Expression of active human P450 3A4 on the cell surface of Escherichia coli by Autodisplay

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The cytochrome P450 enzyme system comprises a large group of enzymes catalyzing a broad diversity of reactions and an extensive substrate specificity, which makes them the most versatile known catalysts. CYP3A4 is one of the important human P450 enzymes and involved in the oxidation of a large range of substrates including toxins and pharmaceuticals. Bottlenecks in studies of this enzyme include the difficulty in expressing it in a bacterial host, its need for membrane surroundings and the limited substrate accessibility of enzymes expressed within the cell. To circumvent these difficulties, human CYP3A4 was expressed on the outer membrane of Escherichia coli using Autodisplay. Transport of CYP3A4 to the cell surface was monitored by SDS-PAGE and Western blot analysis of outer membrane proteins. Localization on the cell envelope was determined by flow cytometry after immunolabeling, a whole cell ELISA and a protease accessibility assay. A HPLC assay confirmed the catalytic activity of displayed CYP3A4, using testosterone as a substrate. This activity required the external addition of electron supplying enzymes, however surprisingly, we found that the external addition of a hemeprotein was not necessary. Our results indicate that human CYP3A4 can be recombinantly expressed by surface display in a gram-negative bacterium.

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1. Introduction

Cytochrome P450 enzymes play a major role in both, drug discovery research and drug development (Spatzenegger and Jaeger, 1995). In the human body they are the most important enzymes in the phase-1-metabolism. They can catalyze the transformation of a drug from a lipophilic into a more hydrophilic form by hydroxylation, N-, O- and S-dealkylation, sulfoxidation, epoxidation, deamination, dehalogenation, peroxidation, and N-oxide reduction (Bernhardt, 2006; Rushmore and Kong, 2002; Sono et al., 1996). They are of growing importance for the synthesis of drug metabolites and have a well established role in toxicity and metabolic pathways (Nagy et al., 2011). Despite the great interest in P450 enzymes and their important roles in the pharmaceutical and fine chemical industry, their use for wide biochemical studies is still hampered due to several technical problems. The vast majority of these enzymes shows a high lack of stability and needs a membrane environment to become active (Nagy et al., 2011), making a purification process very time consuming and challenging.

Secondly, human P450 enzymes tend to show low activity and require several modifications before they can be expressed in significant amounts in bacteria (Gillam, 2008). When expressed in a host such as Escherichia coli, whole cell assays can be conducted as a rapid and efficient method to investigate enzyme activity, however the intracellular location of the enzyme limits the set of substrates to those which are able to cross membranes (Li et al., 2007).

One way to overcome these problems and establish an efficient biocatalytic process is to recombinantly express the P450 enzyme on the cell surface. This strategy enables direct contact between enzyme and substrate without the need for the compounds to cross a membrane. It also eliminates expensive enzyme purification steps and results in the immobilization of the protein in membrane surroundings (Samuelson et al., 2002). The Autodisplay system is an elegant way to secrete proteins in gram-negative bacteria to the cell surface (Jose and Meyer, 2007; Maurer et al., 1997). The recombinant passengers can be transported to the outer membrane by simple insertion of their coding region between a signal peptide and a C-terminal domain called β-barrel. The system is based on AIDA-1, the adhesion involved in diffuse adherence in enteropathogenic E. coli (Benz and Schmidt, 1992), which belongs to the autotransporter family of proteins (Jose et al., 1995; Jose and Meyer, 2007). Enzymes which have been successfully expressed using Autodisplay include esterases, a sorbitol dehydrogenase, a nitralase, a isopenyltransferase, a β-lactamase and a hyaluronidase.
(Detzel et al., 2011; Jose and von Schwichow, 2004a; Kaessler et al., 2011; Kranen et al., 2011; Lattemann et al., 2000; Schultheiss et al., 2002, 2008). Furthermore, the display of the rat NADPH-cytochrome P450 oxidoreductase, containing FMN and FAD (Yim et al., 2006) and a heme and flavin containing P450 BM3 (Yim et al., 2010) using the ice-nucleation protein from Pseudomonas syringae have already been reported. During the Autodisplay of bovine adrenodoxin, which serves as an electron donor for mitochondrial P450s, two major observations were made (Jose et al., 2001, 2002). First, it could be shown, that it is possible to incorporate an inorganic, prosthetic group into an apo-protein expressed by Autodisplay at the cell surface by a simple titration step to yield a functional electron donor without loss of cell viability or cell integrity. Second, after external addition of the purified P450s CYP11B1 and CYP11A1, a functional whole cell biocatalyst was obtained for efficient synthesis of different steroids such as pregnenolone and corticosterone (Jose et al., 2001). While working with the soluble, bacterial P450 106A2 we could show that it is possible to display an active P450 enzyme without the external addition of the heme group, which is most likely exported into the supernatant by the outer membrane channel TolC. After this export the porphyrin is incorporated into the enzyme from the outside (Schumacher et al., submitted for publication). However the surface display of any human, membrane bound P450 of clinical relevance in a bacterial background has yet to be shown and is the aim of the present project.

Cytochrome P450 3A4 (referred to here as CYP3A4) is arguably the most important P450 enzyme in humans as it is involved in the oxidation of the largest range of substrates and belongs to the class II microsomal P450 enzymes (Hannemann et al., 2007). In humans it is predominantly found in the liver and often allows prodrugs to be activated and absorbed. Inhibition or induction of CYP3A4 is a major problem in the daily clinical routine, often leading to drug-drug interactions or side effects. Increased activity of CYP3A4 can lead to the fast inactivation of the applied drug, resulting in low plasma levels and a reduced therapeutic effect. In contrast, inhibition of CYP3A4 can lead to intoxication (Guengerich, 1999). To evaluate these possible risks, it is important to determine which drug candidates are accepted as substrates by CYP3A4, and identify the resulting relevant drug metabolites (Schorer et al., 2010). This urgent demand makes CYP3A4 a logical candidate to test whether human P450 enzymes are functional on the cell surface of an E. coli cell. In this study, we show experimental evidence that it is possible to translocate an active, human P450 3A4 enzyme to the cell surface of E. coli by use of the Autodisplay system.

2. Materials and methods

2.1. Chemicals

Testosterone, 6β-hydroxytestosterone, human Cytochrome P450 reductase, Cytochrome b5 and mouse monoclonal anti-CYP3A4 antibody were purchased from Sigma Chemicals Co (St Louis, MO, USA). Human Cytochrome P450 was obtained from Biozol (Eching, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Carl Roth (Karlsruhe, Germany). Goat anti-mouse IgG conjugated with DyLight647 was obtained from Thermo Scientific (Waltham, MA, USA). The restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA).

2.2. Bacterial strain and growth conditions

E. coli strain UT5600 (DE3) (F- ara-14 leuB6 secA6 lacY1 proC14 tss-67 Δ(ompT-7psecC)266 entA403 trpE38 rfdD1 rpsL109 xyl-5 mtl-1 thi-1 (DE3)) was used for the expression of the autotransporter fusion protein (Jose and von Schwichow, 2004b). E. coli TOP10 (F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 (ara leu) 7697 galU galK rpsL (StrR) endA1 nupG) and the vector pCR4-TOPO which were used for subcloning of PCR products were obtained from Invitrogen (Darmstadt, Germany). E. coli Rosetta cells (F- ompT hsdS(BgII- mB+ ) gal dcm pRARE (CamR) and the plasmid pRARE to adapt the codon usage were obtained from Novagen (Darmstadt, Germany). Cells were routinely grown at 37°C in lysogeny broth (LB) medium, containing 50 μg of carbenicillin per liter, 10 μM ethylenediaminetetraacetate (EDTA) and 10 mM 2-mercaptoethanol. Solid media were prepared by the addition of agar (1.5%, w/v).

2.3. Construction of an artificial gene for the surface display of CYP3A4

For construction of the CYP3A4 autotransporter fusion protein, the gene encoding CYP3A4 was amplified by polymerase chain reaction from plasmid pCW-NF14 (Gillam et al., 1993). This PCR product was inserted into vector pCR4-TOPO from which it was released using the two restriction enzymes Xhol and KpnI before ligation into plasmid pET-SH7 (Petermann et al., 2010), cut with the same enzymes. This yielded an in frame fusion protein consisting of (1) the CtxB signal peptide, (2) CYP3A4 as a passenger, (3) the autotransporter linker region and (4) the autotransporter β-barrel (Fig. 1) under the control of a T7/lac promoter. Construction of the plasmid pST001, used as a control, is described elsewhere (Park et al., 2011). Both plasmids were transformed into UT5600 (DE3) by electroporation-mediated transformation (Sambrook et al., 2001) with standard equipment, and the inserted genes were fully sequenced before use in expression experiments.

2.4. Outer membrane preparation

E. coli cells were grown overnight in LB medium and 20 μl was used to inoculate a 20 ml culture. Cells were cultivated at 37°C under vigorous shaking (200 rpm) until an OD578 of 0.5 was reached. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Roth, Karlsruhe, Germany) to reach a final concentration of 1 mM. After 16 h at 30°C, induction was stopped by harvesting the cells and washing them with buffer (0.2 M Tris–Cl, pH 8). Outer membrane proteins were prepared according to the rapid isolation protocol of Hancke (Hancke, 1981) with modifications as previously described (Jose and von Schwichow, 2004a). A protein accessibility test was used to confirm surface expression, based on the proteinase K mediated degradation of the surface-displayed protein. E. coli cells were harvested, washed and suspended in phosphate buffered saline (pH 7.4). Proteinase K was added to a final concentration of 0.2 mg L−1 and cells were incubated for 60 min at 37°C. Digestion was stopped by washing the cells three times with PBS containing 10% fetal calf serum (FCS). After proteinase K digestion, outer membrane proteins were isolated as described above.

2.5. SDS-PAGE

Outer membrane protein isolates were diluted two-fold with sample buffer (100 mM Tris–Cl, pH 6.8 containing 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol, and 50 mg dithiothreitol). The samples were boiled for 5 min at 95°C and proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a 12.5% acrylamide resolving gel. Proteins were stained with Coomassie Brilliant Blue, and the molecular weight of the proteins estimated using a prestained marker as a standard (Fermentas, St. Leon-Rot, Germany).
2.6. Western blot

For Western blot analysis, gels were electroblotted on polyvinylidene fluoride (PVDF) membranes using standard techniques (Tetra Cell, Bio-Rad, USA). Proteins on the membranes were blocked in 25 mM Tris-base pH 7.4 with 140 mM NaCl, 2.7 mM KCl (TBS) and 3% bovine serum albumin (BSA). For immune detection, membranes were incubated for 3 h with the primary anti-CYP3A4 monoclonal antibody diluted 1:5000 in TBS with 3% BSA. Prior to addition of the secondary antibody, blots were rinsed three times in TBS with 0.1% Tween 20. The secondary antibody was then added and the blots were incubated for 2 h at room temperature, before being washed three times in TBS. The secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase, was used at a dilution of 1:10,000 in TBS with 3% BSA. Antigen-antibody conjugates were visualized by a color reaction with staining solution consisting of 17 mM 4-chloro-1-naphthol and 0.015% H₂O₂ in TBS.

2.7. Flow cytometer analysis

E. coli cells were grown as described above in Section 2.2. Expression of CYP3A4 was induced at an early exponential growth phase (OD₅₇₈ of 0.5) by adding IPTG to a final concentration of 1 mM. Cells were then incubated overnight at 30 °C, 200 rpm in LB medium. Cells were harvested by centrifugation, washed twice with PBS and resuspended to a final OD₅₇₈ of 0.5. 100 μl of the cell suspension was suspended in 500 μl of PBS containing 3% bovine serum albumin (filter-sterilized), and incubated for 10 min at room temperature (RT). Cells were then pelleted in a microcentrifuge (60 s at 18,000 × g, Hettich, Tuttingen, Germany), resuspended in 100 μl of a solution that contained a monoclonal CYP3A4 antibody (diluted 1:100 in PBS [pH 7.4] + 3% BSA) and incubated for another 30 min at RT. Cells were then washed twice with 500 μl of PBS. The second incubation step was conducted in the dark (30 min, RT) using 100 μl of a goat anti-mouse IgG antibody conjugated with DyLight647 (diluted 1:25 in PBS [pH 7.4] + 3% BSA). After washing twice in PBS supplemented with 3% BSA, the cell pellet was resuspended in 1.5 ml of PBS. Samples were then analyzed using a CyFlow® space flow cytometer (Partec GmbH, Münster, Germany) at an excitation wavelength of 647 nm.

2.8. Whole cell ELISA

For a whole cell ELISA as has been described for surface displayed human Ro/SS-A antigen (SD-ELISA; Petermann et al., 2010), E. coli cells were grown and protein expression was induced as described above. Subsequently, cells were washed twice with coating buffer (PBS, pH 7.4) and suspended to a final OD₅₇₈ of 0.5. A 96-well microplate (Maxisorp, Nunc) was coated with 100 μl of the cell suspension overnight at 37 °C. Any nonspecific binding sites were blocked by the addition of 150 μl of PBS (pH 7.4) + 10% FCS for 3 h at RT. After blocking, cells were incubated with a monoclonal anti-CYP3A4 antibody for 1 h at RT. The microplates were rinsed three times with the washing buffer (PBS [pH 7.4] + 0.1% Tween 20), and incubated with a secondary goat anti-mouse IgG conjugated with horseradish peroxidase diluted 1:10,000 in PBS (pH 7.4) + 10% FCS. After a further 45 min of incubation at RT, plates were washed three times, followed by the addition of 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) to each well. Plates were then incubated in the dark at RT for 25 min, and the enzyme reaction was stopped by adding 100 μl of 2 M H₂SO₄. Absorption at 450 nm was measured with a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany).

2.9. Assay for the hydroxylation of testosterone

For activity measurements an enzyme premix was prepared and frozen at −80 °C in 1 ml aliquots (Shaw et al., 1997). The 5× protein premix contained 1 μM NADPH-P450-Reductase, 0.5 μM Cytochrome b5, 50 mM Hepes, 0.5 mg/ml CHAPS and 3 mM glutathione. The enzyme reaction was carried out in 50 mM Tris–Cl pH 7.8 containing 200 mM NaCl and 3 mM magnesium chloride. A typical 100 μl biotransformation reaction was prepared by mixing (on ice) 20 μl of 5× protein premix, 5 mM of glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase (where a unit is described as the amount of enzyme that will reduce 1.0 μmol of NADP per minute at 30 °C, pH 7.8) Additionally the reaction contained either 0.2 μM of purified, reconstituted CYP3A4 enzyme or E. coli pSC001 cells at an OD₅₇₈ of 10 (approximately 2.5 × 10⁹ cells ml⁻¹). Testosterone was dissolved in ethanol and added to the enzymes to a final concentration of 200 μM. The
reaction was initiated by the addition of 600 μM NADPH, proceeded for 72 h at a temperature of 37 °C and was terminated by the addition of 500 μL chloroform. Samples were extracted twice with chloroform and the layers were separated by centrifugation (60 s at 18,000 × g with a microfuge). The organic phases were combined and the solvents removed under vacuum. The dried samples were resuspended in 100 μL of acetonitrile for HPLC analysis (described below).

2.10. Analytical methods

Testosterone conversion was analyzed using a LiChrospher 60 RP-select B column 125–4 (5 μm) from Merck (Darmstadt, Germany) on a LaChrom Elite System from VWR-Hitachi (Darmstadt, Germany). The hydroxylation product was detected at 254 nm. The mobile phase used in the separation, at a flow rate of 1.0 mL/min, consisted of (A) water (0.02% trifluoroacetic acid and (B) an acetonitrile gradient which decreased from 90% to 40% over 20 min. The column was then reequilibrated for 5 min with 90% acetonitrile. The injection volume was 20 μL. These parameters were also used for commercially available 6β-hydroxytestosterone in order to determine its retention time in this HPLC system and for quantification of the enzyme-derived product.

3. Results

3.1. Expression of the CYP3A4 autotransporter protein

The E. coli strains UT5600 (DE3) was transformed with the plasmid pSC001 which encoded the CYP3A4 autotransporter fusion protein. The T7 promoter allowed transcription induced by the addition of IPTG (Studier and Moffatt, 1986). After induction of protein expression the revealed protein band was so weak that it was not visible on the SDS-PAGE. Therefore extracts of outer membrane proteins were applied to Western blot analysis and revealed a protein band with an approximate molecular weight of 110 kDa, as expected for the CYP3A4 autotransporter fusion protein (Fig. 2B, lane 4).

3.2. Protein accessibility test

To test the surface exposure of the CYP3A4 domain, proteinase K was added to whole cells of E. coli expressing the fusion protein. Proteinase K is too large to enter the cell envelope and therefore the degradation of the fusion protein is strong evidence that it is accessible at the cell surface (Fig. 2B, lane 5). As a control, we also examined the molecular weight of OmpA, which has a C-terminal extension in the periplasm that is susceptible to protease cleavage. The lack of OmpA digestion (Fig. 2A, lane 5) is a strong indication that proteinase K had not entered the periplasm.

3.3. Effect of codon usage

The intensity of the CYP3A4 band was low in comparison to the bands corresponding to the native E. coli outer membrane proteins OmpF/C and OmpA (Fig. 2). This suggests that a relatively low amount of CYP3A4 was present at the cell surface, also in comparison to other enzymes expressed by the Autodisplay system, including, e.g. Adx or nitrilease (Detzel et al., 2011; Jose et al., 2001). The 110 kDa band corresponding to the CYP3A4 autotransporter fusion protein was only visible in the Western blot (Fig. 2B, lane 4) and not in the corresponding SDS-PAGE (Fig. 2A, lane 4), indicating an abundance far lower than the natural outer membrane proteins OmpA or OmpF/C. The human CYP3A4 gene contains seven codons that are rare in E. coli (data not shown). To determine if the rare codons were responsible for the low amount of protein detected, we expressed the protein in cells containing extra tRNAs (Rosetta cells containing pRARE) complementing the observed rare codons. In comparison to UT5600 cells, Rosetta cells expressed a higher amount of the 110 kDa protein in the Western blot analyses, suggesting that the rare codons did indeed have a negative effect on total protein expressed (Fig. 2B, lane 8). In both cases, with and without pRARE, the 110 kDa protein band was only found in cells where expression was induced, providing a strong indication that it was the correct fusion protein. The protein accessibility assay with proteinase K confirmed the surface expression of the CYP3A4 protein in Rosetta cells (Fig. 2B, lane 9).

3.4. Flow cytometer analysis

Flow cytometry has previously been used to confirm the surface expression of proteins, and investigate expression efficiency (Petermann et al., 2010). For the cell labeling, we used a primary monoclonal antibody targeting CYP3A4, and a secondary antibody conjugated to DyLight647. The fluorescence intensity of cells expressing the CYP3A4 fusion protein was shifted to higher intensity in comparison to control cells treated identically (Fig. 3) One population of the cells expressing CYP3A4 could not be marked at all and remained negative. Even though the positive population indicates that the CYP3A4 domain was accessible at the cell surface and, as a consequence, surface display of the P450 enzyme by Autodisplay in E. coli was successful, a further method to investigate surface display was performed to confirm these results.

3.5. Whole cell SD-ELISA

A 96-well microplate was coated with E. coli UT5600 (DE3) (negative control), E. coli UT5600 (DE3) pST001 (displaying streptavidin

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**Fig. 2.** Surface expression of CYP3A4 in E. coli. A: Preparations of outer membrane proteins were prepared as described in Section 2. Proteins were separated by SDS-PAGE (12.5%) and stained with Coomassie Brilliant Blue G250. Expression experiments were performed with the E. coli strains UT5600 (DE3) (lanes 2–5) and Rosetta (DE3) (lanes 6–9). 1: marker proteins, 2: cells without plasmid, 3: cells containing pSC001–IPTG, 4: cells containing pSC001 + 1 mM IPTG, 5: cells containing pSC001 + 1 mM IPTG + 1 mM protease K for the degradation of surface displaced proteins, 6: cells containing pRARE, which increases the tRNA corresponding to rare codons, 7: cells containing pSC001 + pRARE–IPTG, 8: cells containing pSC001 + pRARE + 1 mM IPTG, and 9: cells containing pSC001 + pRARE + 1 mM IPTG + 1 mM protease K. B: Western blot with a monoclonal antibody specific to CYP3A4. Proteins were detected using a secondary antibody conjugated to horse-radish peroxidase. Samples were loaded as per A.
on the surface; negative control) (Park et al., 2011) and with E. coli UT5600 (DE3) pSC001 (displaying CYP3A4 on the surface). The second negative control, expressing a different protein, was used to eliminate the possibility of false positive results due to a cross reaction against the linker region or the β-barrel. The cells were incubated with a primary monoclonal anti-CYP3A4-antibody and a secondary horseradish peroxidase detection antibody. The dye TMB (3,3′,5,5′-tetramethylbenzidine) was added to the mixture and was immediately oxidized by the horseradish peroxidase. This reaction, after being stopped by the addition of sulfuric acid, yielded a yellow color, which could be measured as absorption at 450 nm. The cells carrying the plasmid encoding for CYP3A4 showed a significant higher absorption after inducing protein expression in comparison to controls, which is strong evidence that the protein is located at the cell surface (Fig. 3B).

3.6. Hydroxylation assay

To test the activity and with it the correct folding of the displayed CYP3A4 a hydroxylation assay was performed with the substrate testosterone (Fig. 4). P450-mediated catalysis required the external addition of Cytochrome b5. Cells were prepared as described above and hydroxylation of testosterone was carried out for 72 h. The activity of the enzyme CYP3A4 was monitored using an HPLC separation system for steroids, which can demonstrate the CYP3A4-dependent conversion of testosterone into 6β-hydroxytestosterone. The purified enzyme produced only a small product peak (Fig. 5A). Despite the low activity, an enlarged picture of the plot areas revealed enzyme activity of whole cells displaying CYP3A4 (Fig. 5B). UT5600 (DE3) cells containing the plasmid encoding the autotransporter fusion protein but without the addition of IPTG did not show 6β-hydroxylase activity (Fig. 5B, graph 2). After induction of protein expression, cells displaying the fusion protein produced the 6β-hydroxylated product (Fig. 5B, graph 3). Since the product peak was only found in cells where expression was induced this is a strong indication for the activity of the displayed enzyme. The retention time of the product produced by the CYP3A4-expressing cells (~12 min) corresponded very well to the retention time of the commercially available 6β-hydroxytestosterone, which was used as a reference compound (see supplementary Fig. S1) and the product produced by the purified enzyme (Fig. 5A and B). The hydroxylation reaction relies on the addition of NADPH which is supposed to be not able to cross cellular membranes (Gholson et al., 1969) giving further proof that all conversions took place on the cell surface. Human CYP3A4 is known to hydroxylate testosterone at several sites, with the 6β-hydroxylated product being usually formed in the highest quantity. The conversion of the substrate testosterone with the purified enzyme yielded indeed an additional product at a retention time of about 13.9 min (Fig. 5B, graph 1). This peak can also be seen in the conversion mediated by the surface displayed CYP3A4 enzyme (Fig. 5B, lane 3) and it appears – by first sight – to be larger than the main product peak. However, this side product peak is overlapping with a metabolite produced by the bacterial cells without CYP3A4 (Fig. 5B, lanes 2 and 4) and by a component or impurity of the reaction buffer (Fig. 5B, lane 5). This clearly indicates that the peak at 13.9 min obtained by HPLC of the reaction sample with cells displaying CYP3A4 is comprised of the reaction side product obtained by hydroxylation of testosterone, the bacterial metabolite and the buffer constituent. As a consequence, there was no indication that the surface display of CYP3A4 resulted in an altered regio- or stereoselectivity in comparison to the reaction with the purified enzyme. Nevertheless such an alteration has been observed in the surface of sorbitol dehydrogenase (Jose and von Schwichow, 2004b) and nitrilase (Detzel et al., 2011).

To increase the activity of the whole cell biocatalyst the same experiment was performed with the strain Rosetta pRARE pSC001, showing a higher expression of the fusion protein (Fig. 2, lane 8).

Fig. 3. Surface accessibility of CYP3A4 determined by flow cytometry analysis and whole cell ELISA. For both techniques, cells were incubated with a primary monoclonal anti-CYP3A4 antibody. A: Flow cytometry analysis, after staining cells with secondary antibody labeled with DyLight647. Black: UT5600 (DE3) without plasmid, gray: cells displaying CYP3A4 on the surface (UT5600 (DE3) containing the plasmid pSC001, protein expression induced with IPTG). B: Whole cell ELISA, after incubating cells with a secondary antibody labeled with horseradish peroxidase. Reactions were started by the addition of the dye TMB and stopped with sulfuric acid. Absorption was measured at 450 nm.

Fig. 4. Hydroxylation of testosterone at the 6β-position by CYP3A4. When CYP3A4 is displayed on the surface of E. coli cells, redox equivalents from NADPH are transferred to the enzyme by the NADPH-P450-Reductase, included in the protein premix.
Unfortunately the higher expression did not yield a higher enzymatic activity (data not shown).

To verify the functionality of the surface displayed CYP3A4 and to increase the amount of the product 6β-hydroxytestosterone, whole cells of *E. coli* expressing CYP3A4 were incubated with the substrate testosterone in different cell densities. The amount of product formed was measured by HPLC using a standard curve of the commercially available reference compound 6β-hydroxytestosterone product and the results were calculated in nM 6β-hydroxytestosterone as shown by supplementary Fig. S1. The amount of product formed by the whole cell catalyst increased in a linear manner with the optical density (OD578) of the supplied cells (Fig. 6), whereas control cells without a plasmid subjected to this reaction for an identical reaction time did not yield any product peak at all (Fig. 6). This clearly indicated that the product 6β-hydroxytestosterone formed from the substrate testosterone was due to the surface displayed CYP3A4 fusion protein and that the human P450 enzyme was functionally expressed by Autodisplay.

Secondary structure analysis (TMpred program) of the CYP3A4 sequence suggested that the N-terminal part forms a transmembrane helix, which could interfere with the transport of the enzyme across the outer membrane. It could result in a periplasmic degradation of the passenger fusion protein as has been shown before for the Autodisplay passengers CtxB (Jose et al., 1996) and Aprotinin (Jose and Zangen, 2005). To overcome this possible limitation, the N-terminal 17 amino acids were deleted by a PCR-based approach and the resulting truncated CYP3A4 autotransporter fusion protein was analyzed for transport and enzymatic activity as described above. As shown in supplementary Fig. S2, expression of this construct under the control of a constitutive promoter (Jose et al., 2001; Maurer et al., 1997) resulted in an almost 50% higher enzymatic activity of the cells displaying CYP3A4, although in all cases the concentration of the product remained low, in the nm range, as generally expected for CYP3A4, which shows the slowest conversion rates of human first pass metabolizing enzymes. In addition, this clearly indicated that human CYP3A4 expressed on the cell surface of *E. coli* by Autodisplay was displayed in an enzymatically active form.

4. Discussion

Microbial cell surface display has a great potential to be used in a wide range of applications and provides several advantages in biotechnological applications (Lee et al., 2005). Therefore a lot of proteins and peptides have already been brought to the cell surface in several bacteria (Daugherty, 2007; Samuelson et al., 2002). So far the display of bovine adrenodoxin, containing an iron sulfur cluster (Jose et al., 2001, 2002), rat NADPH-cytochrome P450 oxidoreductase, containing FMN and FAD (Yim et al., 2006) and a heme and diflavin containing P450 BM3 (Yim et al., 2010) have already been reported. As far as we know this is the first time, that an active human P450 enzyme was successfully displayed on the cell surface of *E. coli*. The recombinant expression of active human P450 enzymes is necessary to allow analyses of drug metabolism and toxicology. Because many mammalian P450s are not glycosylated, *E. coli* is an attractive host for the rapid production of large quantities of enzyme for research purposes. Despite many
advances in recombinant expression, and in particular recombinant expression in \textit{E. coli}, expression of many P450 enzymes remains difficult (Guengerich \textit{et al}., 1996). After purification from membranes CYP3A4 is notorious for difficulties in reconstitution of activity (Yamazaki \textit{et al}., 1995). Additionally the slow 6β-hydroxylation activity of human P450 3A4 is barely detectable after recombinant expression in \textit{E. coli} (Blake \textit{et al}., 1996). Expression rates in \textit{E. coli} can be improved by modifying the N-terminal part of the enzyme (Gillam, 2008). But even when the enzyme is expressed and active, human P450 3A4 and other P450s require membrane surrounds for activity, increasing the complexity of reconstitution experiments (Hannemann \textit{et al}., 2007).

We have previously shown proof of concept that a soluble, bacterial P450 can be displayed at the surface of the \textit{E. coli} cell with Autodisplay (Schumacher \textit{et al}., submitted for publication). This technique solves many of the typical obstacles of a whole-cell monoxygenase system, including the poor stability of the enzymes without proper membrane surroundings, the inability to test substrates that cannot cross membranes, and the necessity for time-consuming enzyme purification. To determine if a mammalian P450 is also functional at the surface of the cell, in this study we attempt the Autodisplay of human CYP3A4, a class II microsomal P450 enzyme that requires a membrane environment for activity. The enzyme showed full activity without the external addition of the prosthetic group as was necessary in other studies (Jose \textit{et al}., 2001). Expression was verified by SDS-PAGE and Western blot. Flow cytometry, a protease accessibility test and a whole cell SD-ELISA verified surface localization. The functionality of the enzyme was tested by an HPLC assay showing the hydroxylation of the educt testosterone into the product 6β-hydroxytestosterone by the displayed enzyme. Furthermore, the activity was dependent on external addition of substrates that are not thought to cross the \textit{E. coli} membrane, including NADPH-P450-Reductase and NADPH (Gholson \textit{et al}., 1969), supporting the theory that the reaction took place on the cell surface. Unfortunately, the expression of the enzyme as well as the activity of the whole cell biocatalyst obtained thereby was not very high. To improve the expression and with it hopefully the activity the first aim was to improve the codon usage by transforming the plasmid into the strain Rosetta carrying the plasmid pRARE. This strategy yielded into a better expression of the enzyme, as could be seen on the Western blot, but under no circumstances was the fusion protein visible after Coomasie staining of the SDS-PAGE of outer membrane isolations. This clearly indicates that even after improving the codon usage only a small amount of protein was present. The deletion of the N terminal 17 amino acids, which are supposed to form a transmembrane α helix, and therefore interfere with our transport system, resulted in an almost 50% increase of the enzymatic activity of the whole cell biocatalyst displaying human CYP3A4. This indicates that rational enzyme engineering could be a valid tool to improve the activity of CYP3A4 displayed on the surface of \textit{E. coli} (Gillam, 2008).

Nevertheless, towards the synthesis of drug metabolites or for other biotechnological applications, the whole cell biocatalyst displaying CYP3A4 is only the first step. In the present approach, NADPH-P450-Reductase and Cytochrome b5 need to be added from the exterior making this approach rather expensive, and only useful for the production of more expensive metabolites or products in general. The co-factor NADPH can be regenerated by adding glucose-6-phosphate and glucose-6-phosphate dehydrogenase, which is again a burden for a possible commercial use of the system. As a consequence the next steps need to be the co-expression of NADPH-P450-Reductase and Cytochrome b5 with human CYP3A4 on the surface of a single cell of \textit{E. coli} and in order to facilitate efficient co-factor regeneration, to display glucose-6-phosphate dehydrogenase on a second cell of \textit{E. coli} and combine both cell types in one reaction batch. Of course this approach is challenging, but first attempts to co-express enzymes with different functionalities on the surface of \textit{E. coli} were successful (Kraen and Jose, unpublished) and the expansion of this system in this way is under current investigation. The autodisplayed CYP3A4 was active without the addition of an external heme, which has a molecular weight of 616 g mol$^{-1}$ and a structure that is unlikely to pass through the bacterial membrane through passive mechanisms (Tatsumi and Wachi, 2008; Verkamp \textit{et al}., 1993). The most probable explanation is that CYP3A4 passenger molecules were transported to the cell surface by the autotransporter in an unfolded form, without porphyrin, which is in agreement with the original theory regarding secretion by autotransporters. Thereby the proteins are unfolded and need to pass through a small pore in the β-barrel structure (Jose \textit{et al}., 1995; Maurer \textit{et al}., 1997; Pohlenzer \textit{et al}., 1987). The protein folds at the cell surface and incorporates heme groups that were present in the cell supernatant. It has been proposed that porphyrins are exported from \textit{E. coli} cells by a TolC-dependent efflux system (Tatsumi and Wachi, 2008) into the supernatant and recently we were able to show, that a CYP106A2 displaying whole cell biocatalyst exhibited much lower enzymatic activity in a TolC negative host background than in the corresponding TolC positive strain of \textit{E. coli} (Schumacher \textit{et al}., submitted for publication). This makes the export of porphyrins via the outer membrane channel TolC and the incorporation into the CYP3A4 apo-protein at the cell surface very likely and is consistent with the initial model of autotransporter mediated surface translocation of passenger proteins. The heme in the supernatant could also been due to a contamination of the yeast extract used to make LB medium, as described before (Kaur \textit{et al}., 2009).

It cannot be excluded at this point, that CYP3A4 was transported across the outer membrane in a folded, or partially folded state. Although this appears to be inconsistent with most data regarding the transport mechanism of autotransporters, other studies have suggested that other passenger domains can acquire a stable tertiary structure in the periplasm before transport to the cell surface using the Omp85/Bam A pathway (Bernstein, 2007; Tommassen, 2010). In this case the prosthetic group could have been incorporated in the periplasm, where it occurs naturally (Goldman \textit{et al}., 1996; Tommassen, 2010). Considering the diversity of autotransporters in gram negative bacteria, it is possible that different mechanisms exist for the transport of passenger proteins to the surface, and indeed, this may be dependent on the passenger itself.

We conclude that it is possible to immobilize a human P450 enzyme on the surface of \textit{E. coli} in a functional form. This is a first step towards establishing a system to screen and synthesize clinical relevant phase-I-metabolites.

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\textbf{Appendix A. Supplementary data}

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References


