. Besides chemical synthesis and chromatographic separation, the selective production of these metabolites employing whole-cell biotransformations with recombinant microbes has become an emerging strategy. Here, we report for the first time the functional expression of the human cytochrome P450 3A4 (CYP3A4), a key enzyme in drug metabolism, in the methylotrophic yeast Pichia pastoris. Co-expression of the human cytochrome b5 and/or the cytochrome P450 reductase (CPR) yielded in a functional whole-cell monoxygenase catalyst.

RESULTS AND DISCUSSION

Functional expression of CYP2D6, another CYP isoform of pharmacological interest, in P. pastoris was based on the co-expression of the cytochrome P450 reductase (CPR), which is required for the electron transfer between the cofactor NADPH and the P450 enzyme (6). Thus, we have chosen the same strategy for the recombinant production of CYP3A4. To link the integration of both expression cassettes into the P. pastoris genome, a co-expression construct harboring the genes for human CYP3A4 and CPR under the separate control of the strong AOX1 promoter was generated to transform P. pastoris so far [6, 7]. In the present study we report for the first time on the functional expression of CYP3A4 in this yeast. CYP3A4 is the most abundantly expressed P450 enzyme in the human liver and responsible for the metabolism of about half of all drugs currently in use [8]. Thus, CYP3A4 represents a valuable biocatalyst for drug metabolite production and therefore the availability as a robust whole-cell catalyst is desirable.
empty *P. pastoris* and *P. mutS* Cytl.b5). A second light band was found between 35-40 kDa (indicated with an arrow), but this band also appeared in the positive control, a commercially available membrane preparation of Saccharomyces cerevisiae expressing CYP3A4 and CPR. Additionally, Pichia seems to possess endogenous protein(s) that cross-react with the CYP3A4 antibody (bands at ~130 kDa). The observed expression levels are comparable to the CYP3A4 amount found for the commercial membrane preparation, which contains a P450 concentration of 1 nmol/mL according to the manufacturer’s product information.

Although showing suitable CYP3A4 levels in the immunoblot, the expression obtained in *P. pastoris* was too low to be quantified by CO difference spectroscopy. The characteristic peak at 450 nm could not be detected in the spectra recorded from membrane preparations nor from whole cells of recombinant Pichia strains (data not shown). CYP3A4 expression levels typically achieved e.g. in E. coli are ranging from 84 – 890 nmol/L culture (9, 10). Also in yeast systems such as *S. cerevisiae* or *Yarrowia lipolytica* quantifiable amounts of CYP3A4 were produced (11-13). Interestingly, the negative control *P. pastoris* strain showed CYP3A4/CPR and cytochrome b5, respectively. The functionality of the CYP3A4 based monooxygenase system in *P. pastoris* was assessed in whole-cell conversions of typical CYP3A4 substrates such as testosterone and 7-benzyloxy-4-trifluoromethylcoumarin (7-BFC). Whole-cell conversions were conducted by simply incubating the cells in the presence of the substrate in 100 mM KPi buffer, pH 7.4. Since the cell metabolism can be exploited for cofactor regeneration, no costly NADPH had to be added to the reaction mixture. No sugar was added to the reaction mixture for short screening reactions in deep-well plates.

CYP3A4-mediated testosterone hydroxylation was investigated by analyzing the supernatants from the biotransformation by HPLC-MS. As shown in the HPLC-MS chromatogram (m/z 305) one major peak can be observed which corresponds to 6β-hydroxytestosterone, the main metabolite formed by CYP3A4 (Figure 2, panel A). Product formation of up to 9.72 nmol/mgCDW was observed for the strain *P. mutS* CYP3A4/CPR, E7 (Table 1). Besides 6β-hydroxytestosterone 3 further, but minor peaks were observed in the HPLC-MS chromatogram. These peaks might be assigned to the corresponding metabolites resulting from testosterone hydroxylation by CYP3A4 at the 1β-, 2β- and 13β-position as described by Krauser et al. (14).

![Figure 1. Immunoblot analysis for CYP3A4 in membrane fractions of recombinant *P. pastoris* strains. 20 µg of total protein were loaded per lane. (1) *P. pastoris* mutS, (2) *P. pastoris* CYP3A4/CPR, B5, (3) *P. pastoris* CYP3A4/CPR, E7, (4) *P. pastoris* Cytl.b5, (5) *P. pastoris* Cytl.b5 CYP3A4/CPR, E3, (6) *P. pastoris* Cytl.b5 CYP3A4/CPR, D4, and (7) commercial membrane preparation of *S. cerevisiae* CYP3A4/CPR (positive control). PageRuler Prestained Protein Ladder (5 µl) was used for protein size determination (Std.). CYP3A4 has an expected molecular weight of 57.3 kDa.](image1)

![Figure 2. Activities observed in whole-cell conversions of typical CYP3A4 substrates by *P. pastoris* CYP3A4/CPR, E7. (A) Testosterone hydroxylation was performed by incubating whole cells with 10 mM testosterone in 100 mM KPi buffer, pH7.4, for 6 h at 28°C and vigorous shaking. The formation of 6β-hydroxytestosterone (indicated by an arrow) was analyzed by HPLC-MS (m/z 305). (B) Dealkylation of 7-benzyloxy-4-trifluoromethylcoumarin (7-BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC) by *P. pastoris* CYP3A4/CPR, E7 (black squares) was followed by measuring the increase in product fluorescence (λ_{ex} = 410 nm, λ_{em} = 538 nm) for 1 h. The observed activity of the negative control *P. pastoris* mutS is also shown (grey squares).](image2)
enzyme might be a reason for this observation. As CYP3A4 and cytochrome b5 both require heme, there might be a competition for this cofactor, which affects the expression adversely. However, the expression levels of this human P450 enzyme are most probably too low to compete effectively with other heme binding proteins in the cell. At least we can state that cytochrome b5 overexpression is not mandatory to obtain a functional monoxygenase system in P. pastoris and the electron transfer from the cofactor to the P450 enzyme is enabled by the host.

### MATERIALS AND METHODS

#### General

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) with the highest purity available. Zeocin™ was obtained from InvivoGen (San Diego, CA, USA) and 7-benzyloxy-4-trifluoromethycoumarin (7-BFC) from BD Bioscience (Becton, Dickinson and Company, Sparks, USA). Phusion® High Fidelity Polymerase for DNA amplification and further DNA modifying enzymes were purchased from Thermo Fisher Scientific Inc., (Waltham, MA, USA). E. coli Top10 Invitrogen, (Carlsbad, USA) was used for all cloning steps and plasmid propagation. The P. pastoris strain CBS7435 was as well as the plasmids pPpKan_opt and pPp_B1 were obtained from the Pichia pool of TU Graz (17). cDNA clones of human CYP3A4 (Clone ID: 7262313) and human CPR (Clone ID: 3882411) were purchased from BioCat GmbH (Heidelberg, Germany), the cDNA clone of human cytochrome b5 (Clone ID: IRATp970E0218D) from ImaGenes (Berlin, Germany). Microsomal preparations from S. cerevisiae co-expressing human CYP3A4 and CPR were obtained from Berlin Pharma (Montigny le Bretonneux, France).

Lipid Pichia cultures were grown in buffered minimal dextrose medium (BMD) containing 200 mM KP, (pH 6.0), 13.4 g/L yeast nitrogen base and 0.4 mg/L biotin supplemented with 2 % (w/v) glucose. Buffered minimal methanol medium (BMM) has the same composition as BMD, but contains 1 % methanol (BMM2) or 5 % methanol (BMM10) instead of glucose.

#### Plasmid and strain construction

A co-expression plasmid for CYP3A4 and CPR based on the pPp_B1 plasmid was constructed essentially as described earlier (6). This construct was transformed as linear expression cassette into P. pastoris CBS7435 using the condensed protocol by Lin-Cereghino et al. (18). Transformants were selected on YPD agar plates containing 100 mg/L Zeocin™.

In addition, a strain co-expressing CYP3A4, CPR and cytochrome b5 has been generated. Therefore, the gene of the human cytochrome b5 was cloned via EcoRI/NcoI into the multiple cloning site of pPp_Kan_opt. The resulting plasmid was linearized with BglII prior to transformation into P. pastoris and selection of positive clones on YPD plates with 300 mg/L Geneticin®. Colony PCR was performed to identify a clone carrying the cytochrome b5 expression cassette. Subsequently, this strain was transformed with the co-expression plasmid for CYP3A4 and CPR. Transformants were selected for growth on Zeocin™ and Geneticin®.

### CYP3A4 mediated whole-cell conversions of testosterone

Small scale cultivation of positive transformants in 96 well deep-well plates was conducted as described by Weis et al. (19). Shortly, transformants were grown in 250 µL of BMD for approximately 60 h at 28°C, 320 rpm and 80% humidity in an Infors shaker. Protein expression was started by the addition of 250 µL BMM2 per well and kept induced by the further addition of 50 µL BMM10 after 10 h. 24 h and 48 h. At 24 h after the last induction, cells were harvested by centrifugation (5 min, 500xg) and washed once with 200 µL of assay buffer (100 mM KPi, pH 7.4). The centrifugation step was repeated and the cells were resuspended in 200 µL of assay buffer, 10 µL of the thus obtained cell suspension was used for OD600 determination for later corrections. To the cell suspension, 10 µL of a 200 mM testosterone stock solution in 2-propanol were added (precipitation observed). The whole-cell conversions were carried out for 8 h at 28°C and 320 rpm. The reactions were stopped by the addition of 10 µL of 1 mM bordenone as internal standard and by spinning out the cells (10 min, 3200xg). The supernatants were transferred in fresh 96 well microtiter plates and stored at -20°C until analysis by HPLC-MS. Analyses were performed on a HPLC device (1200 series, Agilent technologies, Santa Clara, CA, USA) with an ESI unit equipped with an MSD SL detector with an electron spray ionization (ESI) unit. For high-throughput screening purposes (analysis time 1.5 min), the whole-cell reaction mixtures were separated on an XDB-C18, 1.8 µm, 4.6 x 50 mm column (Agilent technologies) at 60°C. The mobile phase was composed of water and acetonitrile (ACN), both acidified with 0.1% formic acid. Gradient-elution was performed at 1.5 ml/min as follows: 0-0.5 min: 40% ACN, 0.5-0.75 min: 40-85% ACN, 1.2-1.5 min: 40% ACN. To allow a flow rate of 1.5 ml/min, a splitter (2:1 ratio) was implemented in front of the ESI unit. 6β-hydroxytestosterone (m/z 305) eluted after 0.61 min, testosterone (m/z 289) after 1.3 min and boldenone (m/z 287) after 1.2 min.

For metabolite quantification, whole-cell conversions were separated on a Chromolith RP 18-e, 100-4.6 mm column (Merck, Darmstadt, Germany) using a gradient based on water and ACN (0-3 min: 25% ACN, 3-7 min: 25-75% ACN, 7-9 min: 25% ACN). Retention times were 1.2 min for 6β-hydroxytestosterone (m/z 305), 7.0 min for boldenone (m/z 287) and 7.5 min for testosterone (m/z 289).

CYP3A4 mediated whole-cell conversions of 7-benzzyloxy-4-trifluoromethycoumarin (7-BFC)

Cultivation of recombinant Pichia strains in 96 well deep-well plates and induction of protein expression was performed as described above. Cell were harvested by centrifugation (5 min, 500xg), washed once with assay buffer (100 mM KPi, pH7.4) and resuspended in a volume of 300 µL buffer. 100 µL of the cell suspension were transferred to a black microtiter plate and the reaction was started by the addition of 7-BFC to a final concentration of 50 µM. The fluorescence signal of the product formed (λex = 410 nm, λem = 538 nm) was recorded every minute for a period of 1 h using a SynergyMX
Protein expression in shake flasks
Protein expression in P. pastoris was performed essentially as described in (19). Briefly, 200 mL of BMD1% medium in a baffled 2 L flask were inoculated with a single colony and shaken at 28°C and 120 rpm for 60 h. Induction of expression was maintained by daily addition of methanol to 0.5% for 72 h.

Preparation of membrane fractions
Cell disruption of P. pastoris was performed essentially as described in (20). Cells were harvested by 10 min centrifugation at 3000g and 4°C. The pellet was washed once with water before being resuspended in 15-20 mL homogenization buffer (50 mM KPi, pH 7.9) containing 5% glycerol, 1 mM EDTA, 2 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Cell suspensions were mixed with an equal amount of acid-washed glass beads of 0.5 mm diameter and broken in a mechanical homogenizer (B. Braun Biotech International GmbH, Melsungen, Germany). The crude cell lysates were then separated from cell debris by 10 min centrifugation at 10,000xg and 4°C. To recover the membrane the cleared lysates were ultra-centrifuged at 180,000xg and 4°C for 1 h. Total membranes were resuspended in homogenization buffer and stored at -80°C. Total protein concentrations of membrane preparations were determined by CO-difference spectroscopy as described by Omura and Sato (21). Two mL aliquots of isolated membranes in 100 mM KPi, pH 7.4, containing 20% glycerol were supplemented with 100 µL of 200 mM KCN, pH 7.7, to mask the negative peak of cytochrome oxidases at 445 nm (22). A few milligrams of the reductant sodium dithionite were added and the reaction mixture was split into two cuvettes.

Quantification of cytochrome P450
Cytochrome P450 content in membrane preparations was determined by CO-difference spectroscopy as described by Omura and Sato (21). Two mL aliquots of isolated membranes in 100 mM KPi, pH 7.4, containing 20% glycerol were supplemented with 100 µL of 200 mM KCN, pH 7.7, to mask the negative peak of cytochrome oxidases at 445 nm (22). A few milligrams of the reductant sodium dithionite were added and the reaction mixture was split into two cuvettes. A difference spectrum was measured between 400 to 500 nm using a dual-beam spectrophotometer (Specord 205 UV/Visible spectrophotometer, Analytik Jena). The sample cuvette was aerated with carbon monoxide for 1 min and reduced once more with some sodium dithionite. Upon 1 min incubation time, the difference spectrum was recorded again. For CYP quantification in whole cells of P. pastoris, a technical set-up as described by Gudiminch et al. has been used (23). CYP content was calculated based on a molar extinction coefficient of $\epsilon_{409\text{nm}} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$.

CONCLUSIONS
In the present study we describe for the first time the functional expression of the important drug metabolizing enzyme CYP3A4 in the yeast P. pastoris. By co-expressing its electron transfer partners cytochrome b5 and/or CPR an active monooxygenase system was obtained showing activity in the biotransformation of typical CYP3A4 substrates. The achieved CYP3A4 expression levels so far were not quantifiable but definitely lower than those obtained by e.g. E. coli and S. cerevisiae, two host systems well established for CYP expression. However, our work builds the basis for further optimizing the expression of this enzyme in P. pastoris in order to exploit this host in whole-cell biotransformations also for the important human CYP3A4. The ease to produce the biocatalyst in large amounts would be beneficial for preparative applications. One advantage of recombinant P. pastoris whole-cell catalysts might be stability since recently in a comparative study on CYP2D6 expression hosts we have shown that Pichia showed prolonged stability in whole-cell conversion compared to E. coli and S. cerevisiae (24). Strategies to obtain P. pastoris strains with high CYP3A4 activity include fine-tuning copy numbers of expression cassettes and the ratios of expressed CYP3A4:CPR:Cyt.b5, but also protein engineering to develop interesting mutant biocatalysts for the future.

REFERENCES