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

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ABSTRACT

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 studying 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 heme group was not necessary. Our results indicate that human CYP3A4 can be recombinantly expressed by surface display in a gram-negative bacterium.

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-I, the adhesin 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 nitrilase, a isoprenyltransferase, a β-lactamase and a hyaluronidase

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(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 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) 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 (Schroer 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 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1 λ(DE3)*) was used for the expression of the autotransporter fusion protein (Jose and von Schwichow, 2004b). *E. coli* TOP10 (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) 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*$_B$(r_B^- m_B^-) *gal dcm* pRARE (Cam^R)) 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 mg 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 recleaved using the two restriction enzymes *XhoI* 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 OD_{578} 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 Hantke (Hantke, 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).